PROJECT PERIODIC REPORT

Grant Agreement number: 603121

Project acronym: DIVERSIFY

Project title: Exploring the biological and socio-economic potential of new/emerging fish species for the expansion of the European aquaculture industry

Funding Scheme: FP7=KBBE-2013-7-single-stage

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Periodic report: 1st ☐ 2nd ☐ 3rd ☐ X 4th ☐

Period covered: from 31 to 48 mo

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DIVERSIFY

New species for EU aquaculture

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

2. Core of the report for the period:
Project objectives, work progress and achievements, project management

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2.1 Project objectives for the period

Reproduction and Genetics

A total of eight objectives were completed during the 3rd reporting period. All work in WP2 – meagre, was finished and the remaining five objective completed. The completed objectives provided for meagre, *in vitro* fertilisation procedures to make planned genetic crosses and genetic tools, that included the first genetic linkage map for meagre, which identified 731 markers for marker assisted breeding programs. In WP3 - greater amberjack, two objectives were completed to describe the reproductive cycle and compare dysfunctional captive breeders with wild breeders. In WP5 - halibut, work was completed on the objective to improve fecundity and gamete quality in F1/F2 broodstock. In addition to these completed objectives, a large volume of work was made to advance the remaining eight objectives on the following aspects:

- Experiments to improve spawning of greater amberjack, wreckfish and grey mullet,
- Development of techniques to aid description of the reproductive cycle of wreckfish
- Experiments on *in vitro* fertilisation for wreckfish

Assess the effects of captivity on first sexual maturity in grey mullet.

Nutrition

Four types of feeds are used in aquaculture, differing in nutritional and physical characteristics: enrichment products for live preys and dry feeds for weaning, grow out and broodstock diets, all differing in their formulation and production technology. The Project objectives for the third reporting period have focused in first feeding regimes (enrichment products and weaning diets), growth-out diets and broodstock diets. Larval diets have been improved, nutritional requirements for selected nutrients have been determined for juveniles and in those species with bottlenecks in the reproduction information has been obtained to formulate the first broodstock diets to improve spawning quality. Therefore the objectives of this period have been focused in: 8.2. Determination of nutritional requirements to promote feed utilization, consistent growth rates and meagre welfare. 9.2. Develop diets for grow-out of greater amberjack in order to maximize growth potential. 9.3. Design adequate feeding regimes for broodstock to optimize reproduction in amberjack 10.1 Increase knowledge on the effect of nutrients essential for first feeding of pikeperch.10.2. Develop specific enrichment products and formulated diets to improve pikeperch larval performance. 11.4. Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS. 11.5 Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles. 12.1. Live preys and enrichments for wreckfish larvae. 12.2 Determine the influence of broodstock feeds on fecundity and spawning quality of wreckfish. 13.1.Improve enrichment products, weaning, grow out and broodstock diets for grey mullet. 13.2. Determining grey mullet nutritional needs for improved weaning to a dry diet. 13.3. Determining grey mullet nutritional needs for a more cost-effective production. 13.4. Design adequate feeding regimes for grey mullet broodstock to optimize reproduction success.

Larval husbandry

The project objective of WP14 (meagre) (1) to reduce costs by early weaning in meagre larvae and improve growth, survival and larval quality have produced a deliverable and a publication. The project objectives of the amberjack studies (WP15) have been addressed and were (1) Effects of different feeding strategies on larval performance in intensive systems, (2) Development of feeding protocol and rearing system in mesocosm semi-intensive systems, and (3) Development of industrial protocol for larval rearing. The objectives of the pikeperch studies (WP16) were (1) improvement of pikeperch larval rearing protocols by using a multifactorial approach, (2) Reduction of cannibalism rate to increase survival and (3) Development of industrial protocol to improve larval performance during rearing. These aims have been largely addressed. However, the deliverable D16.4, which was initially planned on month 48 will be delivered on month 54 because the experiment will be done in February-March 2018, as a consequence of the delay of the deliverables; D16.1, D16.2 and D16.3. The objective in the studies to improve larval survival and quality during early development of Atlantic halibut (WP17) has been addressed although some analyses are

pending. The objectives in the studies in wreckfish (WP18) were (1) the development of larval rearing protocol based on the most effective prey density, succession of prey type, temperature and culture system and (2) description of ontogeny of digestive system, vision, taste and smell organs in response to larval rearing methods. These objectives have been partially addressed and studies are on-going. The studies on grey mullet larvae had the objectives (1) investigating environmental and nutritional factors that affect larval rearing, (2) determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production and (3) Determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding. These objectives have been largely addressed, except for objective 2, which will be investigated in 2018.

Grow out husbandry

The objectives for WP 20 during this period were (a) to complete the study on the size variability of juveniles (b) to contribute towards the definition of the optimum environment (in terms of depth and ligh intensity) for cage rearing and (c) the development of appropriate feeding methods for cages. For WP 21 the objectives were (a) to contribute towards the development of feeding methods for fry and juveniles by identifying daily rhythms and feeding frequency (b) to define the optimal ranges in terms of environmental temperature during the rearing and also the optimum ranges regarding the stocking density. For WP 22, the objectives were (*a*) to characterize growth, immune and physiological status of pikeperch in farm conditions in order to validate the effects of the best identified rearing conditions (Task 22.2) and (*b*) to assess the effects of pikeperch domestication level and geographical origin on growth and stress sensitivity (Task 22.3).

Fish health

In this reporting period many objectives were to be achieved in the Fish Health GWP, and the majority have been completed on time and DL reports submitted. This included in **WP24** study of the impact of different diets on the severity of Systemic Granulomatosis (SG) in meagre (Task 24.1), attempts to isolate *Nocardia* sp. from SG-affected meagre (Task 24.4), the completion of studies to investigate the cause of chronic ulcerative dermatopathy (Task 24.2), the development of disease challenge models and study of anti-parasitic agents (Tasks 24.3, 24.7), further study of immune responses and how they can be manipulated (Task 24.6), a vaccine trial against *Vibrio anguillarum* (Task 24.4) and further recommendations for diagnosis and treatment of meagre diseases (Tasks 24.7 and 24. 8). In **WP25** objectives included further work to identify the cause of epitheliocystis in greater amberjack (Task 25.1), the impact of prebiotics, stocking density and anti-attachment factors on parasite infestation in amberjack (Tasks 25.2 and 25.4), the modulation of immune responses at mucosal sites and study of antimicrobial defences (Task 25.3), and further contributions to the diagnostic recommendation manual regarding amberjack disease diagnostics and treatments (Tasks 25.5 and 25.6). Lastly, in **WP26**, a vaccine trial was planned to evaluate the efficacy and immune responses elicited following immunisation against a recombinant viral capsid protein from nodavirus, delivered to juvenile halibut by different means (Tasks 26.2 and 26.3).

Socioeconomics

All activities in WP 27 have been completed in the last reporting period. So all other WPs in the Socioeconomics area were able to use the information about the external environment in protocols for further research. In this 3rd RP activities have been done in:

- New product development (WP 28)
- Consumer value perceptions and behavioural change (WP 29)
- Business model and marketing strategy development (WP 30)

In WP 28 work has been done on the following objectives (no. 3 and 4 in the DOW):

3. To monitor the quality of new products in terms of organoleptic characteristics and nutrition-rearing history
4. To make a technical assessment of the products

The consumer study and the product samples developed in this WP have been used in a funnelling approach in WP 29 and 30.

In WP 29 the team worked on the following objectives (no. 3 and 4 in the DOW):

3. To optimize the DIVERSIFY species' newly developed products in terms of ideal extrinsic product attribute combinations that have the potential to generate ideal consumer value perceptions, and
4. To determine the effectiveness of market communication in consumer behaviour change in relation to the DIVERSIFY species considered and the new raw and other value added products developed.

In WP 30 the team has been working on the first objective:

1. To identify business models for sustainable profitability and improved competitiveness of the sector for all the DIVERSIFY species.

Furthermore, work has been done on the following objectives of WP30:

2. To devise marketing strategies for the newly developed products of the DIVERSIFY species, aiming to develop a market that is as large and profitable as possible.
3. To come up with policy/strategy recommendations for further development and market expansion.

2.2 Work progress and achievements during the period

Please provide a concise overview of the progress of the work in line with the structure of Annex I to the Grant Agreement.

For each work package, *except project management, which will be reported in section 3.2.3, please provide the following information:*

- *A summary of progress towards objectives and details for each task;*
- *Highlight clearly significant results;*
- *If applicable, explain the reasons for deviations from Annex I and their impact on other tasks as well as on available resources and planning;*
- *If applicable, explain the reasons for failing to achieve critical objectives and/or not being on schedule and explain the impact on other tasks as well as on available resources and planning (the explanations should be coherent with the declaration by the project coordinator) ;*
- *a statement on the use of resources, in particular highlighting and explaining deviations between actual and planned person-months per work package and per beneficiary in Annex 1 (Description of Work);*
- *If applicable, propose corrective actions.*

Group Work Packages

Reproduction & Genetics

Work was completed to development the tools required for meagre breeding programs. From work in **Tasks 2.3 and 2.4**, a protocol for *in vitro* fertilisation was developed, which included the: characterisation of meagre sperm and ova, protocols for sperm storage and cryopreservation, definition of the timing of stripping to obtain good quality ova and minimum ratios for sperm to egg to obtain optimal fertilisation success. Meagre sperm had a mean sperm density of $3.21 \cdot 10^{10} \pm 1.18$ spzoa/mL, motility duration was 1:43± 0:18 min and different sperm storage methods chilled and cryopreservation were provided. The optimal period for stripping eggs was 38-39 hours after the application of GnRHa and a ratio of 200,000 sperm to egg was recommended. Work in **Task 2.5**, has characterised for the first time the muscle and liver transcriptome and constructed the first genetic linkage map for meagre, which identified 731 markers. This map and markers will aid the selection of breeders with desirable phenotypes in marker assisted breeding programs.

In **Task 3.1**, reproductive dysfunctions in captive breeders were described and compared to wild caught fish, highlighting important differences in pituitary capacity to synthesize and secrete gonadotropins, sex steroids circulating levels, oocytes that undergo maturation, germ cell apoptosis and spermatogenic activity. In **Tasks 3.2 and 3.3**, spawning protocols for greater amberjack have been established and advances have been made to refine the spawning protocols. Work in the Mediterranean Sea produced large volumes of high quality greater amberjack eggs (+20M) with the application of GnRHa treatments on greater amberjack maintained in cages in the and moved to tanks after the hormonal therapy. In **Task 3.4**, a further advance was made in the eastern Atlantic with spontaneous spawning (+20m eggs) of captivity bred (F1) greater amberjack held in tanks with and without GnRHa treatment. Work on pikeperch was completed during the 2nd reporting period.

Work with Atlantic halibut, in **Task 5.1**, found that cultured females had irregular ovulatory intervals compared to captive wild females. In **Task 5.2**, the endocrine regulation of ovulation was studied. However, plasma levels of steroids and gonadotropins did not appear to explain the differences in ovulatory timing. This was the first study of gonadotropin levels in Atlantic halibut.

Unfortunately, little advance was made with **Task 6.1** due to the scarcity of wild wreckfish in the fishery. In **Task 6.2** the reproductive cycle of wreckfish has now been fully described. Wreckfish females can adapt to captivity, mature and produce eggs during a spawning period from March to June. Males produce large amounts of good quality sperm throughout the year. Advances in both female and male maturity were described in association with plasma steroids. However, dysfunctions were observed in some females and low fertilization during spawning suggested a behavioural dysfunctions. Promising advances were made in **Task 6.3**, to induce ovulation for *in vitro* fertilisation procedures. Females were induced to ovulate and eggs fertilized. In **Task 7.3**, the breeding protocol for grey mullet in captivity was optimized in Israel with specific sized breeding groups and out-of-season egg production to provide tens of millions of quality eggs both within and outside of the spawning season. In **Task 7.4**, no differences were found in gonadal development in captive reared wild and hatchery mullet and after 2 years of culture both groups were immature. The 3 year old grey mullet exhibit sex related growth and gonadal development patterns. The 3-year-old hatchery produced mullet females and males exhibited enhanced gonadal maturation than that in the wild-caught captive-reared fish, probably the outcome of domestication. In **Task 7.5**, mullet egg transport was successful for both short and long term transport with a maximal stocking density of 15,000 eggs l^{-1} and a total seawater volume of 10 l.

WP 2 Reproduction & Genetics – meagre

WP No:	2	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Reproduction and Genetics - meagre			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P14. IFREMER	
Lead Scientist preparing the Report (WP leader):	Neil Duncan			
Other Scientists participating:	Juan Manuel Afonso (P2), Costas Tsigenopoulos (P1), Christian Fauvel (P14), Constantinos Mylonas (P1)			

Objectives

1. Evaluate the genetic variation in the available captive broodstocks of meagre (**Completed**),
2. Genetic characterization of fast and slow growers (**Completed**),
3. Development of tools that facilitate the implementation of genetic selection programs (**Completed**),

a. Develop protocols for the paired crossing of breeders with spontaneous spawning (**Completed**),
b. Describe sperm quality and cryopreservation techniques (**Completed**),
c. Develop *in vitro* fertilization protocols to provide planned genetic crosses (**Completed**),
d. Develop a set of SNP markers for genetic selection and stock characterisation (**Completed**).

Summary of work reported in the previous Reporting Period (1-12 Mo):

All tasks planned for the 1st Reporting Period have started and made good progress. **Task 2.1, Evaluation of the genetic variation in captive meagre broodstocks** has been completed with the associated Deliverable D2.2. Over 435 breeders were sampled from broodstocks in 13 centres and 7 countries and studied with 18 microsatellite markers (STRI & SRTS). The broodstocks originated from 3 populations or groups. One broodstock that is held in Turkey was uniquely different from all other broodstocks. The other 12 broodstocks originated from two populations or groups. As a whole, the combined broodstocks appear to have sufficient variation for breeding program(s). However, the majority of broodstocks appear to require an increase in the number of families for a breeding program. New families or stocks could be obtained between centres or from the wild. However, care is required as many broodstocks had the same population of origin and sample size was small from each broodstock. Further information on number of families available in each broodstock is needed to define more precisely the needs to establish breeding program(s). **Task 2.2, The development of protocols for paired crossing in spontaneous spawning** has shown that successful paired spontaneous spawning is possible. Efficacy of spawning was 58%, with 26 pairs spawned out of a total of 45 and the majority of these pairs produced >100,000 hatching eggs. Four pairs that were induced repeatedly each week spawned multiple times for up to 17 weeks with high (>85%) mean hatching and larval survival 5 days post hatch. **Task 2.5, Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers** initiated with the sampling of 16 individual meagre coming from 5 families (formed by 10 breeders). High quality RNA has been extracted from muscle and liver and sent for sequencing. All other tasks are programmed for later in the project as specified in the DOW.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the 2nd Reporting Period all tasks have again progressed. The three deliverables that were due during the first and second reporting periods have been submitted. **Task 2.2, The development of protocols for paired crossing in spontaneous spawning** has been completed, with the associated deliverable D2.3. A total of five experiments were completed for the task. The efficacy of spawning pairs with male rotation was high (76%) and a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success. However, a decline in spawning success that was observed with repeated induced spawning with male rotation was a possible drawback that is highlighted in the deliverable. Work in **Task 2.3, Description of sperm characteristics and cryopreservation methods,** has been completed using ImageJ CASA system to describe meagre sperm characteristics. Sperm motility was approximately 60% at 10 sec after activation, and both speed and percentage motility declined to 0 in approximately 60 seconds. Different mediums tested to use for sperm storage and cryopreservation techniques already used for European seabass (*Dicentrarchus labrax*) were modified to provide protocols for meagre sperm. For **Task 2.4, Development of *in vitro* fertilization methods for planned crosses,** trials have been made to induce ovulation, and sperm management protocols from **Task 2.3** have been used for *in vitro* fertilisation. More work is needed, but initial results indicate that ova stripped 39 hours after the application of GnRHa to induce ovulation were successfully fertilised with sperm stored in a modified Leibovitz medium (identified in **Task 2.3**). **Task 2.5, Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers** is advancing towards completion. During the second reporting period, Deliverable D2.1 was completed and submitted. The DNA has been extracted for 400 meagre that were grown to harvest size with varying growth rates. The genetic marker library from Deliverable D2.1 is being used to genetically characterize fast and slow growers within the population. There has been little deviation in the planned tasks and the remaining three deliverables are progressing to be completed as specified in the DOW.

Summary of progress towards objectives (31-48 Mo):

All the work for WP2 has been completed. During the 3rd Reporting Period all remaining tasks were completed and associated deliverables were submitted. **Task 2.3, Description of sperm characteristics and cryopreservation methods,** was completed and **D2.6 Description of sperm characteristics and cryopreservation protocol of meagre sperm** was submitted. Meagre sperm had a mean sperm density of $3.21 \cdot 10^{10}$ ± 1.18 spzoa/mL, motility duration was 1:43± 0:18 min, mean percentage of initial motility of spermatozoa was 48.17 ± 2.80 and the mean initial VAP was 90.69 ± 5.76 μm/ s. Different sperm storage methods chilled and cryopreservation techniques were modified to provide protocols for meagre sperm. **Task 2.4, Development of *in vitro* fertilization methods for planned crosses,** was completed and **D2.7 Protocol for the strip spawning of meagre females and in vitro fertilisation** was submitted. A total of 24 different strip spawning trails were completed and tests were made to determine the sperm to egg ratio and period of viability of stripped eggs. The optimal period for stripping eggs was 38-39 hours after the application of GnRHa and a ratio of 200,000 sperm to egg was recommended. **Task 2.5, Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers** was completed and the two associated deliverables submitted, **D2.4 Construction of a genetic linkage map in meagre** and **D2.5 Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre through QTL mapping.** The work characterised for the first time the muscle and liver transcriptome and constructed the first genetic linkage map for meagre using the ddRAD methodology which identified 731 markers organized in 27 linkage groups. The model mapping from the two larger families identified 5 QTLs on only two LGs which exhibited significant evidence of linkage at the genome level and multiple QTLs appeared to be related to differences in body weight and length.

Details for each Task

Task 2.1 Evaluation of the genetic variation in captive meagre broodstocks (led by FCPCT, Juan Manuel Afonso).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 2.2 Genetic characterization of different meagre captive broodstocks and evaluation of available variability.***

Task 2.2 Development of protocols for paired crossing in spontaneous spawning (led by IRTA, Neil Duncan).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 2.3 Development of protocols for paired crossing in spontaneous spawning.***

Task 2.3 Description of sperm characteristics and cryopreservation methods (led by IFREMER, Christian Fauvel).

This task has been completed during the 3rd reporting period and the full description of the work and results have been since submitted as "**D2.6 Description of sperm characteristics and cryopreservation protocol of meagre sperm**".

The characteristics of meagre sperm were described, mean sperm density during the experimental period was $3.21 \cdot 10^{10}$ ± 1.18 spzoa/mL, the mean motility duration was 1:43± 0:18 min, the mean percentage of initial motility of spermatozoa was 48.17 ± 2.80 and the mean initial VAP was 90.69 ± 5.76 µm/ s. Percentage of motility was maintained without variations until 55 s after activation and after which a quick decrease was observed with time until the movement ceased. In contrast, VAP values quickly decreased with time, so that initial values only remained without variation until 35 s after activation (**Fig. 2.3.1**). Different storage methods, fresh (control), chilled and cryopreserved were tested. There were no significant differences between the duration of motility nor initial VAP values from sperm subject to different kinds of storage, but initial motility of sperm stored in Leibovitz for 24 h was significantly the lowest (**Table 2.4.1**). Out of 4 experiments on artificial fertilization using both fresh and frozen samples of sperm, only one showed significant drop of fertility due to cryopreservation while in any case, chilled conservation did not allow to maintain high fertility until 24h.

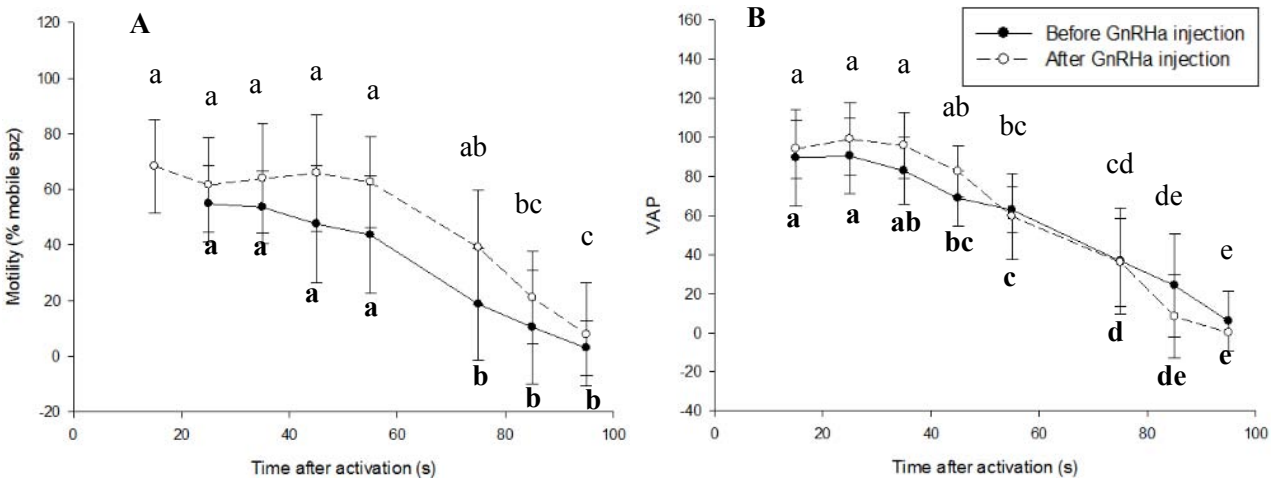

Figure 2.3.1 Effect of GnRHa treatment on (A) percentage of mobile spermatozoa (%) and (B) Average Path Velocity of spermatozoa (µm/ s) during the experimental period when data before and after treatment were available. Data are expressed as mean ± standard deviation. Different lowercase bold letters mean significant differences with time after activation before GnRHa injection. Different lowercase letters mean significant differences with time after activation after GnRHa injection. Data was from five different males with three repeated measures on different weeks for each male (n=15).

Table 2.4.1. Weekly sperm quality parameters of sperm samples used in experiment 3 (comparison of fertilisation by fresh, cryopreserved sperm and sperm stored in Leibovitz for 24h). Data from 5 males was used and values are expressed as mean ± SD. Different lowercase letters mean significant differences between each parameter from different storage types in the same week. Different capital letters mean significant differences between each parameter from fresh sperm used in fertilisation among three weeks (read in columns).

Experiment	Sperm storage	Total duration (s)	Initial motility (%)	Initial VAP (µm/s
3a	Fresh for storage	1.66 ± 0.59^{a}	55.16 ± 13.81^{a}	109.37 ± 15.00^{a}
	Cryopreserved	1.29 ± 0.32^{a}	50.24 ± 15.90^{a}	93.73 ± 15.59^{ab}
	Chilled Stored for 24 h	2.07 ± 0.51^{a}	8.91 ± 6.79^{b}	68.00 ± 3.73^{b}
	Fresh for fertilisation	**1.82 ± 0.29$^{a\ A}$**	**80.51 ± 13.05$^{c\ B}$**	**81.29 ± 12.72$^{b\ A}$**
3b	Fresh for storage	1.77 ± 0.04^{a}	42.66 ± 3.78^{a}	72.28 ± 11.18^{ab}
	Cryopreserved	1.07 ± 0.31^{b}	24.16 ± 18.32^{ab}	50.26 ± 8.09^{a}
	Chilled Stored for 24 h	0.61 ± 0.04^{c}	2.60 ± 1.00^{b}	55.72 ± 5.1^{a}
	Fresh for fertilisation	**1.34 ± 0.08$^{b\ B}$**	**48.80 ± 8.08$^{a\ A}$**	**83.65 ± 5.38$^{b\ A}$**
3c	Fresh for fertilisation	**0.60 ± 0.09 C**	**70.29 ± 7.30^{AB}**	**260.15 ± 7.77 B**

Task 2.4 Development of in vitro fertilization methods for planned crosses (led by IRTA, Neil Duncan).

This task has been completed during the 3rd reporting period and the full description of the work and results have been since submitted as "**D2.7 Protocol for the strip spawning of meagre females and in vitro fertilisation**".

Task 2.4, used the sperm management protocols developed in ***Deliverable D2.6 Description of sperm characteristics and cryopreservation protocol of meagre sperm*** to obtain high quality sperm, which was stored for a short time (1-7 hours) in modified Leibovitz medium until eggs were obtained from the female meagre. Females with advanced stages of maturity were induced to ovulate with a single 15 µg/kg GnRHa injection. The injections were applied at 20:00-22:00 hours and the females held separate from males in darkness until being checked for ovulation. Checks for ovulation were made every 2.5 hours from 35 to 45 hours post GnRHa injection. When ovulated eggs were obtained, *in vitro* fertilisation was made and egg quality assessed by determining the percentage of developing eggs. An injection of GnRHa was also applied to males, and sperm requirements and quality were assessed. Ratios of sperm to eggs were tested from approximately 3,000 to 500,000 sperm per eggs. Ovulated eggs were observed from 35 hours onwards. Optimal eggs quality was observed at 38-39 hours after the GnRHa injection (**Fig. 2.4.1**).

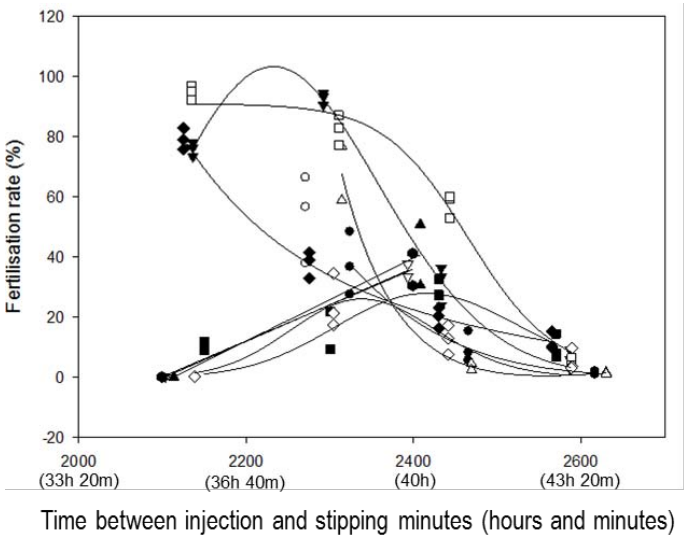

Figure 2.4.1. The fertilisation rate (%) of each batch of eggs stripped from each female meagre (*Argyrosomus regius*) at different times after the GnRHa (15 µg/kg) injection. n = 6 females and 11 ovulations.

From 35 to 38-39 h there was a slight increase in eggs quality and the ease with which eggs could be stripped indicating that from 35-38 hours there was a possibility that eggs were not fully ovulated. After 38-39 hours, there was a decline in eggs quality to 43 – 44 hours. Sperm quality was maintained without decline for up to 7 hours in Leibovitz medium and sperm quality did not appear to affect fertilisation success. The *in vitro* fertilisation was made by rapidly mixing eggs, sperm and seawater at the same time to ensure sperm were activated and in contact with eggs during the first 30 seconds after activation, which was identified as the optimal period for fertilisation in ***Deliverable D2.6***. The optimal ratio of sperm to eggs to obtain high percentage of fertilisation was above 200,000 sperm per egg (**Fig. 2.4.2**). The protocol was successfully used in a large factorial cross of 120 *in vitro* fertilisations using either fresh or cryopreserved sperm.

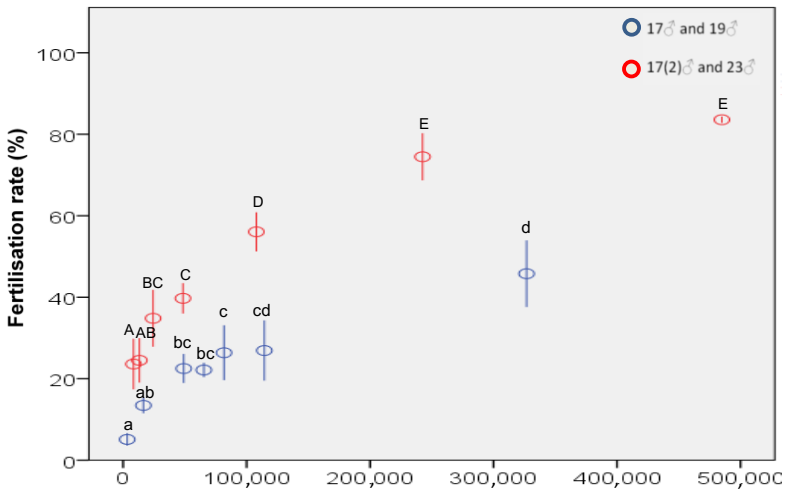

Figure 2.4.2. Mean fertilisation rates of meagre (*Argyrosomus regius*) eggs fertilised *in vitro* at different sperm: egg ratios. The horizontal error bars indicate the standard deviation of mean. Significant differences are indicated by different letters (capital letters for males 17♂ and 19♂, and lowercase letters for males 17(2)♂ and 23♂) ($P < 0.05$).

Task 2.5 Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers (led by HCMR, Costas Tsigenopoulos).

This task has been completed during the 3rd reporting period and the full description of the work and results have been since submitted as "**D2.4 Construction of a genetic linkage map in meagre**" and "**D2.5 Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre through QTL mapping**".

D2.4 Construction of a genetic linkage map in meagre: four hundred meagre fish were sampled from a large fish-cage that formed part of a commercial farm site on the Spanish coast in the community of Valencia. The juveniles were from the largest grade of fish that came from the same group of spawns collected from a broodstock that contained 19 breeders (8 females and 11 males) that were injected with GnRHa to induce spawning. Total length and weight was measured for all 400 sampled fish. All fish were genotyped in order to infer parentage allocations which were finally based on nine loci; single parentage assignment (match) was successfully described for 345 of those fish (86.25%) and the rest had multiple matches. Fish belonged to 17 families (out of the 88 theoretically expected). Only 5 out of the 8 females were identified as probable parents of the offspring; likewise, six out of the 11 males were identified as probable parents of the offspring.

Then, the ddRAD (double-digest restriction associated DNA) library preparation protocol was followed to construct a library and sequence it. Sequencing of the constructed ddRAD libraries yielded 765,712,194 and 788,654,246 total raw reads. Following demultiplexing and quality control we obtained 542,447,568 and 566,148,132 reads for the two libraries respectively. For parental samples, we obtained ~6 million reads on average, while for progeny samples we got ~4 million reads on average and after following STACKS analysis, we obtained in total 87,522 ddRAD loci in the parental samples which constituted the ddRAD catalogue. The progeny contained on average 19,402 ddRAD loci, and the number of SNPs identified seems to be proportional to the sequencing depth (reads sequenced per fish).

Following the ddRAD analysis in STACKS, the two largest families were chosen for building the linkage map. Following filtering of the markers that deviated significantly from the Mendelian ratios in total 950 markers were maternally informative, while 929 were paternally informative summing up to 1,008 total markers used to build a sex-averaged linkage map. The constructed linkage map included 731 markers organised in 27 linkage groups (**Fig. 2.5.1**), that means 3 LGs-chromosomes more than the haploid number determined in the karyotype of this species; it is anticipated that when more markers are added, some LGs will merge into larger ones, and the number of LGs should eventually match the number of chromosomes. The LGs range from 14,001 to 67,333 cM in length and the whole map spans 1,158.299 cM. The loci included in the LGs range from 11 to 54 ddRAD loci per LG.

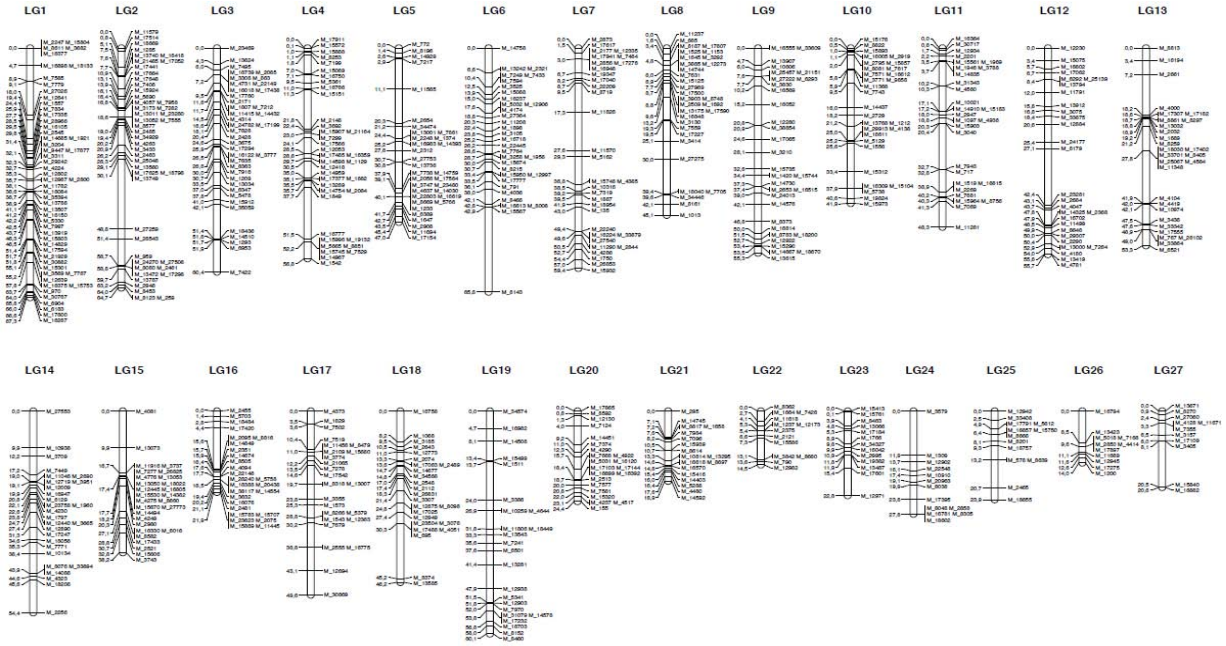

Figure 2.5.1. The genetic linkage map of meagre (*Argyrosomus regius*)

The mapped loci were used for a comparative genomic analysis against the genome of European seabass. The similarity search revealed that more than one third (36.66%) and 268 out of 731 loci have a homologous region in the genome of European seabass. The homologous loci revealed extensive conservation of synteny (regions or fragments are conserved of order within two sets of chromosomes that are being compared with each other) between the two species. Based on the comparison of the meagre linkage map against the European seabass genome map, a reduction of the number of LGs to 24 is possible. Fusion of LG20 with LG24 is suggested since both map to seabass LG8. Likewise, LG22 should join LG26, as markers from both LGs are found on the same seabass LG6. Lastly, the LG25 might not be supported since it comprises markers from different seabass LGs and could potentially in the future disintegrate when linkage map are enriched.

D2.5 Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre through QTL mapping: Average weight for all 400 fish sampled above was 2.35 kg (ranging from 1.33 to 3.71 kg) and average total length 62.3 cm (ranging from 53.0 to 74.0 cm). The 15 families were ranked according to their median weight and body length (**Fig. 2.5.2**). From the above fish, 232 fish from the five families that exhibited the greatest phenotypic variation together with their seven breeders (three females and four males5) were selected for the construction of two above mentioned ddRAD libraries. For the QTL mapping purposes, the two bigger families were used; 731 SNP markers spread on 27 LGs that were common to both families were used for a genome wide QTL scan in meagre. The three other smaller families were used to crosscheck and validate the existence of certain SNP combinations that could explain hereditary variation in BW and TL.

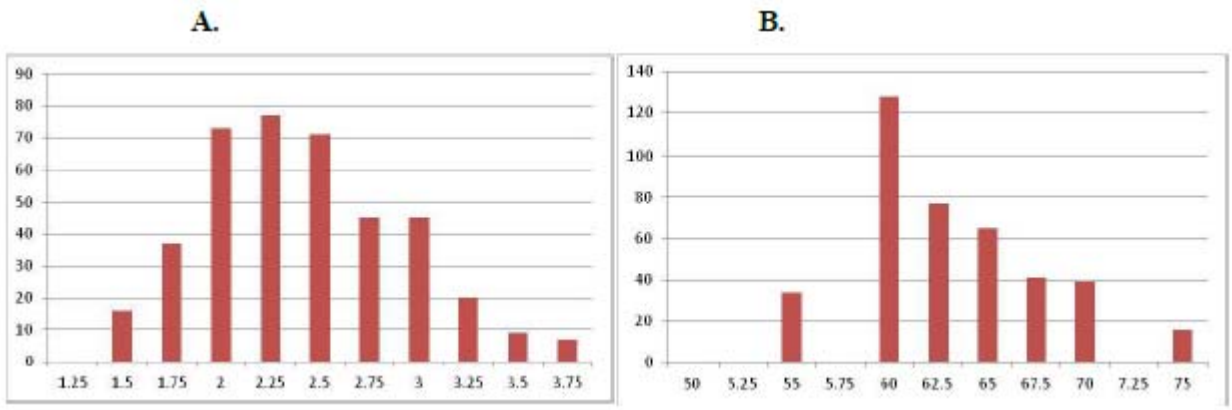

Figure 2.5.1. A. Weight (in kg) and B. total length (in cm) of the 400 meagre fish sampled.

The genome-wide scan revealed two areas containing QTL on LG 11 and 20 for both traits under investigation. For both traits the likely position of the QTL was the same. The highest test-statistic (Log Likelihood Ratio) was observed at the beginning of LG 11 followed by the LG 20 (18.745 to 22.042cM). In **Figure 2.5.3**, the likelihood ratios over all the LGs are presented

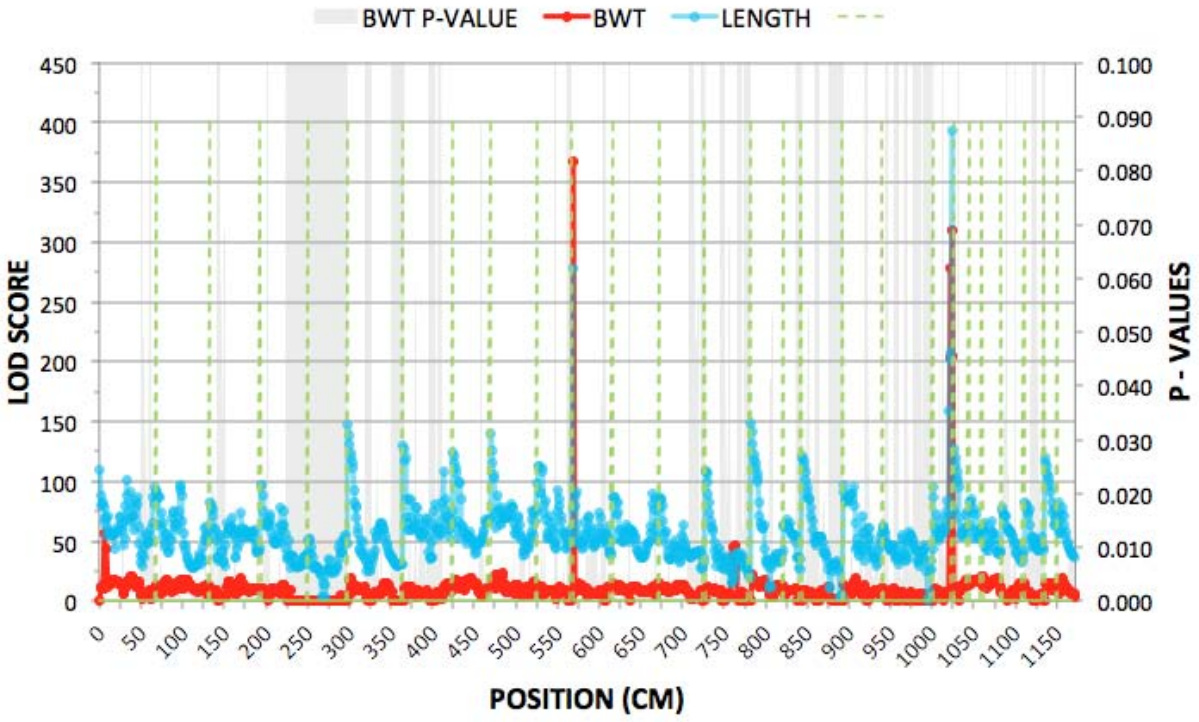

Figure 2.5.3. Likelihood Ratio scores, every cM, from a QTL genome scan. The green dashed lines are for separating the different linkage groups. The gray shaded areas indicate those areas where the likelihood ratio is not significant.

Therefore, we completed a genome scan for QTLs that affect body weight (BW) and total length (TL) in meagre from five full-sib families using the markers developed for the linkage map of meagre distributed across 27 linkage groups. Model mapping from the two larger families identified 5 QTLs on only two LGs (11 & 20), which exhibited significant evidence of linkage at the genome level ($P < 0.05$). Multiple QTLs on LG20 seem to affect both BW and TL and are located at close positions, suggesting that the same genetic factors may control variability in these traits and are expected to be of great value in future Marker Assisted Selection (MAS) programmes.

Deviations from Annex I and their impact:
No deviations existed.

WP 3 Reproduction & Genetics – greater amberjack

WP No:	3	**WP Lead beneficiary:**		P13. UNIBA
WP Title (from DOW):	Reproduction and Genetics – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P4. IOLR	P8. IEO
P14. IFREMER	P15. ULL	P23. ARGO	P40. GMF	
Lead Scientist preparing the Report (WP leader):	Aldo Corriero			
Other Scientists participating:	Constantinos Mylonas (P1), Ioannis Fakriadis (P1), Hipolito Fernandez Palacios (P2), Hanna Rosenfeld (P4), Salvador Jerez Herrera (P8), Rosa Zupa (P13), Crysovalentinos Pousis (P13), Christian Fauvel (P14), Covadonga Rodriguez (P15), Tasos Raftopoulos (P23), Kalliopi Tsakoniti (P40),			

Objectives

1. Describe the endocrine control of reproduction in captive broodstocks, and the nutritional status of fish during the reproductive season,
2. Assess reproductive potential of wild vs. captive amberjack broodstocks and identify possible reproductive/metabolic dysfunctions during gametogenesis,
3. Develop spawning induction methods for captive-reared and F1 broodstocks of both the Mediterranean and Atlantic stocks,
4. Apply the developed spawning induction methods for broodstocks maintained in cages, and examine the efficiency of an egg collector to obtain fertilized eggs,
5. Develop a Computer Assisted Sperm Analysis method (CASA) for the evaluation of greater amberjack sperm during the reproductive season, and evaluate the possible effects of captivity.

Summary of work reported in the previous Reporting Period (1-12 Mo):

In **Task 3.1 Description of the reproductive cycle of greater amberjack**, wild-caught broodstock was established in ITTICAL, but after two months an infestation of the parasite *Amylodinium ocellatum* caused a massive mortality. Consequently, it was decided to move the sampling activity of captive-reared greater amberjack to ARGO. Sampling of wild greater amberjack started in Y1 with 17 individuals caught around Lampedusa (Pelagie Islands, Sicily, Italy). Wild-caught fish were acclimatized to captivity at ARGO (tanks) and HCMR (tanks and cage). Other wild-caught individuals were maintained at ITTICAL, FORKYS and Galaxidi Marine Farms (GMF, a collaborator from outside the consortium).

In **Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean,** preliminary experiments by using a single dose of GnRHa controlled-release delivery systems (implants), resulted in the production of eggs for larval rearing experiments and provided valuable information for the further development of spawning induction protocols.

Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic. Greater amberjack of the Atlantic stock were kept at FCPCT in order to investigate the occurrence of (a) natural spawning, (b) spawning induced by GnRHa injection and (c) spawning induced by GnRHa controlled-release delivery systems (implants). Naturally spawning individuals produced the highest amount of eggs compared to the treated ones. Moreover, eggs obtained by natural spawning showed

the highest percentage of fertilization, viability at 24 hours and hatching, and provided the highest percentage of larval survival at 4 and 8 days.

In **Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic**, a greater amberjack broodstock of the Atlantic stock born in captivity (F1 generation) at IEO was divided between an outdoor 500-m^3 raceway and a 50-m^3 circular tank. The fish were hormonally-induced for spawning. The broodstock in the raceway tank spawned from August till September whereas no spawning event was recorded in the circular tank.

In **Task 3.5 Spawning induction of greater amberjack and egg collection in cages**, egg collection devices were mounted in cages of 40-m perimeter at HCMR, ARGO and Galaxidi Marine Farms (GMF), which is an SME not in the DIVERSIFY consortium, but which contributes its stock and facilities for our experiments. The egg collector consisted of two sections, a lower section starting at about 30 cm above the water line and going down to about 3.5 m in depth, and an upper section hanging from the rails of the cage and draping down the cage over the lower section. Following the spawning induction with GnRHa implants, egg collection was successful but limited in numbers. Presumably, most of eggs were swept outside of the cage by the currents, before they could rise to the surface where the collector would have prevented them from escaping.

Summary of work reported in the previous Reporting Period (13-30 Mo):

Major improvements of our understanding of confinement effects on greater amberjack reproductive activity were obtained during the second reporting period. Moreover, during this period, large-scale egg productions were obtained both thanks to spontaneous spawning and after hormonal induction trials of the different broodstocks located in the Mediterranean Sea and in the eastern Atlantic, providing important results for the setup of optimized spawning induction protocols. Large amounts of eggs were also obtained after hormonal treatment of greater amberjack hatchery-produced generation. During Mo 31 (although outside the scope of this report, and the results will be reported fully in the 3rd Periodic Report) we had great success in inducing spawning of three broodstocks maintained in sea cages, and obtained a large amount of eggs (~50 million), which allowed the production for the first time, of a large number of fingerlings for the implementation of grow out studies in the Mediterranean region (See also a brief mention in WP 15 larval husbandry – greater amberjack.

In **Task 3.1 Description of the reproductive cycle of greater amberjack**, sampling of wild and captive-reared greater amberjack was accomplished and the comparative analyses of fish reproductive and nutritional state were carried out. Results showed that a severe impairment of gametogenesis occurred in captive-reared greater amberjack that were manipulated a few times during the reproductive season, since these fish exhibited poor gonadal development, low pituitary gonadotropin expression, low gonadotropin and sex steroid plasma concentrations, extensive atresia of vitellogenic follicles and high level of male germ cell apoptosis. Moreover, gonads, liver and muscle of captive reared fish showed lower content of specific lipid classes and fatty acids compared to their wild counterpart.

In **Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean,** it was observed that greater amberjack caught from the wild and confined in captivity undergo gametogenesis and complete vitellogenesis, but necessitate hormonal therapies to induce oocyte maturation and spawning. The applied GnRHa treatments were more effective in females maintained in cages during gametogenesis and moved to tanks after the hormonal therapy, with a better fecundity and fertilization success compared to females maintained in tanks throughout the year.

In **Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic**, comparative trials between spontaneous spawning and spawning induced by GnRHa injections were performed, showing better performances of natural spawning in terms of fertilization and larval survival.

In **Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic**, excellent progresses were made with hatchery-produced greater amberjack (F1

generation) induced spawning. Repeated spawning for 3 months and almost 15 million eggs were obtained after treatment with three consecutive GnRHa implants.

In **Task 3.5 Spawning induction of greater amberjack and egg collection in cages**, small amount of eggs was collected in cages equipped with the *ad hoc* designed egg collector probably due to low buoyancy of eggs immediately upon spawning and loss of the eggs through the bottom and side of the cage, before eggs could be trapped by the collector system. The method is not performing adequate yet, and further modifications and improvements are necessary before it can be recommended for commercial use.

Summary of progress towards objectives (31-48 Mo):

Important life history traits of wild greater amberjack, such as growth (mean length-at-age) and size/age at first sexual maturity were determined. The reproductive cycle of greater amberjack reared in a sea cage in the Mediterranean Sea was described and severe reproductive dysfunctions were identified, which involved a reduced pituitary capacity to synthesize and secrete gonadotropins, with consequent reduction of sex steroids circulating levels. In females, the hormonal dysfunction finally resulted in failure of oocytes to undergo maturation after completion of the vitellogenic process. Males showed a reduced capacity of spermatogonia to proceed toward meiosis, an increase of germ cell apoptosis and an early cessation of the spermatogenic activity. The observed spermatogenesis alterations finally resulted in the production of low quality sperm. However, another broodstock was maintained under identical conditions in the same and it reached advanced stages of gametogenesis to be able to be induced to spawn and produce fertilized eggs whose quality was equivalent to those of wild specimens. We suppose that the repeated sampling operations in the rearing cage might have played a major role in the observed reproductive dysfunction, thus underlying the extreme susceptibility of this species to the handling stress and the need for a careful management of greater amberjack broodstocks.

During the 3rd reporting period, large-scale egg productions were obtained both thanks to spontaneous spawning and after hormonal induction trials in the Mediterranean Sea and in the eastern Atlantic, providing important results for the setup of optimized spawning induction protocols.

In the Mediterranean, GnRHa administration through EVAc implants proved to be more effective compared to injections in terms of relative and total fecundity. Between the two investigated GnRHa doses, i.e. 25 and 75 μg kg^{-1} body weight, the former determined the best results in term of fertilization success. The best response to GnRHa treatments was obtained when the hormone was administrated between the end of May and the first week of June.

In the eastern Atlantic, hatchery produced greater amberjack (F1 generation) were able to finalize vitellogenesis and spermiation, and, after treatment with 75 μg/kg GnRHa, they underwent repeated spawning for 4 months with a total production of almost 22 million eggs. In addition, during the spawning season 2017, from a single untreated female, more than 25 million eggs were produced during 21 spontaneous spawning events.

Details for each Task

Task 3.1 Description of the reproductive cycle of greater amberjack (led by UNIBA, Aldo Corriero).

This Task has been accomplished and the full description of the work and results is provided in the following submitted Deliverables: D3.1 Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor); D3.2 Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack; D3.3 Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating and apoptotic germ cells, vitellogenin accumulation, yolk content in the oocytes and nutritional status; D3.4 Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of greater amberjack sperm; D3.5 Description of the process of

oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones and Vg, and (e) nutritional status; D3.6 Description of the process of spermatogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of testicular development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones, (e) proliferation and apoptosis of germ cells, (f) sperm quality, (g) fish nutritional status.

The work carried out within this task has resulted in three publications to international scientific journals:

Pousis, C., Mylonas, C.C., De Virgilio, C., Gadaleta, G., Santamaria, N., Passantino, L., Zupa, R., Papadaki, M., Fakriadis, I., Ferreri, R., and Corriero, A. (2018). The observed oogenesis impairment in greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is not related to an insufficient liver transcription or oocyte uptake of vitellogenin. Aquaculture Research 49, 243-252.

Zupa, R., Fauvel, C., Mylonas, C.C., Pousis, C., Santamaria, N., Papadaki, M., Fakriadis, I., Cicirelli, V., Mangano, S., Passantino, L., Lacalandra, G. M., and Corriero, A. (2017). Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack *Seriola dumerili* (Risso, 1810) Journal of Animal Science *95*, 4085-4100

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., and Corriero, A. (2017). Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLoS ONE 12, e0169645.

3.1.1 Materials and methods

3.1.1.1 Age, growth and sexual maturity

For the analyses of age, growth and sexual maturity, 5 juvenile greater amberjack sampled during the 2nd Reporting Period, 18 juveniles sampled during the 3rd Reporting Period (**Table 3.1.1**) as well as all the wild adults sampled during the 1st and the 2nd Reporting Periods (D3.3) were used. The sex of these fish was determined by macroscopic observation of the gonads: 8 were females, 10 males and 5 were classified as "Indeterminate" because their sex was not discernible by simple visual inspection of the gonads. Fish biometric data (fork length, FL in cm; body mass, BM in kg; gonad mass, GM in g) were recorded, gonado-somatic index was calculated (GSI=100GM/BM) and gonads and scales were sampled for reproductive state assessment and age determination.

Scales were removed from the skin taken from a body area between the pectoral and first dorsal fin, rinsed in tap water and in 70% ethanol and finally placed between two microscope slides. Subsequently, they were observed with a binocular lens microscope Wild M3C (Leitz, Heerbrugg, Switzerland) under transmitted light, connected through a digital camera DC 300 (Leica, Wetzlar, Germany) to the image analyser Quantiment 500 W (Leica, Wetzlar, Germany). The age of the fish was then estimated based on the number of annuli counted on their scales (Meunier, 2002).

Estimate of greater amberjack theoretical growth in length was obtained by fitting the von Bertalanffy growth model (Bertalanffy von, 1938) to the mean lengths at estimated age and the theoretical longevity of the species was calculated using Pauly and Munro's (1984) formula: AGE MAX = 3/k.

For the estimate of female first sexual maturity each specimen was classified as immature or mature on the basis of the histological appearance of the gonads. For this purpose, gonad samples were fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were than stained with haematoxylin-eosin, and observed under light microscope. Females were classified as mature if their ovaries contained: a) late vitellogenesis and/or more advanced oocyte stages; b) primary growth oocytes along with signs of previous reproductive activity (atretic vitellogenic follicles and/or post-ovulatory follicles). Males were classified as mature if their testes showed all the stages of spermatogenesis and abundant luminal spermatozoa. Body length at median sexual maturity (L_{50}) was estimated for female greater amberjack by fitting a logistic function to the fraction of mature fish per 5 cm FL intervals by nonlinear regression. L_{50} was defined as the theoretical length in which 50% of the specimens were mature.

Table 3.1.1. Biometric data and estimated age of wild juvenile greater amberjack sampled around the Pelagie Islands (Italy) during the reproductive seasons 2015 and 2016.

Fish origin	Sampling Date	Sex	FL (cm)	BM (kg)	GM (g)	GSI (%)	Estimated Age (years)
wild	29/06/2015	Indeterminate	35.0	1.0	nd	nd	1
		Indeterminate	35.0	1.0	nd	nd	1
		Indeterminate	36.0	1.0	nd	nd	1
		Indeterminate	38.0	1.1	nd	nd	1
		Indeterminate	39.0	1.2	nd	nd	1
wild	06/06/2016	f	59.0	3.09	6	0.19	2
		f	60.0	3.53	5	0.14	2
		f	66.0	3.89	10	0.26	2
		f	69.0	4.95	11	0.22	2
		f	69.0	4.20	15	0.36	2
		m	58.0	2.99	3	0.10	2
		m	61.0	3.38	4	0.12	2
		m	65.0	3.89	2	0.05	2
		m	68.5	4.85	9	0.19	2
		m	71.5	5.10	17	0.33	2
wild	10/06/2016	f	86.0	8.69	43	0.49	3
		f	87.0	8.88	27	0.30	3
		f	87.0	9.17	288	3.14	3
		m	80.0	6.69	67	1.00	3
		m	84.0	8.58	182	2.12	3
		m	84.0	7.65	73	0.95	3
		m	88.0	9.37	220	2.35	3
		m	89.0	10.40	194	1.87	3

BM = body mass; nd = not determined; FL = fork length; GM = gonad mass; GSI = gonado-somatic index.

3.1.1.2 Reproductive cycle and reproductive dysfunctions of captive-reared greater amberjack

For the study of the reproductive cycle of captive-reared greater amberjack, 12 female and 12 male adults sampled during the 2nd Reporting Period were used (**Table 3.1.2**). The gonado-somatic index was calculated and the reproductive state was histologically evaluated as described in D3.5 and D3.6. Proliferating and apoptotic germ cells were identified through the immunohistochemical detection of Proliferating Cell Nuclear Antigen (PCNA) and the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method, respectively. The density of anti-PCNA and TUNEL positive cells were determined through a computerized image analysis system as described in D3.6.

Pituitary gonadotropin gene expression levels were analysed through quantitative real-time polymerase chain reaction (qPCR); pituitary and plasma LH levels were measured using the heterologous ELISA developed for striped bass LH (Mañanós et al., 1997) and validated for the greater amberjack (D3.2); pituitary and plasma FSH levels were measured using an homologous ELISA that was developed in the framework of this study (D3.2); sex steroid and vitellogenin plasma levels were determined using already established enzyme-linked immunoassays (ELISAs). The relative oocyte area occupied by yolk granules was measured in oocytes at early and late stage of vitellogenesis through computerized image analysis of histological sections.

Table 3.1.2. Biometric data of adult greater amberjack reared in sea cages in Salamina (Greece) during the 2nd Reporting Period and analysed for the reproductive cycle description and the identification of reproductive dysfunctions.

Fish origin	Sampling Date	Sex	FL (cm)	BM (kg)	GM (g)
Early Gametogenesis (EARLY)					
captive	24/04/2015	f	87	10	85
		f	96	14	125
		f	97	14	155
		f	100	14	160
		m	92	12	65
		m	94	12	60
		m	94	13	60
		m	101	15	95
Advanced Gametogenesis (ADVANCED)					
captive	04/06/2015	f	97	13	335
		f	97	13	920
		f	101	12	660
		f	106	17	305
		m	90	9	370
		m	97	14	295
		m	98	13	600
		m	103	15	690
Spawning (SPAWNING)					
captive	02/07/2015	f	92	8	95
		f	95	11	135
		f	96	12	130
		f	97	12	140
		m	91	10	70
		m	95	11	155
		m	96	13	140
		m	96	12	130

BM = body mass; FL = fork length; GM = gonad mass.

3.1.1.3 Egg composition

Samples of eggs produced by the greater amberjack broodstock reared at P23.ARGO within Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean, were analysed for the determination of moisture, total lipid (TL), lipid class (LC) composition, fatty acid profiles and carotenoids as described in D3.5.

3.1.1.4 Sperm quality

Sperm quality analyses were carried out at the sampling site (P23. ARGO), using a microscope (Nikon Eclipse 50i, Japan) equipped with a video camera (SONY SSC-DC58AP, Japan) recording 25 frames per second (FPS). Spermatozoa concentration, sperm motility, spermatozoa ATP content were analysed as described in D3.4.

3.1.1.5 Nutritional state

Fish body condition was assessed through the Fulton's condition factor ($K = BM\ 100/FL^3$). The analytical determination of leptin plasma levels could not be accomplished because the obtained antibodies exhibited high specificity to fish and human recombinant leptin but they failed to detect greater amberjack leptin

extracted from liver samples (D3.2). Liver leptin transcript levels were analysed through qPCR (D3.3, D3.5 and D3.6).

3.1.1.6 Statistical analysis

Differences in GSI, GTHs and leptin gene expression, as well as GTHs and sex steroid plasma concentrations mean values between specimens sampled in consecutive phases of the reproductive cycle were assessed by a two tailed Student's t-test. Prior to the Student's t-test, the raw data of GSI and apoptotic surfaces were arcsine-transformed, as appropriate with proportions (Sokal and Rohlf, 1981). Differences in the sperm quality indexes among sampling phases were assessed either by ANOVA (after angular transformation in the case of % of motile spermatozoa), or by nested design ANOVA (in the case of sperm velocity where individual spermatozoa performances were taken into account for each male at the different sampling periods). Means were compared using Duncan's New Multiple Range (DNMR) post hoc test.

Fulton's condition factor (K) was compared with that of wild specimens sampled for the study of the reproductive cycle of the wild population (D3.3) by two tailed Student's t-test. All the results are presented as means ± SE; the statistical probability significance was established at the $P \leq 0.05$ level.

3.1.2 Results

3.1.2.1 Age, growth and sexual maturity

Among the 23 juvenile greater amberjack sampled for the study of the first sexual maturity, 5 belonged to the age class 1 (all those classified as "Sex Indeterminate"), 10 (5 females and 5 males) to the age class 2 and 8 (3 females and 5 males) to the age class 3 (**Fig. 3.1.1; Table 3.1.1**).

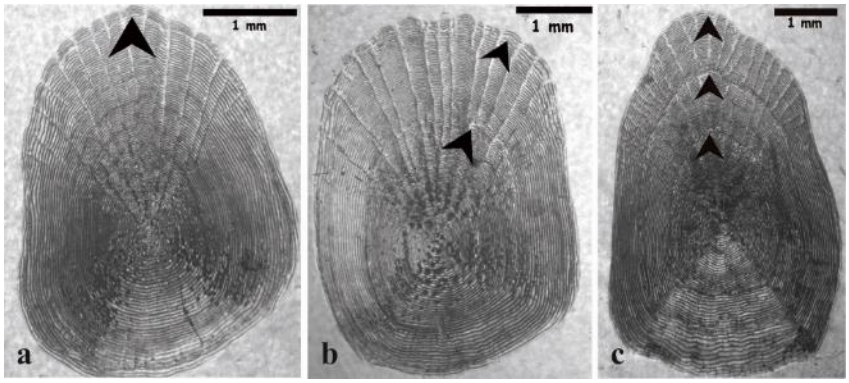

Figure 3.1.1. Images of scales from juvenile wild greater amberjack sampled in the Mediterranean Sea. (a) Sex indeterminate; age 1; 35 cm fork length. (b) Female; age 2; 59 cm fork length. (c) Female; age 3; 87 cm fork length. Magnification bar = 1 mm. Arrowheads: growth marks (annuli).

The parameters of the von Bertalanffy growth equation derived from the observed FLs-at-age of juvenile and adult female greater amberjack were: FL_∞ = 121.5 cm; k = 0.40; t_0 = -0.09 (**Fig. 3.1.2**). The theoretical longevity was 8 years.

The parameters of the von Bertalanffy growth equation derived from the observed FLs-at-age of juvenile and adult male greater amberjack were: FL_∞ = 127.1 cm; k = 0.35; t_0 = -0.05 (**Fig. 3.1.3**). The theoretical longevity was 9 years.

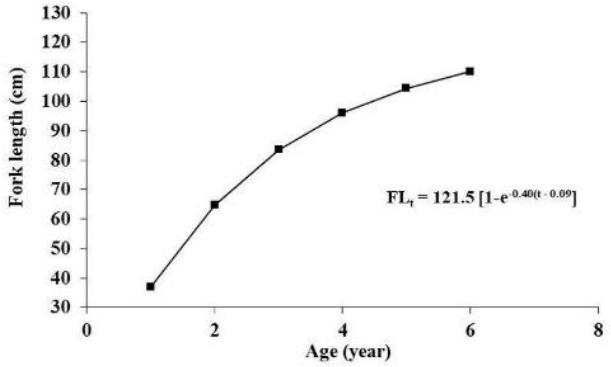

Figure 3.1.2. Von Bertalanffy growth curve of female greater amberjack. FL_t= predicted fork length at age t.

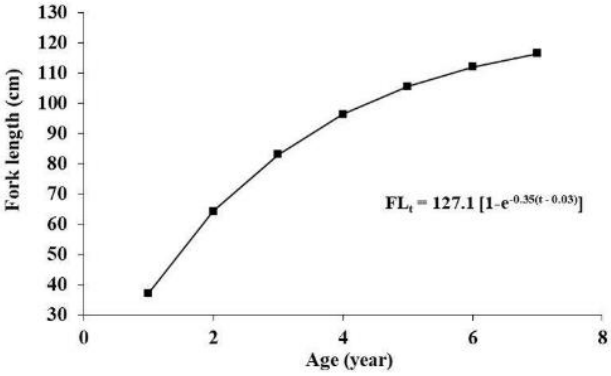

Figure 3.1.3. Von Bertalanffy growth curve of male greater amberjack. FL_t = predicted fork length at age t.

All the females belonging to age class 2 showed only perinucleolar oocytes in their ovaries and were classified as immature. Among the females belonging to age class 3, two had primary growth oocytes and were classified as immature and one showed late vitellogenesis oocytes and it was classified as reproductively active (sexually mature). Based on the above maturity results, the theoretical body length at median sexual maturity (L_{50}) of female greater amberjack was 88.3 cm FL and 100% maturity was reached above 110 cm FL (**Fig. 3.1.4**).

All the males belonging to age class 2 were immature since they showed testes in quiescent/early spermatogenesis stage. All the fish belonging to the age class 3 were classified as mature as their testes were in a histological condition similar to those of adults in advanced spermatogenesis stage, showing all the stages of spermatogenesis and abundant luminal spermatozoa.

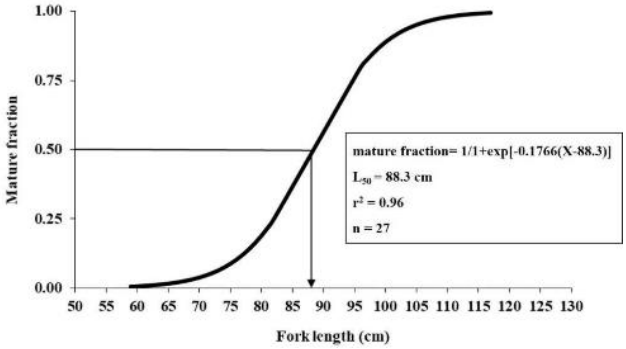

Figure 3.1.4. Fraction of mature female greater amberjack by fork length. Arrow indicates body length at median sexual maturity (L_{50}); n = sample size.

3.1.2.2 Reproductive cycle and reproductive dysfunctions in captive-reared greater amberjack

Females

In the examined ovaries of captive-reared greater amberjack, oogonia along with the following oocyte developmental stages were observed: chromatin-nucleolus, perinucleolar, lipid, cortical alveoli, early vitellogenesis, late vitellogenesis (**Fig. 3.1.5**). Neither hydrated oocytes nor postovulatory follicles were found in any of the examined specimen. A description of female germ cells morphology has been provided in D3.5.

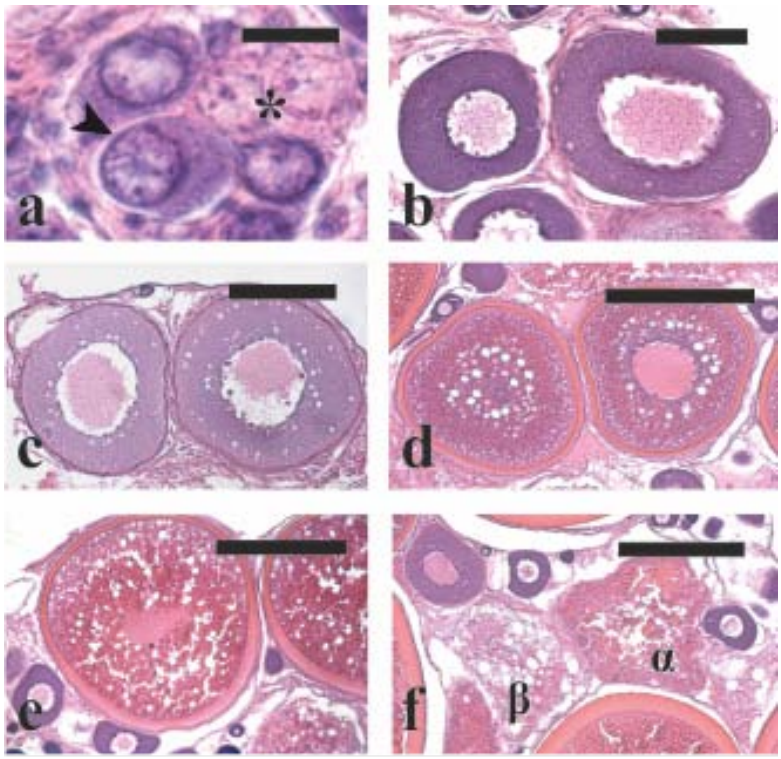

Figure 3.1.5. Micrographs of ovary sections from different captive-reared greater amberjack sampled at P23. ARGO showing oogonia and oocytes in different developmental stages. a) Oogonia (asterisk) and chromatin-nucleolus stage oocytes (arrowhead). b) Perinucleolar stage oocytes. c) Cortical alveoli stage oocytes. d) Early vitellogenic oocytes. e) Late vitellogenic oocyte. f) Alfa (α) and beta (β) atretic vitellogenic follicles. Haematoxylin-eosin staining. Magnification bars = 10 µm in (a), 50 µm in (b), 100 µm in (c), and 200 µm in (d), (e), (f).

Among the four greater amberjack specimens sampled during the EARLY phase (late April), one had ovaries with primary growth oocytes and three showed few early vitellogenic oocytes. In the ADVANCED phase, the four sampled specimens had oocytes at late vitellogenesis and three of them displayed major α atresia (> 50% of vitellogenic oocytes were in α atresia). In the SPAWNING phase, among the four sampled fish, three showed ovaries with late vitellogenic oocytes undergoing extensive atresia and one showed only perinucleolar oocytes, indicating that all these animals were in a regressed condition.

Gonado-somatic index (**Table 3.1.3**) changed according to the oocyte maturation stage. Increasing GSI values were found in individuals showing primary growth, early vitellogenesis and late vitellogenesis stage oocytes, respectively. The lowest GSI values were observed in spent fish sampled in July.

Pituitary FSH content did not vary significantly ($P > 0.05$) in captive reared greater amberjack females during all the examined reproductive phases (**Table 3.1.3**). Likewise, circulating FSH levels, were steady and relatively low in females at early and late vitellogenesis, however, significantly ($P < 0.05$) increased in the regressing females. Although not statistically significant, the pituitary FSHβ transcript levels exhibited an opposed trend; maximal and minimal levels coinciding with early vitellogenic and regressing females, respectively.

The pituitary LH content (**Table 3.1.3**) gradually increased reaching its maximum (53.91 ± 9.36 ng/pituitary/kg BW) in the regressing females. The circulating LH levels were steady and relatively low during early and late vitellogenesis. Maximal circulating LH levels were detected in regressing females (63.7 ± 44.8 ng/ml). Nonetheless, the latter result lacks statistical significance due to markedly high variability within the specific sampling group. Thus, the plasma LH levels in the regressing females ranged between 10 to 200 ng/ml. The pituitary LHβ gene expression did not vary significantly during the examined reproductive phases.

Sex steroids plasma concentrations of captive-reared greater amberjack females are reported in **Table 3.1.3**. Testosterone and E_2 plasma concentrations increased constantly from primary oocyte growth to late vitellogenesis stage and then significantly decreased in specimens having ovaries in regressed conditions. A constant increase was shown by 17,20β-P from primary oocyte growth to the regression phase.

A significant increase ($P < 0.05$) of vitellogenin plasma concentration was observed from early to late vitellogenesis stage, followed by a significant decrease ($P < 0.05$) in females with regressed ovaries (**Table 3.1.3**).

No difference in the surface occupied by yolk granules was found between captive-reared and wild specimens (D3.5).

Table 3.1.3 Gonado-somatic Index, intra-pituitary gonadotropin concentrations, plasma concentrations of sex steroids and vitellogenin, and transcript levels of gonadotropins and leptin in female greater amberjack reared in captivity at P23. ARGO.

	Ovarian development stage			
	Primary growth (n = 1)	Early Vitellogenesis (n = 3)	Late vitellogenesis (n = 4)	Regressing (n = 4)
Gonado-somatic index (GSI, %)	0.8	1.0 ± 0.07	4.2 ± 1.23	1.2 ± 0.02*
Pituitary FSH (ng/pituitary/kg BW)	8.8	6.4 ± 2.1	6.8 ± 2.5	9.1 ± 1.7
Pituitary LH (ng/pituitary/kg BW)	24.8	19.8 ± 13.8	36.8 ± 12.1	53.9 ± 9.4*
Plasma FSH (ng ml^{-1})	7.38	7.3 ± 1.3	8.3 ± 0.9	14.9 ± 2.3*
Plasma LH (ng ml^{-1})	49.1	36.1 ± 23.4	12.5 ± 3.3	63.7 ± 44.8
Pituitary FSHβ mRNA	7.8	10.6 ± 6.6	5.3 ± 2.9	1.8 ± 0.4
Pituitary LHβ mRNA	5.9	5.13 ± 2.7	3.6 ± 1.5	3.1 ± 1.1
Plasma T (ng ml^{-1})	0.2	0.3 ± 0.1	0.7 ± 0.2	0.2 ± 0.1*
Plasma E_2 (ng ml^{-1})	0.5	0.7 ± 0.1	1.9 ± 0.5	0.4 ± 0.2*
17,20β-P (ng ml^{-1})	0.1	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1*
Plama vitellogenin (mg ml^{-1})	2.5	2.4 ± 0.1	4.3 ± 0.1*	2.7 ± 0.2*
Plasma Leptin	ND	ND	ND	ND
Liver leptin mRNA	21.71	27.82 ± 17.90	2.34 ± 0.86	75.79 ± 32.80*

FSH, follicle stimulating hormone; LH, luteinizing hormone; ND, not determined. Asterisks = statistically significant difference versus the previous ovarian development stage ($P < 0.05$).

Males

In the examined testis sections single spermatogonia, spermatogonia contained in cysts, primary and secondary spermatocytes, spermatids and spermatozoa were observed. (**Fig. 3.1.6**). A detailed morphological description of male germ cells has been reported in Deliverable 3.6.

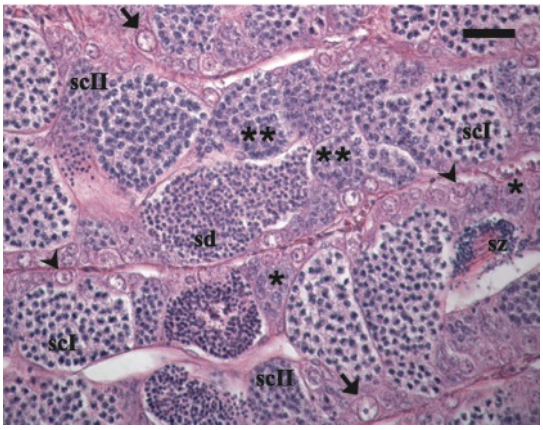

Figure 3.1.6. Micrograph of a greater amberjack testis section showing the different germ cell types. Haematoxylin-eosin staining. Magnification bar = 25 μm. Single A spermatogonia are indicated by black arrows and arrowheads (presumptively differentiated and undifferentiated stem spermatogonia, respectively). Asterisk: type A spermatogonial cyst; double asterisk: type B spermatogonial cyst; sd: spermatid cyst; scI: primary spermatocyte cyst; scII: secondary spermatocyte cyst; sz: spermatozoa.

The testes of the four captive-reared specimens sampled during the EARLY period contained germ cells in all spermatogenic stages, as well as spermatozoa in the seminiferous lobules. The testes of the fish sampled during the ADVANCED phase had seminiferous lobules in different conditions: in some cases, the germinal epithelium was in active spermatogenesis showing all the spermatogenic stages, in other cases the spermatogenic activity was ceased and only residual sperm cysts were visible. All the testes sampled in this period had a moderate/abundant amount of luminal spermatozoa. In the presumed SPAWNING phase, all the four captive-reared males had already ceased their spermatogenic activity, still showing a moderate amount of spermatozoa in the lumen of seminiferous lobules.

Gonado-somatic index (**Table 3.1.4**) changed according to testicular maturity stage. Mean GSI increased significantly ($P<0.05$) from the EARLY to the ADVANCED spermatogenesis phase and decreased significantly ($P<0.05$) during the SPAWNING phase.

Anti-PCNA immunostaining was observed in the nuclei of single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (**Fig. 3.1.7a**). A weak staining of the nuclei of secondary spermatocytes was also observed, but these cells were not included in the quantitative analysis. The density of anti-PCNA single A spermatogonia was stable throughout the EARLY and the ADVANCED phase and decreased dramatically in the SPAWNING phase ($P < 0.05$); a progressive decrease of anti-PCNA positive spermatocysts density was observed throughout the examined phases of the reproductive cycle ($P < 0.05$) (D3.6).

All the captive-reared greater amberjack showed TUNEL-positive germ cells. Apparently, the TUNEL reaction involved mainly single A spermatogonia, spermatogonia contained in cysts and primary spermatocytes (**Fig. 3.1.7b**). No significant difference in the surface occupied by apoptotic cells was observed during the three sampling phases ($P <0.05$.) (D3.6).

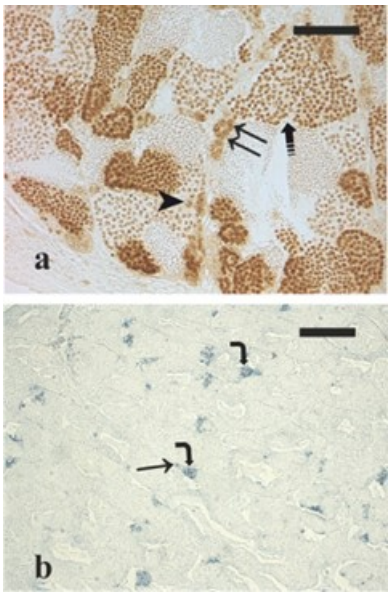

Figure 3.1.7. Micrographs of greater amberjack testis sections. (a) Testis section immunostained with antibodies against the Proliferating Cell Nuclear Antigen (PCNA), which stains brown the nuclei of proliferating cells. Magnification bar = 40 µm. Arrowhead: anti-PCNA positive single spermatogonium; double arrow: anti-PCNA positive spermatogonial cyst; dashed arrow: primary spermatocyte cyst. (b) Testis section stained with the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method, with apoptotic cells appearing as dark blue dots. Magnification bar = 150 µm. Arrow: TUNEL positive single spermatogonium; curved arrow: TUNEL positive spermatocysts

Pituitary and circulating levels of FSH (**Table 3.1.4**) as well as pituitary expression levels of the FSHβ (**Table 3.1.4**) did not vary significantly ($P > 0.05$) during the three examined reproductive phases of greater amberjack reared in captivity. Conversely, the pituitary LH content gradually increased reaching its maximum (23.42 ± 3.68) at the spent stage (**Table 3.1.4**). However, cognate pituitary LHβ gene expression (**Table 3.1.4**) and plasma LH levels (**Table 3.1.4**), exhibited no significant variation during the inspected reproductive stages.

The trend of sex steroid plasma levels during the three examined phases of captive-reared greater amberjack reproductive cycle is reported in **Table 3.1.4**. Testosterone, 11-KT and 17,20β-P plasma levels showed a progressive decrease from the EARLY to the SPAWNING phase. Unexpectedly high E_2 plasma levels were observed during the EARLY phase, but these levels decreased significantly in the following phases.

Table 3.1.4. Pituitary and plasma gonadotropin concentrations, steroid plasma concentrations, pituitary gonadotropin and liver leptin gene expression in male greater amberjack reared in captivity at P23. ARGO.

	Testicular maturity stage		
	Early spermatogenesis (n = 4)	Advanced spermatogenesis (n = 4)	Spent (n = 4)
Gonado-somatic index (GSI, %)	0.5 ± 0.03	3.8 ± 0.59*	1.0 ± 0.13*
Pituitary FSH (ng/pit/kg BW)	7.09 ± 0.04	4.5 ± 1.38	8.51 ± 1.26
Pituitary LH (ng/pit/kg BW)	8.45 ± 1.04	22.3 ± 5.11	23.42 ± 3.68*
Plasma FSH (ng ml^{-1})	8.57 ± 1.11	7.52 ± 1.79	11.34 ± 1.65
Plasma LH (ng ml^{-1})	15.71 ± 5.75	8.56 ± 2.94	16.96 ± 5.94
Testosterone (T, ng ml^{-1})	0.7 ± 0.16	0.4 ± 0.10	0.2 ± 0.04
11-Ketotestoterone (11-KT, ng ml^{-1})	2.3 ± 0.63	0.8 ± 0.15	0.2 ± 0.04*
17β-estradiol (E_2, ng ml^{-1})	5.4 ± 1.95	0.7 ± 0.26*	1.1 ± 0.65
17,20β-dihydroxypren-4-en-3-one (17,20β-P, ng ml^{-1})	0.4 ± 0.36	0.1 ± 0.01	0.5 ± 0.13*
Pituitary FSHβ (relative units)	6.24 ± 3.1	6.91 ± 0.56	3.6 ± 1.1
Pituitary LHβ (relative units)	3.02 ± 1.51	4.11 ± 1.46	3.98 ± 1.37
Leptin (relative units)	7.22 ± 2.04	4.56 ± 2.2	67.7 ± 49.3
Plasma Leptin	ND	ND	ND

FSH, follicle stimulating hormone; LH, luteinizing hormone; ND, not determined. Asterisks = statistically significant difference versus the previous testicular maturity stage (Student' s t-test, $P < 0.05$).

3.1.2.3 Egg composition

Analytical data of total carotenoid content, moisture and lipids from eggs of captive-reared females have been reported in details in D3.5. Among total polar lipids (TPL) phosphatidylcholine (PC) followed by phosphatidylethanolamine were found to be the most relevant lipid classes. In terms of the fatty acid profile of total lipids (TL), monounsaturates (MUFA; mainly 18:1n-9) followed by n-3 HUFA fatty acids, with a triplicate DHA (22:6n-3) content compared to EPA (20:5n-3), were found to be the most abundant. Finally saturated fatty acids, whose main representative is 16:0 were also prominent in eggs. At the individual level, 18: 2n-6 was also particularly high. When this profile was compared with that of polar lipids (TPL), it was observed that the presence of saturates is enhanced, and that n-3 HUFA, which becomes the most abundant group, and particularly the DHA content in this lipid fraction was also particularly enhanced. Arachidonic acid (20: 4n-6) also evidenced this increase in the eggs polar lipids.

3.1.2.4 Sperm quality

Spermatozoa concentration of captive-reared greater amberjack was stable throughout the EARLY ($2.3 \pm 0.5 \times 10^{10}$ spz ml^{-1}) and ADVANCED ($3.6 \pm 0.4 \times 10^{10}$ spz ml^{-1}) phases, and increased significantly during the SPAWNING period ($4.6 \pm 0.6 \times 10^{10}$ spz ml^{-1}; ANOVA, $P < 0.05$). For all the three sampling phases, the highest spermatozoa motility (%) was reached within the first 20 s after activation, and was followed by a progressive decrease until complete cessation of movement. However, sperm movement within the first 20 s presented variations linked to the sampling time, with the highest mean percentage of swimming spermatozoa recorded in the ADVANCED phase (59 ± 16.9 % of motile spz), and the lowest mean value registered in the SPAWNING phase (21 ± 9.7 % of motile spz). The mean VAP of the spermatozoa varied during the three different phases, with the highest mean value 10 s after activation recorded in the ADVANCED phase (102.7 ± 7.0 μm s^{-1}) and the lowest mean VAP during the SPAWNING phase (36.5 ± 3.3 μm s^{-1}); the highest maximum value of individual velocity was reached during the ADVANCED phase (164 μm s^{-1}). Finally, a progressive significant decrease ($P < 0.05$) of sperm motility duration was observed from the EARLY to the SPAWNING phase. The ATP level of captive-reared greater amberjack sperm was generally very low, and close to the detection threshold level.

3.1.2.5 Nutritional state

The body condition of captive-reared greater amberjack, expressed as Fulton's condition factor, was not significantly different from that of wild specimens of the same age class ($P > 0.05$).

Recombinant leptin was produced using the *P. pastoris* yeast recombinant DNA expression system. Following purification on an affinity column, the recombinant leptin was used to immunize rabbits and generate the specific polyclonal antibodies (D3.2). As attested by Western blot analyses the obtained antibodies exhibited high specificity to fish (produced herein) and human (ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel) recombinant leptin, yet they failed to detect greater amberjack leptin extracted from liver samples and then it was not possible to establish an ELISA to measure leptin plasma levels (D3.2).

The leptin analysis, performed at the gene expression levels, showed that liver leptin transcripts reach their minimum in females undergoing late vitellogenesis and climax in the regressing females (**Table 3.1.3**). In males, liver leptin mRNA levels appear to be relatively low at early and during spermatogenesis and elevated at the spent stage (**Table 3.1.4**).

3.1.3 Conclusions

The age analysis showed that the 23 juvenile greater amberjack specimens sampled for the study of age, growth and sexual maturity were 1 to 3 years old. The age of wild adults had been previously estimated (D3.3) and ranged between 4 and 6 years. The parameters of the von Bertalanffy equation, calculated using all the available juveniles and adult females, indicate a theoretical maximum length of 121.5 cm and 127cm FL for females and males, respectively. The theoretical longevity was 8 and 9 years for females and males,

respectively. The theoretical length-at-age data, confirm a very rapid growth of both sexes during the first years of age, with a body weight of about 0.7 kg, 3.2 kg and 6.3 kg at age 1, 2 and 3, respectively. Greater amberjack starts to reproduce at the age of 3 years and the whole population is sexually mature by the age of 5 years. The median at first sexual maturity (L_{50}) of female greater amberjack is 88 con FL and all the females larger than 110 cm FL are reproductively active.

The comparative analysis of GSI, histological observations, pituitary and circulating gonadotropins and sex steroid plasma levels in the present study indicate a severe adverse effect of confinement in captivity on the reproductive axis of both female and male greater amberjack, with consequent gametogenesis impairment.

The gonadotropin analyses indicate relatively low pituitary FSH and LH content and, consequently, reduced levels of these hormones in the circulation of captive-reared greater amberjack undergoing gametogenesis compared to levels measured in wild fish during the equivalent reproductive periods.

The negative effects of confinement were glaring in females with oocytes in late vitellogenesis stage, perhaps because the fish sampled at this stage had already been manipulated once, as they were kept together in the same sea cage, and resulted in an extensive oocyte atresia that prevented any further oocyte development. The observed reproductive dysfunction was not related to an impairment of the vitellogenic process because Vg plasma concentrations were found to be in the normal range of the wild population during the reproductive season and no difference was found in the amount of yolk accumulated in oocytes of wild and captive-reared greater amberjack. It is possible that the observed extensive atresia affecting oocytes in late vitellogenesis in greater amberjack reared in captivity, was related to the low 17, 20β-P plasma concentration (about half than in wild specimens).

Male greater amberjack reared in captivity showed lower GSI and sex steroid plasma levels than wild fish. Coherently with the low sex steroid circulating levels, captive-reared greater amberjack showed a limited spermatogonial capacity to proceed toward meiosis and a precocious cessation of the spermatogenic activity. Abnormally high E_2 plasma levels were found at the early stage of spermatogenesis and they were associated to a high density of apoptotic male germ cells. The sperm analysis showed the lack of proper sperm hydration in captive-reared greater amberjack, probably in response to low sex steroid levels. Moreover, the percentage of motile spermatozoa, motility duration and velocity and sperm ATP content declined drastically during the supposed spawning phase.

Egg biochemical analysis demonstrated that the dietary regime of captive-reared greater amberjack (Vitali-Cal, Skretting), covered even in excess the amount of carotenoids found in wild specimens. In addition, except for the high contents of 18:2n-6 and the lower levels of 20:4n-6, the dietary regime and the resultant eggs were quite similar in terms of total polar lipids, EPA and DHA levels and ratios, to those present in the wild counterparts (Rodríguez-Barreto et al., 2014).

The observed gametogenesis impairment was related to a malfunctioning of the reproductive axis, which involved low levels of both, FSH and to a greater extent, LH and as a result low plasma steroid concentrations, particularly 17, 20β-P, the hormone responsible for oocyte maturation/spermiation and spawning. However, another broodstock of the same source and age was maintained under identical conditions in the same facility for the execution of **Task 3.2** and, during June, it reached advanced stages of gametogenesis to be able to be induced to spawn and produce fertilized eggs whose quality, in terms of total polar lipids, EPA and DHA levels and ratios, was equivalent to those of wild specimens. We suppose that the repeated sampling operations in the rearing cage might have played a major role in the observed reproductive dysfunction, thus underlying the extreme susceptibility of this species to the handling stress and the need for a careful management of greater amberjack broodstocks.

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Task 3.2. Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (led by HCMR, Constantinos Mylonas).

As was mentioned in 2nd Periodic Report, the comparison between multiple injections and implants was implemented during months 31-32 (June-July 2016) and could not be analysed and reported in time within that report. This experiment, together with those planned for Y3 and Y4, i.e. those aimed at the identification of the most effective GnRHa dose and those aimed at understanding the best timing for the hormonal treatment, are reported below.

3.2.1 Broodstock maintenance

A total of 109 breeders fish in 5 stocks were used for the spawning induction experiments in 2016, and 95 breeders in 2017, respectively (**Table 3.2.1**):

HCMR tanks (AQUALABS): Breeders (n=19-26) were kept in two 35-m tanks under simulated natural temperature and photoperiod. Fish were fed on dry pellets (Vitalis Cal, Skretting, Spain).
HCMR cage: Breeders (n=7) were kept in a 40-m perimeter cage at the Souda Bay pilot cage farm, and were fed on moist pellets (Vitalis Cal, Skretting, Spain).
ARGO cage: Breeders (n=28-29) were kept in a 40-m perimeter cage at Salamina Island, Greece, and were fed on dry pellets (Vitalis Cal, Skretting, Spain).
FORKYS tank: Breeders (n=15-19) were kept in a 25 m^3 tank in FORKYS' hatchery in Siteia, Crete, Greece. Fish were under natural temperature and photoperiod and fed with raw fish and squid.
GMF cage: Breeders (n=26-28) were kept in a 40-m perimeter cage at Galaxidi, Greece and fed with live juvenile fish (seabass and seabream) in 2016 and moist pellet in 2017 (Vitalis Cal, Skretting, Spain).

Table 3.2.1. Description of the various broodstocks maintained for this task.

2016

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	29	11.2-23.6	pellet
GMF	sea-cages	28	11.8-21.5	live fish
SOUDA	sea-cages	7	14.0-20.7	moist pellet
AQUALABS	land-based	26	9.8-18.5	pellet
FORKYS	land-based	19	12.6-20.3	raw fish, squid

2017

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	28	11.8-26.0	pellet
GMF	sea-cages	26	11.5-22.2	moist pellet
SOUDA	sea-cages	7	14.2-22.1	moist pellet
AQUALABS	land-based	19	12.1-22.7	pellet
FORKYS	land-based	15	11.1-20.5	raw fish, squid

3.2.2 Evaluation of reproductive stage

The evaluation of the reproductive stage begun in June 2016 in various broodstocks, based on the local temperature and observations on the maturation stage from the previous years. For the evaluation of the reproductive stage, fish were fully anaesthetized and:

- Gonadal biopsy was taken from female fish to evaluate the reproductive stage under an optical microscope, and a portion of the biopsy was stored in fixative solution for histological evaluation.
- Sperm sample was taken from male fish using a catheter (since it is difficult to obtain sperm with abdominal pressure) to estimate quality parameters such as motility percentage, motility duration and density.

2016

HCMR tank (3/6/2016 and 5/7/2016): Males were mostly immature and only in four of them milt collection was possible only using a catheter, hence it was classified as intra-testicular sperm (IT sperm) as the previous years. Sperm motility was 60-85%, motility duration 8.95-10.27 min, sperm survival 4-8 days and sperm density 2.22-3.17 x 10^{10} szoa ml^{-1}. Females either contained primary oocytes (PO) in their gonadal biopsies or oocytes in early and vitellogenic stage (eVg - Vg) (**Fig. 3.2.1A)**. The early Vg-Vg females were three out of 16 fish, having also increased percentage of atresia (AT) and oocyte diameter maximum of 450 μm.

FORKYS tank (23/6/2016): Five out of 11 males were spermiating after abdominal pressure. Sperm collection was also possible at the rest males, using a catheter to collect IT sperm. Females on the other hand were mostly immature, having POs in their gonadal biopsies and only two fish had eVg or Vg oocytes (300 μm or 650 μm, respectively) (**Fig. 3.2.1B)**.

ARGO cage: Fish in ARGO facilities were evaluated prior the implementation of the comparison of multiple injections vs implants, so their reproduction stage is reported below in the specific session (**3.2.4 Multiple GnRHa injections vs GnRHa implants**).

GMF cage (16/6/2016): Mid-June males did not release any sperm quantity after abdominal pressure, but IT sperm collection was possible. Sperm motility was 0-90%. At the same time, females were in Vg stage with oocytes of 670-740 μm in diameter (**Fig. 3.2.1C)**. Only one female was found to contain atretic oocytes in its ovarian biopsy.

HCMR Souda cage (6/7/2016): Early July males had IT sperm of low sperm motility (20-35%). Females on the other hand had mostly PO in their ovarian biopsies (**Fig. 3.2.1D)**. At the same time, AT was present at 75% of the females, while in one female there were signs of possible previous ovulation.

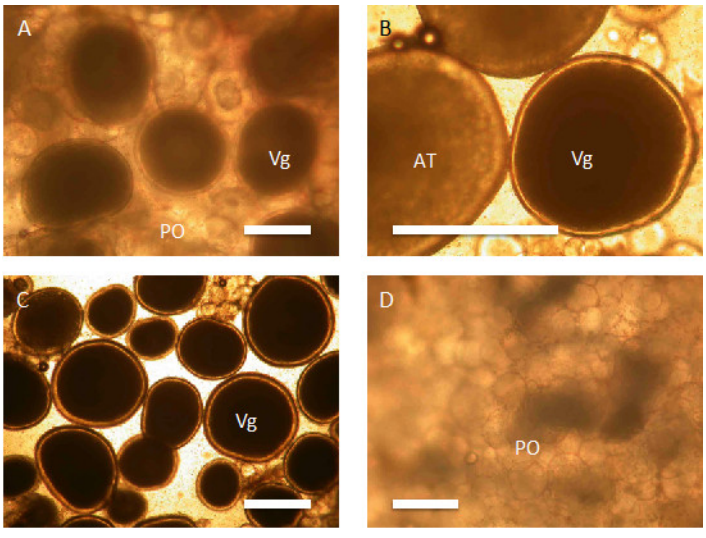

Figure 3.2.1. Female greater amberjack maintained in land-based tanks and sea cages during 2016. Wet mount photographs (A) from HCMR in land based tanks, (B) from FORKYS, (C) from GMF and (D) HCMR sea cages broodstock. A: Female on 5/7/2016, in vitellogenesis (Vg) with a large number of primary oocytes. B: Female on 23/6/2016, with Vg oocytes and increased percentage of atresia (AT), C: Female on 16/6/2016, in Vg. D: Female on 6/7/2016 having only PO. Bar = 500 μm.

2017

HCMR tank (11/5/2017 and 12/6/2017): All males had IT sperm. Four females had eVg or Vg oocytes (divided equally in each month) and the rest four females had only PO or gonadal biopsying was not possible. Oocytes in eVg stage were 410-440 μm and Vg 600-620 μm, respectively (**Fig. 3.2.2A, B**).

FORKYS tank (23/6/2017): Males had only IT sperm. Females were mostly immature, having PO or gonad was not accessible for biopsy. Two of the females were in Vg stage, the first with oocytes of 450 μm in diameter but with increased number of PO and the second with oocytes of 630 μm in diameter but signs of early AT (**Fig. 3.2.2C**).

ARGO cage: Fish in ARGO facilities were evaluated prior the implementation of the comparison of two GnRHa doses, so their reproduction stage is reported below in the specific session (**3.2.5 Comparison of two GnRHa doses**).

GMF cage: Fish in GMF facilities were evaluated prior the implementation of the experiment to find the optimum timing of GnRHa treatment, so their reproduction stage is reported below in the specific session (**3.2.6 Timing of GnRHa treatment application**).

HCMR Souda cage (28/6/2017): Males had IT sperm with sperm motility 35-75% and motility duration 1.77-2.12 min. Females had mostly POs and AT was present. There were signs of possible previous ovulation in two females (**Fig. 3.2.2D**).

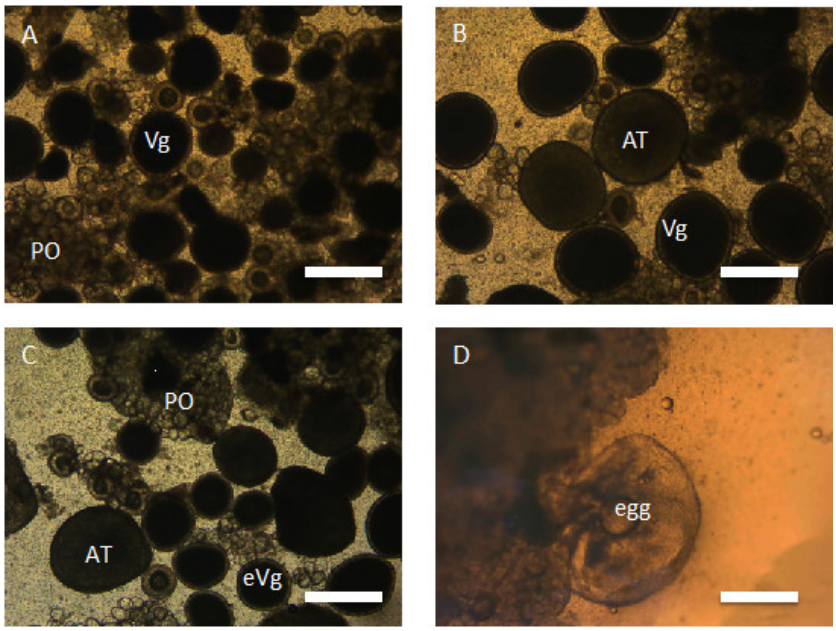

Figure 3.2.2. Female greater amberjack maintained in land-based tanks and sea cages during 2017. Wet mount photographs (A,B) from HCMR in land based tanks, (C) from FORKYS and (D) HCMR sea cages broodstock. A,B: Females on 11/5/2017, in vitellogenesis (Vg) with a large number of primary oocytes and atresia (AT). C: Female on 23/6/2017, in early Vg with a large number of PO and AT. D: Female on 28/6/2017, having a post ovulated egg. Bar = 500 μm.

3.2.3 Spawning induction

When fish were in the appropriate stage of oocyte development, they were administered with GnRHa implants, depending on their size, to obtain an effective dose of ~50 μg GnRHa kg^{-1} body weight. A single dose of GnRHa controlled-release delivery systems (implants) was used, chosen based on previous experiments with greater amberjack, but also other marine fish (Mylonas et al., 2004, 2010). Tanks were

fitted with passive egg collectors, which were monitored for eggs every day. Fecundity and fertilization success were estimated after transferring the eggs in a 10 l bucket, and taking a sub sample of 10 ml. Also, the stage of egg development was determined.

Of the stocks examined for their reproductive stage (see Section 3.2.2 above), a number of females reached a stage that was appropriate to be given a hormonal therapy to induce maturation, ovulation and spawning:

GMF cage (16/6/2016 and 30/6/2016): On 16/6 twenty fish were treated with GnRHa implants. Six females and seven males were transferred in two land-based tanks (3:3, 3:4) while the rest were left in the cage to spawn. Fish started spawning two days later and eggs were collected from the sea cage once, while in land-based tanks eggs were collected for 10 days. Fertilization was variable (0-100%) and daily fecundity was less than 1,600,000 for each tank (**Fig. 3.2.3)**. Only 200,000 floating eggs were possible to be collected in cage. After two weeks (30/6), a 2nd treatment was given to different fish from the cage according to their reproductive evaluation. A total number of 8 females and 8 males were transferred in two land-based tanks. The maximum daily fecundity was observed two days after (almost 4,000,000 eggs), while the fish spawned for four consecutive days. The fertilization for this period was 0-77%.

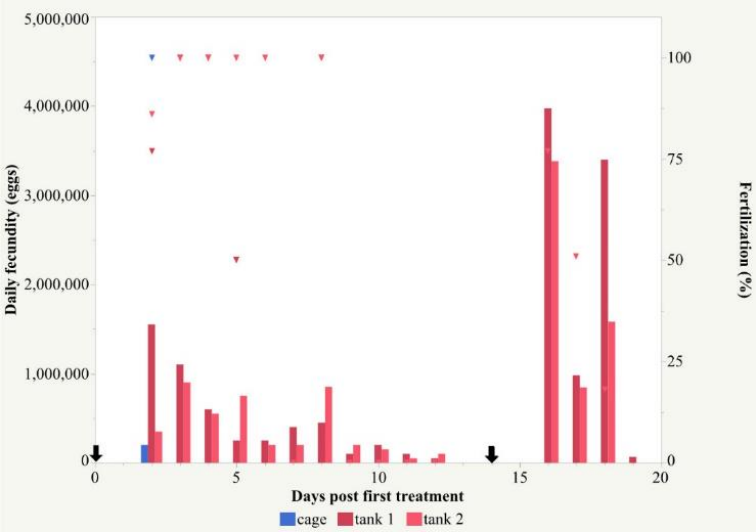

Figure 3.2.3. Fecundity (eggs, bars) and fertilization (%, marks) from greater amberjack stock maintained at the GMF sea cage facility, induced with GnRHa implants at 16/6/2016 and 30/6/2016 (marked with arrows).

3.2.4 Multiple GnRHa injections vs GnRHa implants

3.2.4.1 Broodstock maintenance

Amberjack broodstock was kept at the facility of Argosaronikos Fish Farm S.A., on the island of Salamina (Greece). Stock consisted of 28 fish, captured at the juvenile stage and acquired in May 2014 from Asteras SA (Astakos, Greece). Broodstock was maintained in a 1000 m^3 sea cage, 300 m offshore from the inland facility. At the time of the 2016 reproductive season (late spring-summer), the stock consisted of 14 female (mean ± SD body weight 18.8 ± 2.1 kg), and 14 males (mean ± SD body weight 15.1 ± 3.0 kg). Feed was given to apparent satiation 6 days a week using Skretting Vitalis CAL (22 mm) and fish were starved one day prior to handling. For the spawning induction trial, fish were transferred to the inland facility, to 23 m^3 flow through round tanks, accordingly to the treatment received, and maintained at a 1:1 sex ratio. Each treatment was conducted in duplicates, with 6 and 8 fish per tank, respectively. Tanks were supplied with constant oxygen flow, a mixture of surface (~16% h^{-1}) and well water (~6% h^{-1}) and exposed to ambient photo-thermal conditions, with temperature ranging from 20.1 °C to 24.1 °C in the course of the experiment. Measurements of temperature and oxygen saturation were conducted twice a day (AM, PM). Tank overflows were fitted with 250 L passive egg collectors. Feeding in the tanks was done to satiation between samplings 5 days a week.

3.2.4.2 Evaluation of reproductive stage and broodstock selection

Once the fish were lightly anaesthetized with the use of clove oil to a concentration of 0.01 mL L^{-1} they were moved for complete sedation to an anaesthetic bath, at a concentration of 0.03 mL L^{-1}. Ovarian biopsies were obtained by inserting an endometrial catheter (Pipelle de Cornier) into the ovarian cavity and applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100×) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10), and mount pictures were taken for further measurements. A portion of some biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Female fish were considered eligible for spawning induction if they contained only fully vitellogenic oocytes. Because of the hard musculature surrounding of the abdominal cavity of the greater amberjack, milt samples were obtained by cannulation as described above for the females. Milt was kept on ice until examination.

3.2.4.3 Spawning induction experiments

Spawning induction trial was conducted between 7 June and 28 June 2016. Female fish were treated either with GnRHa injection (20-25 µg GnRHa kg^{-1}) or with EVAc GnRHa implant (Mylonas and Zohar, 2001), loaded with 750–1000 µg of Des-Gly^{10},D-Ala^{6}-Pro-$NEth^{9}$-mGnRHa (H-4070, Bachem, Switzerland), for an effective dose of 49-69 µg GnRHa kg^{-1}. In order to enhance spermiation and ensure the adequate sperm production, males were treated with at the start of the experiment with EVAc GnRHa implant at a dose of 45-70 µg GnRHa kg^{-1} and divided randomly between the stocks. First hormonal treatment with GnRHa was given calculating the dose accordingly to an estimated 30 % growth rate from the previous year (15 June 2015). This caused variations in the effective GnRHa first dose applied to each fish. For EVAc treatments, further variations were due to the fact that implants are loaded with fixed amounts of GnRHa. Even though combinations of two implants loaded with different amounts of GnRHa were used when necessary, it was still not possible to adjust the dose exactly to the different body weight of the fish. During the initial treatment, females were divided in four experimental groups in order to obtain two duplicates per treatment method (3 and 4 fish per duplicate, respectively). Fish were divided equally between the two treatment methods according to their gonadal maturation stage. Before transferring the broodstock to experimental tanks, weight of each individual was recorded in order to calculate further doses accordingly to the actual fish weight (injection dose: 20 µg GnRHa kg^{-1}; EVAc dose: 50 µg GnRHa kg^{-1}). Females were treated weekly only in injected stocks, and in both stocks on the third week, on 21 June 2016 (a total of 3 injections and 2 implants). Treatments and samplings in the facility were implemented with the same procedure used for the first sampling. Water level was reduced in the tank, fish were slightly anaesthetized and moved to an anesthetic bath for complete sedation. Three weeks after the start of the experiment, on 28 June 2016, final sampling was conducted and the fish were returned to the cage.

3.2.4.4 Evaluation of egg/larval quality

Egg collectors were examined three times a day (8:00AM, 3:00PM, 8:00PM) and eventual eggs were processed as following: for each spawn, date, collection time and developmental stage (Tachihara et al., 1993) were recorded, in order to identify different spawns and estimate an approximate spawning time. This allowed calculating the time between treatment administration and first spawn (latency period). All eggs were collected and transferred into a 10 L bucket of water proceeding from the same tank. Their number (fecundity) was estimated using a stereoscope by counting the total number of eggs in a sub-sample of 10 mL, collected with a pipette after vigorous agitation. Eggs contained in the sample were the thousand part of the number of eggs spawned. Fecundity of the spawn was obtained adding up the amount of viable (alive and fertilized, L) and unviable (dead, unfertilized or showing anomalies in the development, D) eggs counted and multiplying by 1000 [(L + D) x 1000]. Fertilization percentage was evaluated at the same time by examining each egg and dividing the amount of viable eggs by the total [L $(L + D)^{-1}$]. After collecting the sub-sample, each bucket was transferred into a commercial 500 L conical tip tank-incubator fitted with an overflow filter (250 µm mesh size) and mild micro-bubbles aeration provided with a wooden air-stone and positioned beneath the overflow filter, in order to prevent eggs from sticking to the mesh. Incubators were

supplied with seawater and flow was adjusted to ~90% h^{-1}. From 23 June 2016 onwards, due to overheating of seawater, a mixture of seawater and well water in equal ratio was provided, in order to maintain a similar temperature to the tanks.

With the purpose of monitoring embryo and larval survival, eggs from each spawn were collected from the tank incubators and placed individually in 96-well microtiter plates (in duplicates) according to the procedure of Panini et al. (2001) with some modifications. Briefly, sample of floating (~100% fertilized) eggs were taken from the tank incubators with a 250 μm mesh sieve, rinsed with clean water proceeding from the incubator inlets and poured in 2 L beakers filled with clean water. Using the sieve, 100-200 floating eggs were scooped from the beaker and placed in a Petri dish. Together with 200 μL of seawater, fertilized eggs were aspirated with a micropipette one by one and transferred individually to the 96-wells of a mct plate. Plates were checked under a stereoscope and eventual unviable eggs were replaced. Each mct plate took 10-15 min to load, and, once loaded, it was covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 7 days at temperatures ranging between 21 and 23.5 °C, imitating the water temperature of the tanks. Using a stereoscope, embryonic and early larval development was evaluated daily, recording the number of live embryos 24 hours after egg collection (or ~30 hrs after spawning), hatched larvae (examined ~55 hrs after spawning) and viable larvae on day 5 after egg collection (near the time of yolk sack absorption). At 21-23.5 °C, hatching of amberjack eggs took place in 40-55 hrs. Embryo survival was calculated as the number of eggs having live embryos 1 d after egg collection/number of fertilized eggs initially loaded in the microtiter plates. Hatching success was calculated as number of hatched larvae/24 hrs embryos, and 5 d larval survival was calculated as number of live larvae 5 d after egg collection/hatched larvae. Estimating percentage survival (%) by using as denominator the number of individuals that survived to the previous developmental stage was considered as a more accurate evaluation of survival within specific developmental stages, without the potential of a distortion effect of the previous stage (Mylonas et al., 1992, 2004).

3.2.4.5 Histological analysis

The samples were fixed in 4% formaldehyde: 1% glutaraldehyde (McDowell and Trump, 1976), dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Serial sections were obtained at a thickness of 3–5 μm on a microtome (Leica RM2245, Germany) using disposable blades. After drying, slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976), examined under a light microscope (50i Eclipse, Nikon, Japan) and photographed using a digital camera (Progres, Jenoptik AG, Germany).

3.2.4.6 Statistical analysis

Differences in mean relative fecundity and egg/larval performance parameters (fertilization success, 24 hrs embryos survival, hatching, and 5 d larval survival) among fish induced to spawn with multiple GnRHa injections or implants were examined using one-way ANOVA followed by Tukey HSD test, at a $P \leq 0.05$ significance level. Data was examined for normality in the distribution of variances, in order to comply with the prerequisites of ANOVA. Percentages were arcsin transformed before statistical analysis. All analyses were performed with a statistics software (JMP 12, SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± SEM, unless otherwise stated.

3.2.4.7 Results

Decrease in diameter of the largest vitellogenic oocytes was observed among sampling weeks (2-way ANOVA, Tukey HSD, P=0.022) from 810±29 μm before 1st treatment to 653±31 μm at 3rd week (**Fig. 3.2.4**). Fish that contained fully vitellogenic oocytes decreased from 7 to 6 for the GnRHa implanted and from 7 to 3 for the injected fish, respectively, from the initial to the final sampling.

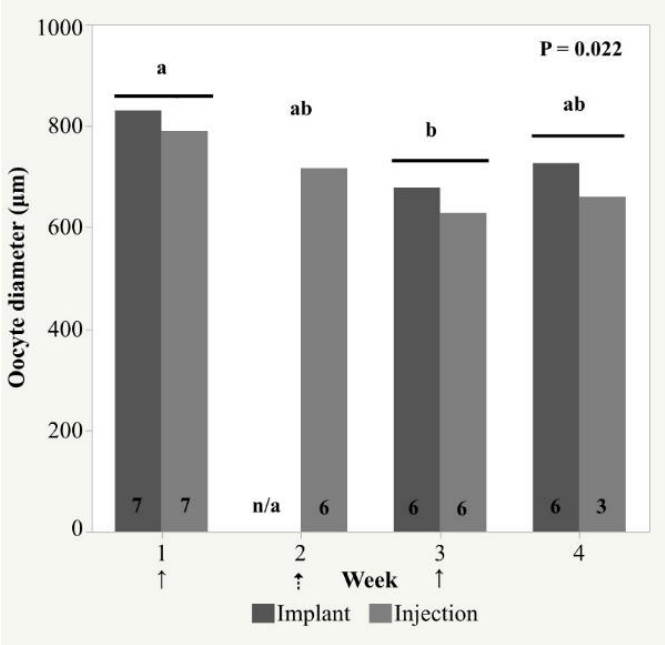

Figure 3.2.4. Mean diameter (± SEM) of the largest oocytes of biopsies of female greater amberjack treated either with EVAc GnRHa implants (dark grey) or GnRHa injections (light grey), respectively. Lowercase letters indicate significant differences among different sampling weeks (2 way ANOVA, Tukey HSD, P=0.022). No sampling was done during the 2nd week for the GnRHa implanted group. Numbers in bars indicate the number of fish biopsies used for the mean calculation. Solid arrows indicate treatment with both methods and dashed arrow indicates treatment only with GnRHa injection. n/a = non applicable.

Before the first treatment, females had mainly vitellogenic oocytes, while 4 fish out of 14 were in advanced maturational stage (**Fig. 3.2.5**, **first row**) and only a minor number of apoptotic oocytes was observed. During the second sampling only injected fish were biopsied and post ovulated eggs and apoptotic oocytes were observed in almost all biopsies taken. In parallel, vitellogenic oocytes were still present, except one case where only post ovulated eggs and apoptotic oocytes were observed (**Fig. 3.2.5**, **second row**). At the third sampling, still vitellogenic oocytes were visible in both treatment groups (**Fig. 3.2.5**, **third row**). One fish from each treatment method had only apoptotic oocytes and post ovulated eggs, while the number of primary oocytes was increased. At the final sampling, the implanted fish still had vitellogenic oocytes and some of them in maturational stages, and the proportion of atresia was minimal. On the other hand, 4 of 7 injected fish had finalized their reproductive period since only primary and early vitellogenic oocytes were obvious concomitantly with the remnants of the immature-unreleased oocytes (**Fig. 3.2.5**, **fourth row**). The remaining injected fish still had some vitellogenic oocytes, but with increased number of apoptotic oocytes.

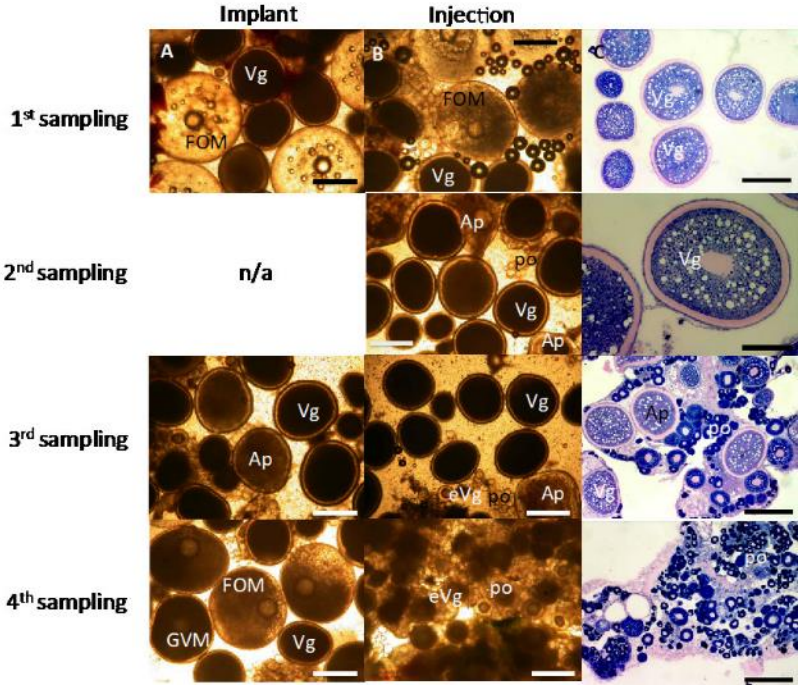

Figure 3.2.5. Microphotographs of representative ovarian biopsies from greater amberjack at different sampling times, presented as wet mounts or after histological processing. Fish were treated either with GnRHa implants or GnRHa injections. po=primary oocyte, Vg=vitellogenic oocyte, GVM=Germinal Vesicle Migration, FOM=Final Oocyte Maturation, Ap=apoptotic oocyte. Bars=500µm.

In both treatment methods, spawning started one day after 1st application because of the existence of oocytes in maturation stage (**Fig. 3.2.6**). Implanted fish spawned for 9-10 times after 1st treatment, while only 4 times after 2nd treatment. On the contrary, injected fish spawned for 7 times after the 1st treatment, 3-5 times after 2nd treatment and 1-3 times after the 3rd treatment, respectively. The higher egg production was observed in implanted fish with 4,242,000 eggs $tank^{-1}$ two days after 1st treatment, while in injected 2,454,000 eggs $tank^{-1}$, respectively.

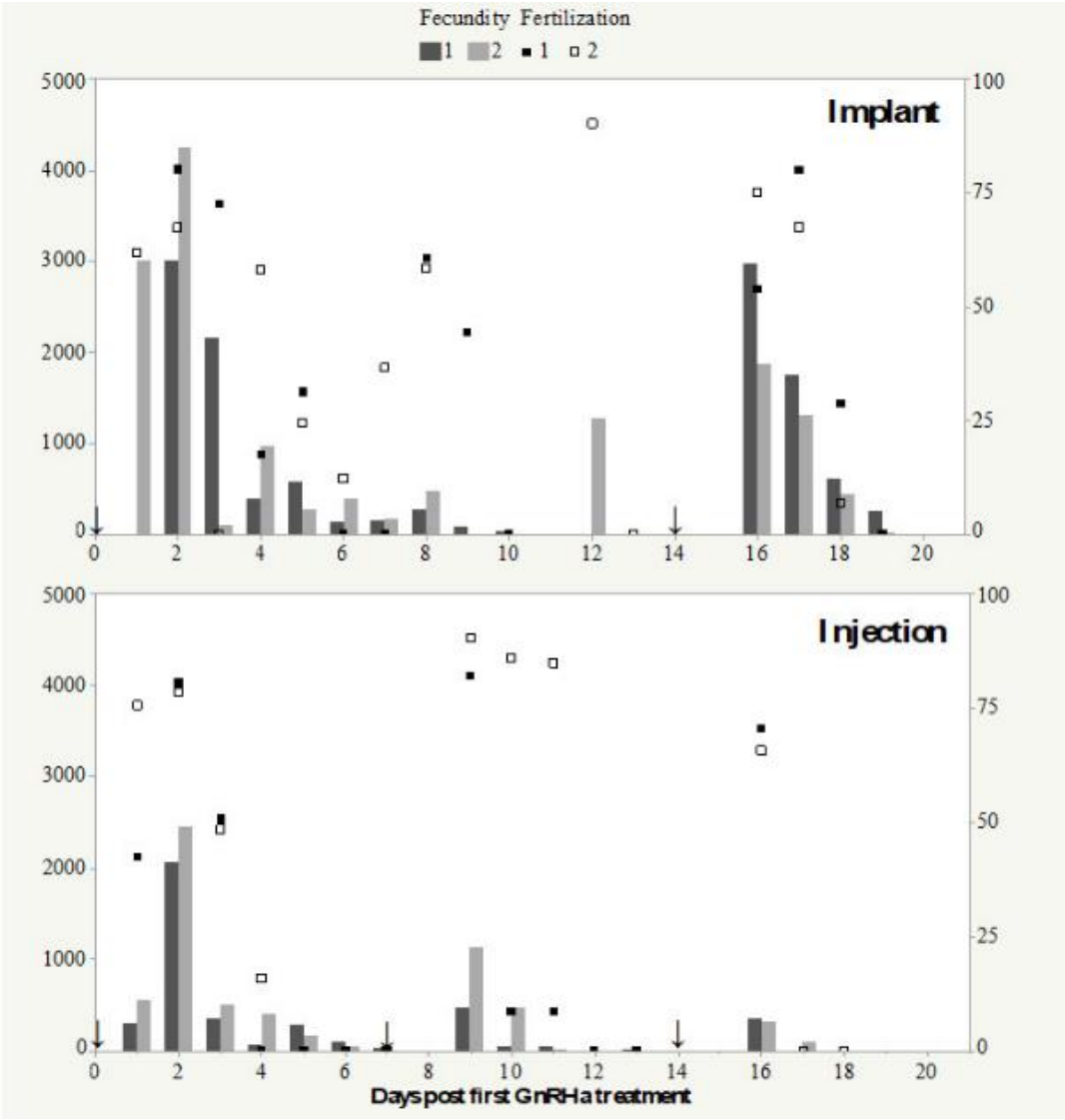

Figure 3.2.6. Daily fecundity (bars, x1000 eggs) and fertilization success (marks, %) of GnRHa implanted or injected greater amberjack. Arrows (n=2 for GnRHa implanted and n=3 for injected, respectively) indicate the time of treatment. First application was done on 7 June 2016.

Mean daily relative fecundity was higher in implanted fish (15,170±2,738 eggs $kg^{-1}day^{-1}$) compared to the injected fish (6,119±2,790 eggs $kg^{-1}day^{-1}$) (**Fig. 3.2.7**). Total relative fecundity was also higher in implanted fish (102,402±20,337 eggs $kg^{-1}tank^{-1}$) compared to the injected (26,517±9,938 eggs $kg^{-1}tank^{-1}$), respectively (**Fig. 3.2.8**). Total egg production was decreasing in injected fish after consecutive GnRHa treatments, while in implanted no statistical differences were observed among treatment number (P = 0.17).

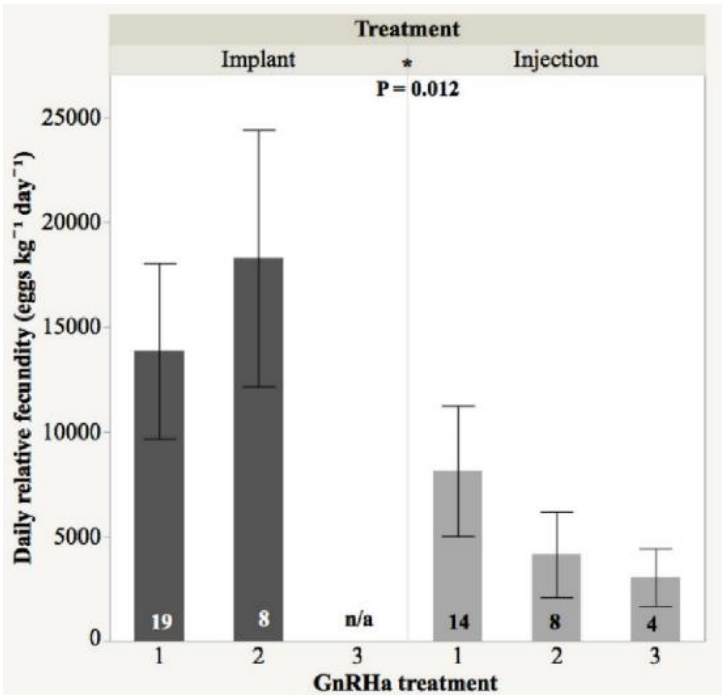

Figure 3.2.7. Mean daily relative fecundity (± SEM) of GnRHa implanted (dark grey) or GnRHa injected (light grey) greater amberjacks. Numbers in bars are the spawns constitute each mean. Asterisk indicates differences between treatment methods (t test, P = 0.012).

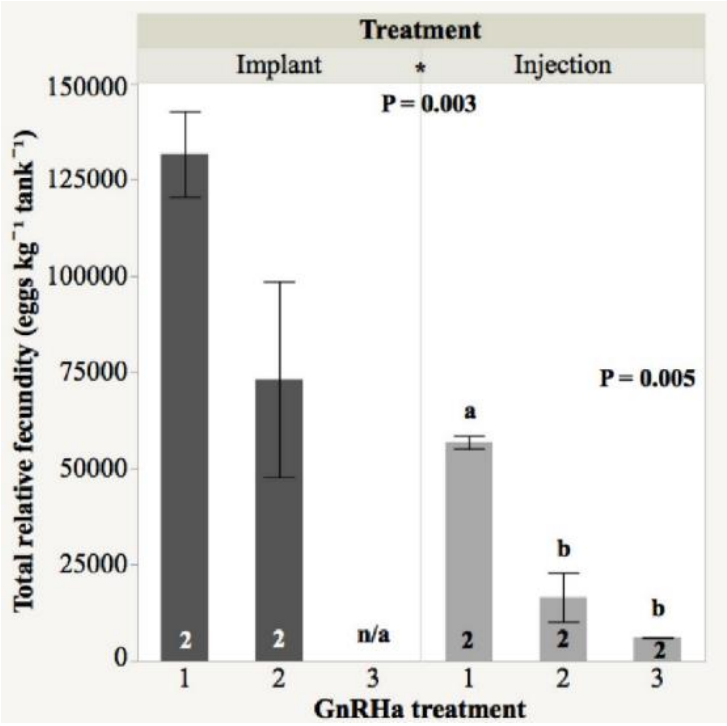

Figure 3.2.8. Mean total relative fecundity (± SEM) of GnRHa implanted (dark grey) or GnRHa injected (light grey) greater amberjacks. Numbers in bars are the tanks constitute each mean. Asterisk indicates differences between treatment methods (t test, P = 0.003) and lowercase letters between treatment number (ANOVA, Tukey HSD, P = 0.005).

Fertilization success, 24 h embryo survival, hatching and 5d larval survival was similar among treatment methods, while no statistical differences were observed among different treatment number (**Fig. 3.2.9**). Mean fertilization success was 36 ± 5% and 24 h embryo survival 53 ± 7%, for both treatment methods. Additionally, hatching was 70±4% and 5d larval survival 20±4%, respectively.

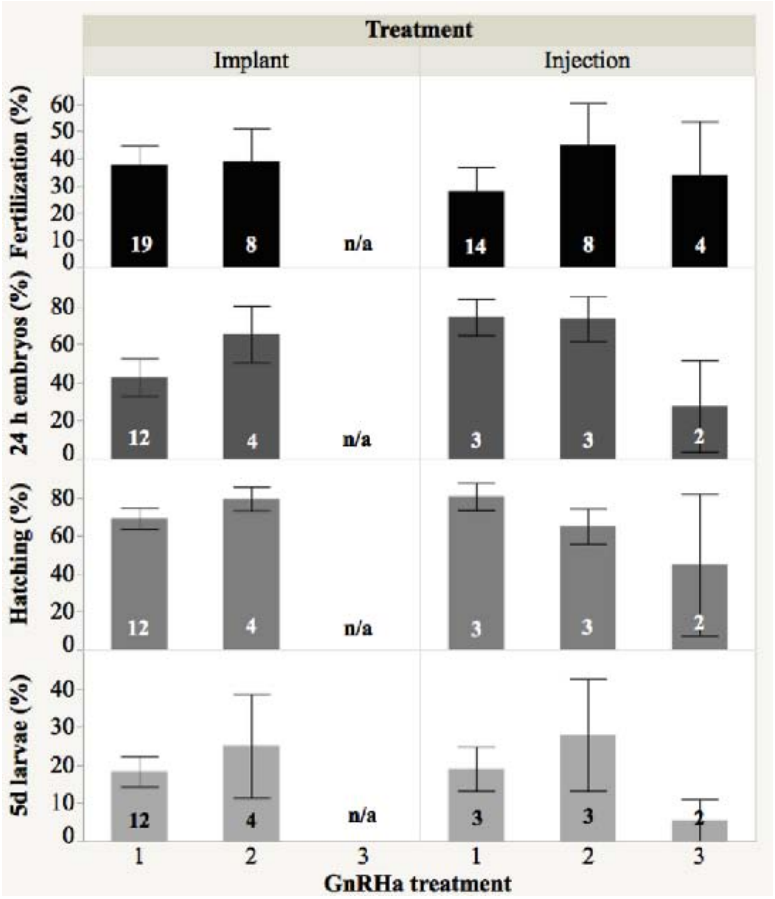

Figure 3.2.9. Mean (±SEM) fertilization (black), 24h embryo survival (dark grey), hatching (grey) and 5d larval survival (light grey) after consecutive treatments with GnRHa implants or injections. The numbers in bars indicate the spawns constituting each mean. No statistical differences were observed between different treatment methods or different treatment numbers (ANOVA, $P<0.05$).

3.2.5 Comparison of two GnRHa doses

Evaluation of reproductive stage and broodstock selection, evaluation of egg/larval quality, statistical analysis are common procedures used in sessions **3.2.4 Multiple GnRHa injections vs GnRHa implants, 3.2.5 Comparison of two GnRHa doses** and **3.2.6 Timing of GnRHa treatment application.** They are reported in 3.2.4 section.

3.2.5.1 Broodstock maintenance

Amberjack broodstock was kept at the facility of Argosaronikos Fish Farm S.A., on the island of Salamina (Greece). The same stock that was used for the purposes of **3.2.4 Multiple GnRHa injections vs GnRHa implants** in 2016, was also used for the comparison of two GnRHa doses. Twenty fish were used, 10 females (mean ± SD body weight 23.0±2.2 kg) and 10 males (mean ± SD body weight 18.4±1.9 kg) and maintained at a 1:1 sex ratio in the land-based tanks that were transferred.

3.2.5.2 Spawning induction experiments

Spawning induction trial was conducted between 7 June and 5 July 2017. Female fish were treated either with EVAc GnRHa implant of ~25 µg GnRHa kg^{-1} (group named as "LOW") or EVAc GnRHa implant of ~75 µg GnRHa kg^{-1} (group named as "HIGH") (Mylonas and Zohar, 2001), using implants loaded with 500–1000 µg of Des-Gly^{10},D-Ala^{6}-Pro-$NEth^{9}$-mGnRHa (H-4070, Bachem, Switzerland). In order to enhance spermiation and ensure the adequate sperm production, males were treated at the start of the experiment with EVAc GnRHa implant at a dose of 58.3±17.7 µg GnRHa kg^{-1} and divided randomly between the stocks. First hormonal treatment with GnRHa was given calculating the dose accordingly to an estimated 20 % growth rate from the previous year (7 June 2016). This caused variations in the effective GnRHa first dose applied to each fish. For EVAc treatments, further variations were due to the fact that implants are loaded with fixed amounts of GnRHa. Even though combinations of two implants loaded with different amounts of GnRHa were used when necessary, it was still not possible to adjust the dose exactly to the different body weight of the fish. The actual dose was 22.4 ±2.4 µg GnRHa kg^{-1} for LOW group and 74.4±4.5 µg GnRHa kg^{-1} for HIGH group, respectively. During the initial treatment, females were divided in four experimental groups in order to obtain two duplicates per treatment dose (2 and 3 fish per duplicate, respectively). Fish were divided equally between the two treatment methods according to their gonadal maturation stage. Before transferring the broodstock to experimental tanks, weight of each individual was recorded in order to calculate further doses accordingly to the actual fish weight. Females and males were treated again two weeks after (21 June 2017) with EVAc GnRHa implants of the same effective dose, since the actual dose was very close to the scheduled one. Four weeks after the start of the experiment, on 5 July 2017, final sampling was conducted and the fish were returned to the cage.

3.2.5.3 Results

Before the first treatment, females had mainly vitellogenic (Vg) oocytes, while 2 fish out of 10 were in advanced maturational stage. Some percentage of atresia was observed in almost all ovarian biopsies (**Fig. 3.2.10**, **first row)**. At the same time, males had IT sperm. During the second sampling, three fish from LOW group had still Vg oocytes, but also apoptotic oocytes and post ovulated eggs, while in one female ovarian biopsy was not feasible (**Fig. 3.2.10**, **second row)**. The last female from LOW group had mostly primary oocytes, and apoptotic oocytes as well. The 4 females from the HIGH group had Vg oocytes, with apoptotic oocytes and post ovulated eggs in their ovarian biopsies. One female was found with ovulated eggs and oocytes in maturation stage. The last female from this group had mostly primary oocytes with increased percentage of atresia. Males were still having IT sperm. On the last sampling, one female from each group had Vg oocytes, with increased percentage of POs and early signs of atresia. The rest were out of Vg oocytes, having mostly POs, post ovulated eggs, signaling the end of the reproductive season for the specific females (**Fig. 3.2.10**, **third row)**.

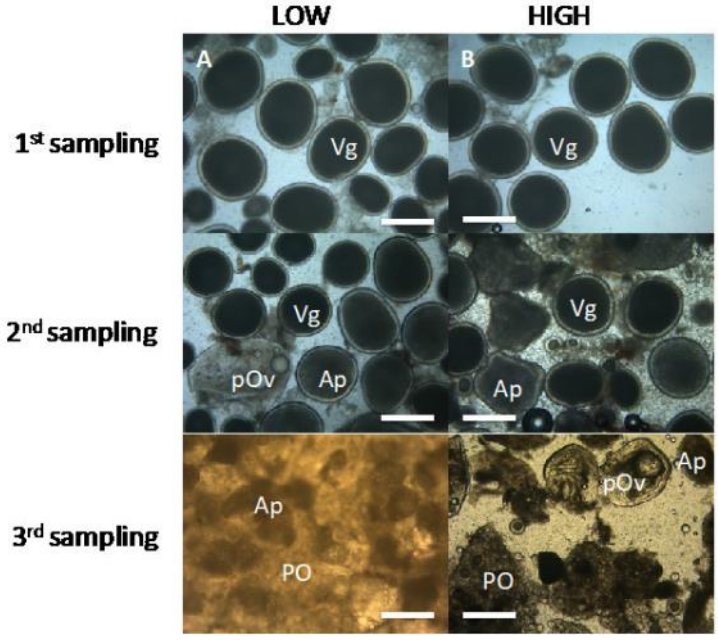

Figure 3.2.10. Microphotographs of representative ovarian biopsies from greater amberjack at different sampling times, presented as wet mounts. Fish were treated either with (A) LOW (~25 µg kg^{-1}) or (B) HIGH (~75 µg kg^{-1}) GnRHa dose using EVAc implants. PO=primary oocyte, Vg=vitellogenic oocyte, pOv=post Ovulated egg, Ap=apoptotic oocyte. Bars=500µm.

In both treatment doses, spawning started one day after 1st application because of the existence of oocytes in maturation stage. Fish spawned for 7-9 times after 1st treatment, while only 5 times after 2nd treatment. The higher egg production was observed in LOW group with 33,826 eggs kg^{-1} two days after 1st treatment, while in injected 30,206 eggs kg^{-1}, respectively (**Fig. 3.2.11)**.

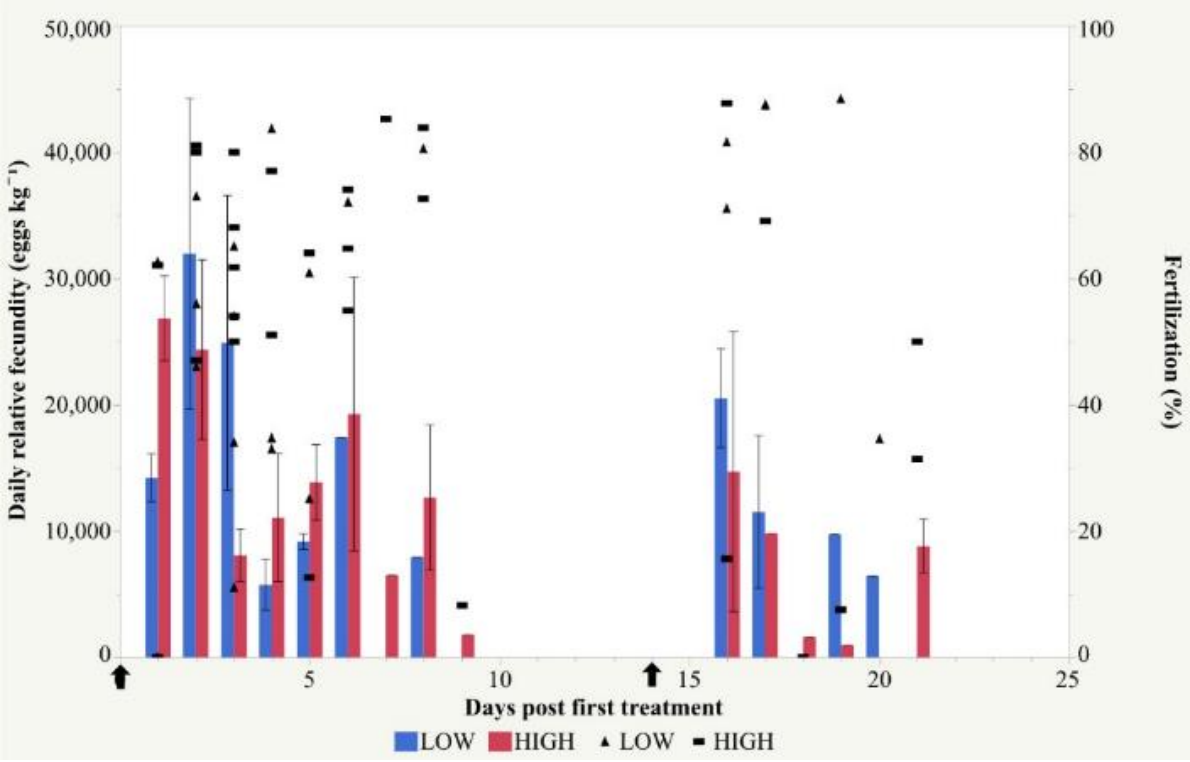

Figure 3.2.11. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of LOW (blue bars) and HIGH (red bars) groups of greater amberjack implanted with different doses of GnRHa. Arrows indicate the time of treatment. First application was done on 7 June 2017.

Mean daily relative fecundity was not significantly different among the LOW and HIGH dose groups. LOW group produced 17,801±4,127 eggs $kg^{-1}day^{-1}$ and HIGH group 14,648±2,285 eggs $kg^{-1}day^{-1}$ after the 1st treatment, respectively (**Fig. 3.2.12)**. After the 2nd treatment, LOW group spawned 13,373±3,022 eggs $kg^{-1}day^{-1}$ and HIGH group 8,484±3,228 eggs $kg^{-1}day^{-1}$. Fertilization success found to be different among the two doses at the 2nd treatment. On the other hand 24 h embryo survival, hatching and 5d larval survival was similar among treatment methods, while no statistical differences were observed among different treatment number (**Fig. 3.2.12)**.

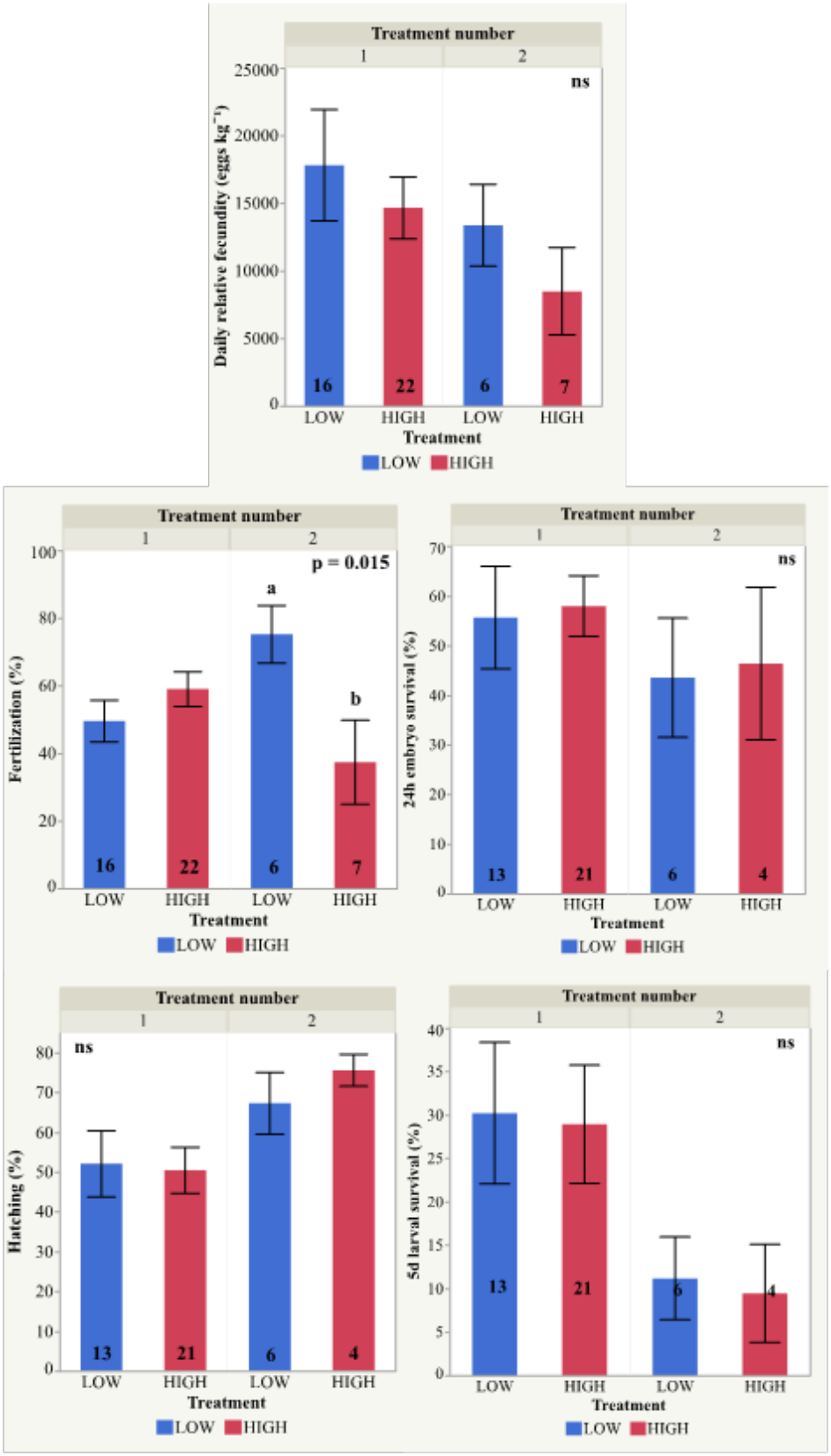

Figure 3.2.12. Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of LOW (blue) or HIGH (red) GnRHa dose group of greater amberjacks. Numbers in bars are the spawns constitute each mean. Lowercase letters indicate significant differences between different doses groups in 2nd treatment for fertilization percentage (t test, p=0.015). No other statistical differences were observed between different treatment methods or different treatment numbers (t test, $P < 0.05$).

3.2.6 Timing of GnRHa treatment application

Evaluation of reproductive stage and broodstock selection, evaluation of egg/larval quality, statistical analysis are common procedures used in sessions **3.2.4 Multiple GnRHa injections vs GnRHa implants, 3.2.5 Comparison of two GnRHa doses** and **3.2.6 Timing of GnRHa treatment application.** They are reported in 3.2.4 section.

3.2.6.1 Broodstock maintenance

Amberjack broodstock was kept at the facility of Galaxidi Marine Farms S.A., Galaxidi (Greece). Stock consisted of 26 fish, captured at the juvenile stage and acquired in May 2014 from Asteras SA (Astakos, Greece). Broodstock was maintained in a 1000 m^3 sea cage, 500 m offshore from the inland facility. On 29/3/2017, fish were split in two cages at a 1:1 sex ratio (12 fish in cage A and 14 fish in cage B), in order to minimize the stress for the fish (please see below for further explanation). Feed was given to apparent satiation 6 days a week using Skretting Vitalis CAL (22 mm) and fish were starved one day prior to handling. For the spawning induction trial, fish were transferred to the inland facility, to 23 m^3 flow through round tanks, accordingly to the treatment received, and maintained at a 1:1 sex ratio. Tanks were supplied with constant oxygen flow, surface seawater and exposed to ambient photo-thermal conditions. Measurements of temperature and oxygen saturation were conducted twice a day (AM, PM). Tank overflows were fitted with 250 L passive egg collectors. Feeding in the tanks was done to satiation.

3.2.6.2 Spawning induction experiments

Spawning induction trial was conducted between 30 May and 18 July 2017. Female fish were treated with EVAc GnRHa implant (Mylonas and Zohar, 2001), loaded with 750–1000 μg of Des-Gly^{10},D-Ala^{6}-Pro-NEth^{9}-mGnRHa (H-4070, Bachem, Switzerland), for an effective dose of 58 ± 9 μg GnRHa kg^{-1}. In order to enhance spermiation and ensure the adequate sperm production, males were also treated with EVAc GnRHa implant at a dose of 67 ± 6 μg GnRHa kg^{-1}. In order to estimate the best timing for GnRHa application, fish were split in four spawning induction groups. On 30 may 2017, three females and three males from cage A after reproductive evaluation were treated with GnRHa and transferred to the land-based tank for spawning (1st period). A week later, on 7 June 2017 the rest fish from cage A, were treated with GnRHa and transferred to a different land based tank for spawning (2nd period). In the same manner, three females and three males from cage B, after reproductive evaluation on 20 June 2017 and GnRHa treatment, fish were transferred to a land based tank (3rd period). The same number of fish, following the same procedure and using six from the rest fish in cage B were transferred for spawning in land based tank on 4 July 2017 (4th period). Each group remained in land based facilities for 14 days and then transferred back in sea cage, except from the fish from the 1st period that were transferred after 21 days, respectively. Prior to transfer back to the sea cage, evaluation of the reproductive stage of the fish was done.

3.2.6.3 Results

Before the first treatment, the females had mainly vitellogenic (Vg) oocytes of 740-760 μm in diameter. Atresia was present in one female, while signs of a past spawning event was visible in a second female fish (**Fig. 3.2.13**, **first row)**. Three weeks later, on 20/6/2017, there was variability in ovarian biopsies, since one was found to be fully apoptotic and primary oocytes were visible (PO), the second had Vg oocytes of 700 μm and no biopsy was obtained from the 3rd female. The reproductive evaluation on 6/6/2017 showed that the females were mostly in Vg stage of 730-760 μm in diameter and one of them had ovulated eggs (**Fig. 3.2.13**, **second row)**. Two weeks later that fish had still some Vg oocytes of 650-700 in diameter, and post ovulated eggs and atresia were present. In one fish oocytes in maturation stage were found. At the beginning of the 3rd period the females were mostly in Vg stage and early signs of atresia were present. (**Fig. 3.2.13**, **third row).** One female was more progressed and had oocytes in early maturation of 800 μm in diameter. At the end of this period the females had apoptotic oocytes and post ovulated eggs. One female had only POs (spent) and Vg oocytes of lower diameter were present in another one. The last period started with the

reproductive evaluation of the fish on 4/7/2017, where the females had again Vg oocytes of 650-680 μm in diameter (**Fig. 3.2.13**, **fourth row)**. At the end of this period, two weeks later, the females were spent, having POs, apoptotic oocytes or ovulated eggs. Males had IT sperm from the first sampling to the last one.

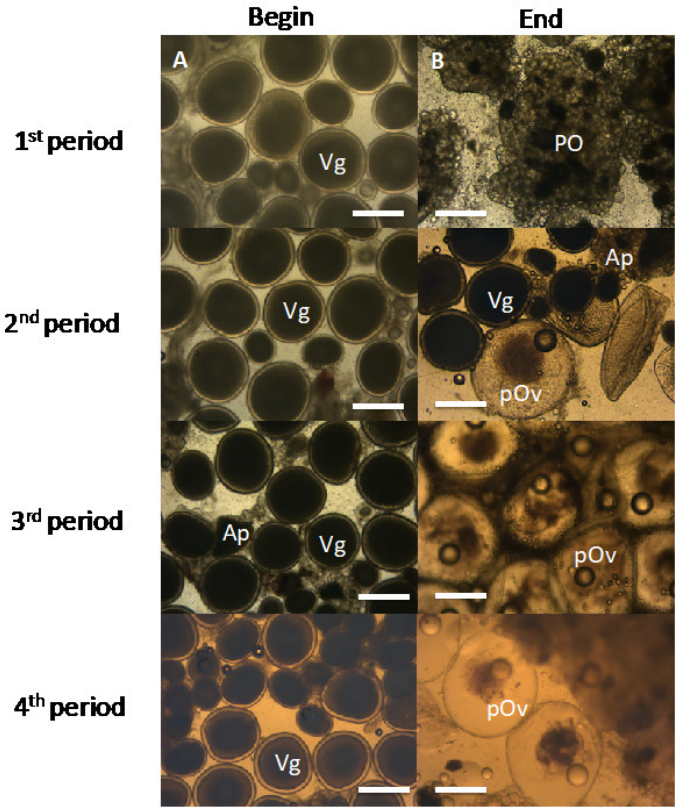

Figure 3.2.13. Microphotographs of representative ovarian biopsies from greater amberjack at different sampling times, presented as wet mounts. Fish were treated with GnRHa implants for spawning at 1st period (30/5/2017), 2nd period (6/6/2017), 3rd period (20/6/2017) and 4th period (4/7/2017). Column (A) presents pictures during the ovarian evaluation before each treatment and (B) after the end of spawning period in land-based tank. PO=primary oocyte, Vg=vitellogenic oocyte, pOv=post Ovulated egg, Ap=apoptotic oocyte. Bars=500μm.

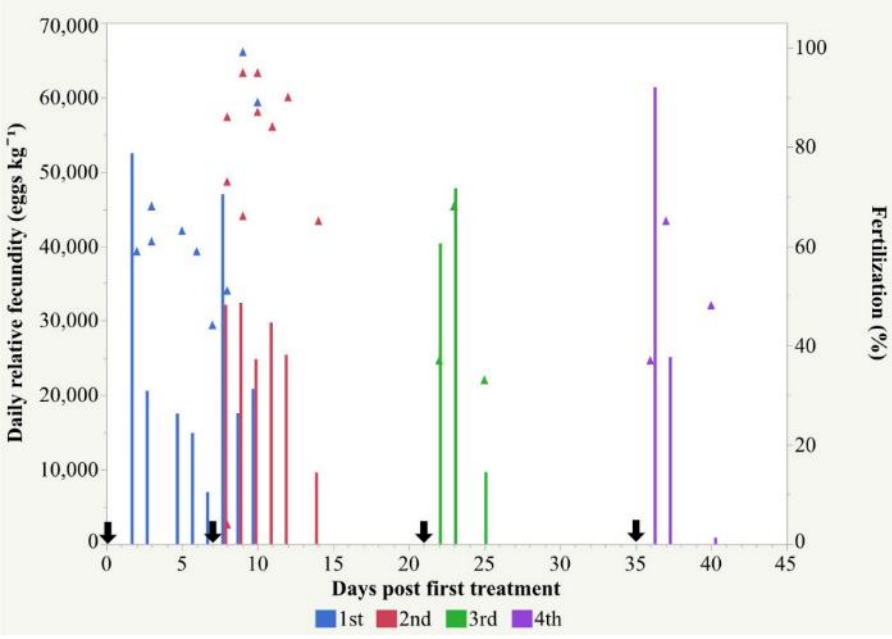

Figure 3.2.14. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of 1st (blue bars), 2nd (red bars), 3rd (green bars) and 4th (purple bars) period of GnRHa treatment of greater amberjack. Arrows indicate the time of treatment. First application was done on 30 May 2017.

Mean daily relative fecundity was not significantly different between the four periods of GnRHa treatment and was 27,173±3,144 eggs $kg^{-1}day^{-1}$ (**Fig. 3.2.14 and Fig. 3.2.15)**. The first two periods the number of spawns was higher (9 and 10, 1st and 2nd period, respectively) compared to the last two periods (3 for 3rd and 4th period, respectively). Mean fertilization was 65±4%, mean 24h embryo survival 79±5%, mean hatching 60±8% and 5-days larval survival 23±5%, respectively (**Fig. 3.2.15)**.

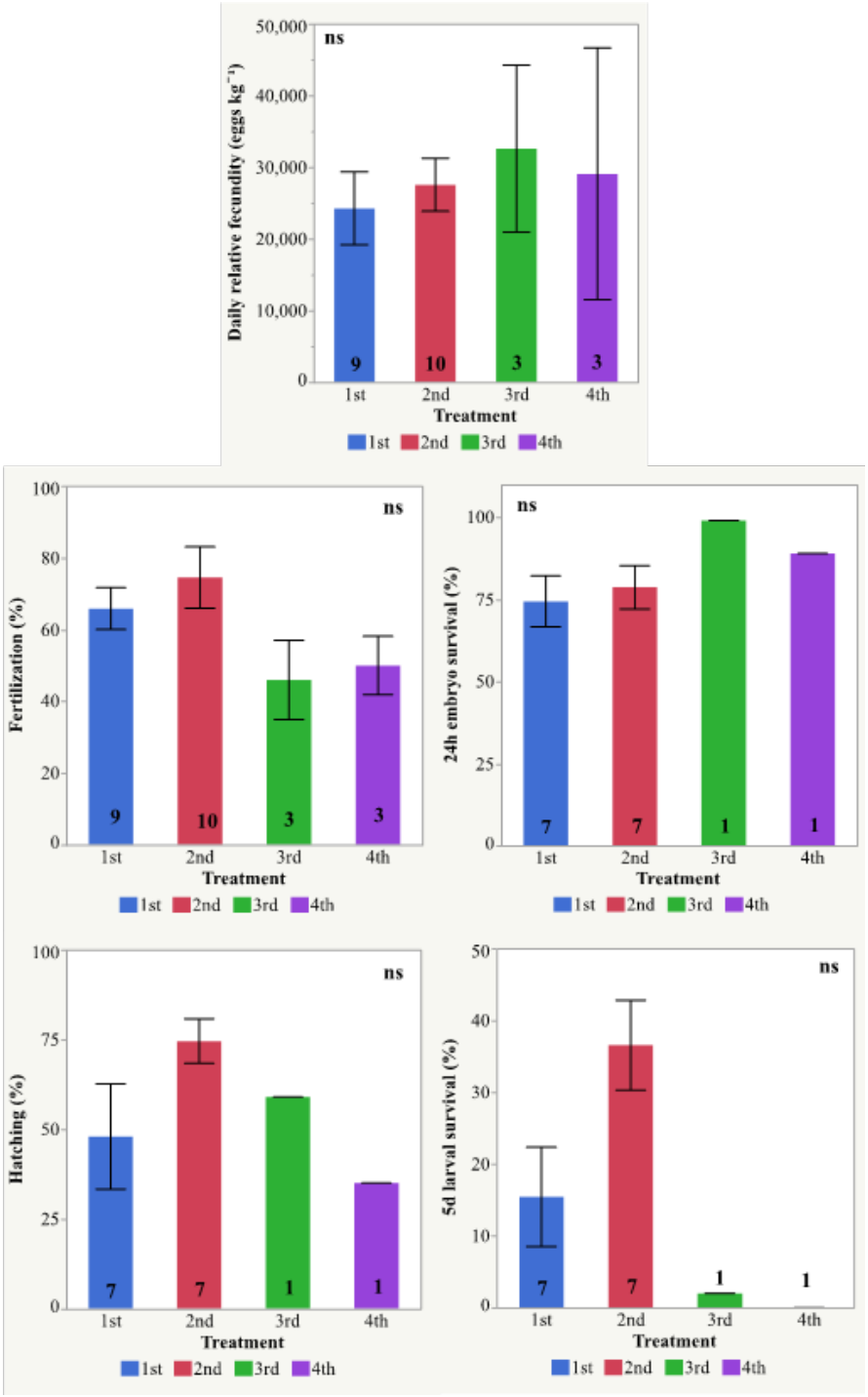

Figure 3.2.15 Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of 1st (blue bars), 2nd (red bars), 3rd (green bars) and 4th (purple bars) period of GnRHa treatment of greater amberjack. Numbers in bars are the spawns constitute each mean. No statistical differences were observed between different periods of GnRHa application (ANOVA, $P < 0.05$).

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Task 3.3 Development of an optimized spawning induction protocol for captive greater amberjack in the eastern Atlantic (led by FCPCT, Marisol Izquierdo).

This task was accomplished and fully described in the 1st and 2nd Periodic Reports and in ***Deliverable 3.7 Comparative effectiveness of a GnRHa injection vs GnRHa implant treatment for the induction of spawning of greater amberjack in the eastern Atlantic.***

Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic (led by IEO, Salvador Jerez Herrera).

3.4.1 Spawning induction protocol for F1 greater amberjack during 2016 spawning season

3.4.1.1 Experimental conditions

The experiments carried out in the present subtask involved IEO and HCMR staff and were developed during the 2016 spawning season. A group of 9 greater amberjack breeders born in captivity (average weight 18.3±6.3 kg) were maintained in a 500 m^3 outdoor covered raceway tank with continuous water supply (6 renewals day^{-1}) under natural photoperiod in the facilities of IEO in Tenerife, Canary Islands (Spain). Broodstock (3 males and 5 females) were tagged with passive integrated transponders (PIT tags).

All fish were sampled and weighed five times during the spawning season (June, July, August, September and October). Ovarian biopsies for the evaluation of oocyte development were obtained and a wet mount of the biopsy was examined under a compound microscope to evaluate the stage of oogenesis and measure the mean diameter of vitellogenic oocytes. A portion of the biopsy was fixed in a solution of 4% formaldehyde:1% glutaraldehyde for further histological processing. Maturation of males was examined by the release of sperm upon application of gentle abdominal pressure. If this was not possible, a sperm sample was obtained by a plastic catheter. The collected sperm was stored at 4°C until quality evaluation. In order to measure sex steroid hormone concentrations and biochemical parameters, blood was collected at each sampling from the caudal vessels using heparinized syringes and centrifuged at 1400 rpm for 20 min. Plasma was then collected and stored at −80 °C until analysis.

Fish were treated with an Ethylene–Vinyl acetate (EVAc) GnRHa implant loaded with Des-Gly10, D-Ala6-Pro-NEth9-mGnRHa (H-4070, Bachem, Switzerland) in June, July, August and September. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and intra-testicular sperm was observed in males. The selected females were administered the GnRHa implants to obtain an effective dose

of ~75 µg GnRHa kg^{-1} body weight (**Table 3.4.1**). The dose of GnRHa implanted to males was about 60 µg GnRHa kg^{-1} body weight in each monthly treatment, from June to September (**Table 3.4.1**).

Table 3.4.1. Number of biopsied and treated fish and body weight (mean ± SEM) at each treatment sampling. All fish were treated with a GnRHa implant, with a dose of ~75 µg GnRHa kg^{-1} body weight for females and ~60 µg GnRHa kg^{-1} body weight for males.

Sex		Females				Males			
Sampling (Month)	Treatment	Treated (Biopsied)	Weight (mean±sd)			Treated (Biopsied)	Weight (mean±sd)		
June	First	5 (5)	20.1	±	6.8	3 (3)	15.3	±	4.8
July	Second	5 (5)	18.5	±	6.2	3 (3)	14.4	±	4.2
August	Third	5 (5)	18.8	±	6.0	3 (3)	15.4	±	4.9
September	Fourth	2 (5)	19.6	±	5.3	3 (3)	15.2	±	4.6
October		0 (5)	17.0	±	6.0	0 (3)	14.2	±	3.9

Sperm quality parameters included (a) sperm concentration (number of spermatozoa ml^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (sperm motility, %), (c) duration of forward sperm motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4°C (sperm survival, days).

At the expected onset of the spawning season (May 2016), a passive egg collector was placed in the outflow of each spawning tank and checked daily, in order to collect the spawned eggs. Eggs were collected every morning and their number (fecundity) was estimated by counting the total number of eggs. Fertilization success was evaluated by the presence of a viable embryo using a stereoscope. The diameter of eggs was measured using a binocular microscope. Each spawning was incubated in a 90 l tank with gentle aeration and a supply of filtered water.

To monitor embryo and larval survival, eggs from each spawn were periodically placed individually in 96-well microtiter plates according to the procedure of Panini et al. (2001), with some modifications. The number of (a) live embryos, was recorded 1 day after egg collection (or ~36 h after spawning, day 1), (b) hatched larvae, was recorded 2 and 3 days after egg collection (>60 h after spawning) and (c) viable larvae, was recorded 4 and 5 days after egg collection (~ yolk sac absorption). Embryo survival was calculated as the number of eggs having live embryos 1 d after egg collection / number of fertilized eggs initially loaded in the microtiter plates. Hatching success was calculated as number of hatched larvae / number of live embryos, and 2-5-d larval survival was calculated as number of live larvae 2-5 d after egg collection / number of hatched larvae.

Total erythrocytes and leucocytes were determined by counting using a Neubauer haemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation. Plasma levels of protein, triglycerides, cholesterol, glucose, lactate and enzymes (alkaline phosphatase and amylase) were measured in duplicates by enzymatic colorimetric assays (Biosystems, Spain). Plasma concentrations of sodium (Mg-Uranylacetate Method) and potassium (TPB-Na Method) were determined using standard spectrophotometric assays (Spinreact, Spain).

3.4.1.2 Results

Mean sperm motility percentage was higher than 40% and showed the maximum value at the middle of the samplings period (August) while the duration of sperm motility was 2.3 ±1.0 min. Mean sperm density was 14.3 x 10^{10} spermatozoa ml^{-1} and it ranged between 8.6 x 10^{10} in July to 22.4 x 10^{10} spermatozoa ml^{-1} in October, although no significant differences were observed among samplings. Mean sperm motility was 54±29% during the reproductive period and it was highest in August (85±7%) but no differences were

observed among samplings. On the contrary, motility duration was significantly higher in September and October samplings (4.4±1.1min) comparing to the previous samplings in June and August (**Figure 3.4.1**).

The number of spawning obtained in the successive post treatment periods remained unchanged throughout consecutive samplings (from June to August), as well as the number of eggs released (**Fig. 3.4.2**). Moreover, the spawning events were concentrated immediately after the application of each treatment. After the first 2 treatments the eggs were collected during 31 days in 20 and 23 spawning events, respectively. However, after the 3rd treatment a total of 17 spawning were recorder during the first 22 days and no eggs were collected after further treatments.

Mean fertilization and hatching exhibited similar trends during the three spawning periods, reaching their highest values in the second period (July) (**Fig. 3.4.2**).

The oocyte diameter during the successive samplings ranged between 440 μm in July to 720 μm in October, but no significant differences was observed.

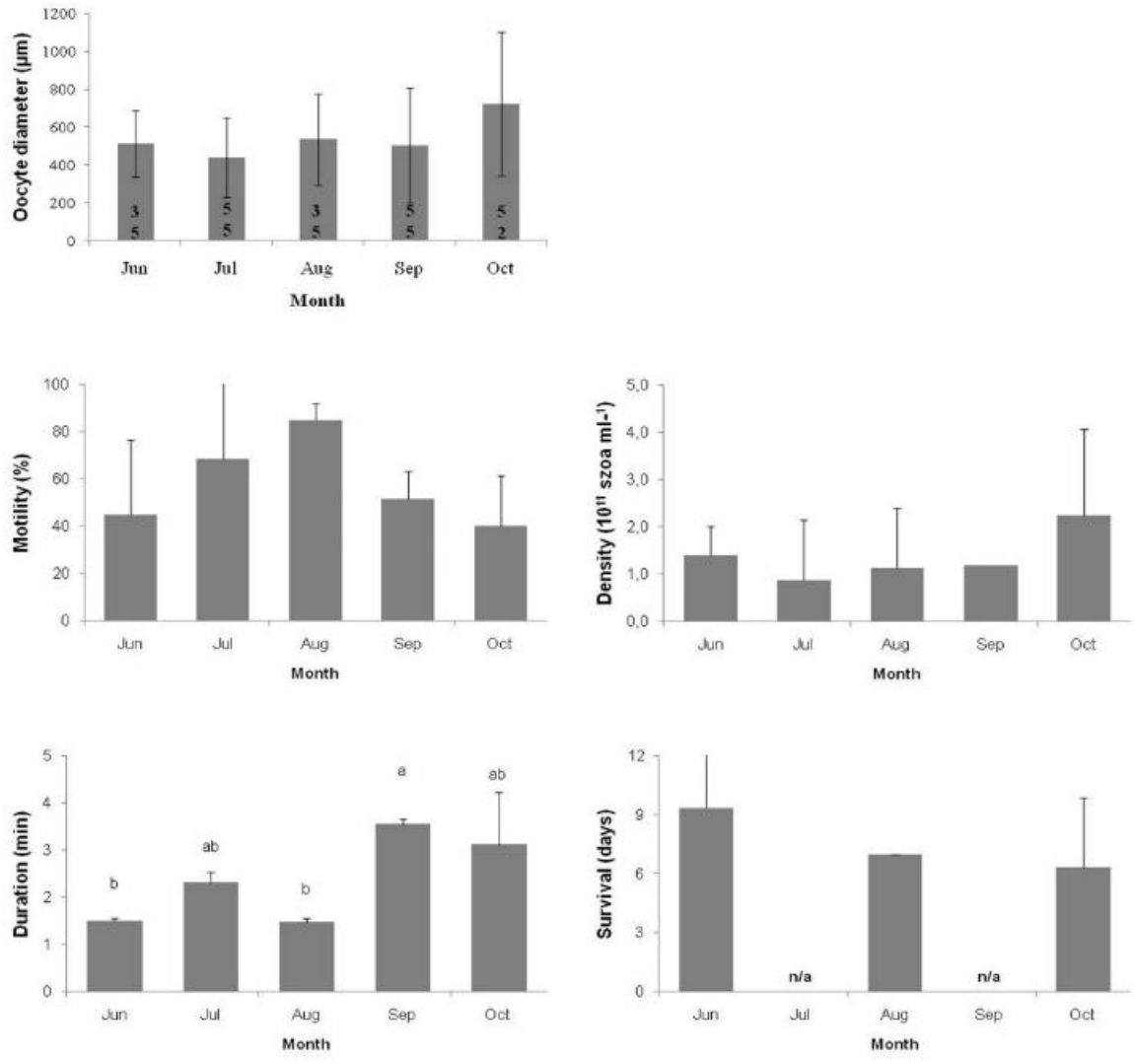

Figure 3.4.1. Mean (± SE) oocyte diameter and sperm quality parameters of greater amberjack at each sampling-treatment. Statistically significant differences among months are indicated by different lower case letters ($P < 0.05$).

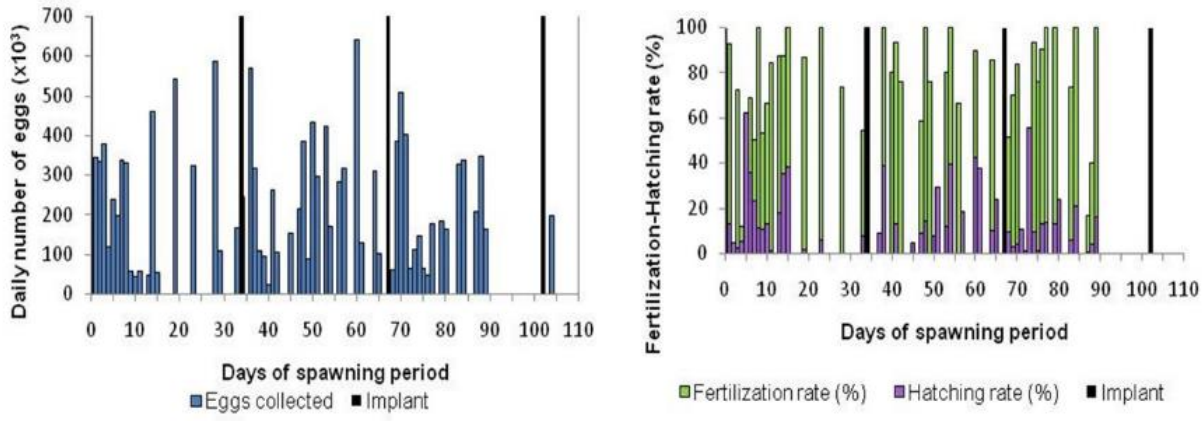

Figure 3.4.2. Daily number of eggs collected and fertilization and hatching rate registered during the experimental spawning period in 2016. The black bars indicate the day of the implant treatment - sampling.

All blood parameters studied remained constant along the study and only erythrocytes, leucocytes and plasma protein changed slightly during the experimental period (**Table 3.4.2**).

Table 3.4.2. Erythrocytes (x10^4), leucocytes (x10^3), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), alkaline phosphatase (U/L), amylase (U/L), along the spawning season. Values are means ± SEM. (ANOVA, P<0.05).

Females	June				July				August				September				October			
Erythrocytes	244.88	±	30.04	b	211.5	±	40.21	b	465.00	±	31.21	a	161.75	±	30.42	b	243.38	±	57.67	b
Leucocytes	143.20	±	17.66	a	127.05	±	50.41	ab	81.06	±	10.43	ab	28.05	±	6.96	b	28.20	±	8.63	b
Hematocrit	45.44	±	4.61		46.33	±	6.60		45.58	±	6.07		39.88	±	5.75		47.61	±	7.15	
Cholesterol	165.83	±	10.91		190.60	±	9.35		206.62	±	13.86		209.70	±	29.59		210.32	±	31.49	
Protein	52.47	±	5.21	b	62.81	±	5.40	b	48.13	±	3.01	b	91.33	±	5.16	a	45.00	±	2.44	b
Triglycerides	243.00	±	34.97		228.99	±	37.03		296.43	±	20.83		278.93	±	88.02		251.64	±	51.09	
Alkaline phosphatase	98.34	±	6.46		65.76	±	6.31		78.93	±	17.88		86.97	±	12.57		126.78	±	22.15	
Amylase	11.19	±	1.98		10.97	±	1.66		13.35	±	2.73		10.10	±	1.13		7.68	±	1.10	

Stress secondary responses included changes in plasma ions and metabolite levels (e.g., increased in glucose, lactate, and decreased in plasma sodium and potassium). In this study, an increase in plasma lactate together with a drop in plasma sodium (**Figure 3.4.3**) was observed in August, concomitantly with the 3rd treatment.

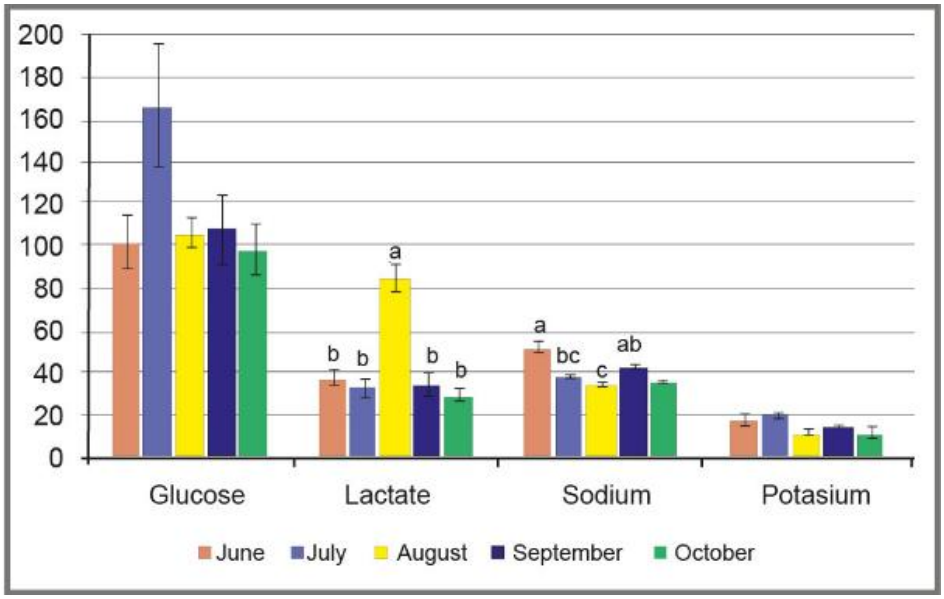

Figure 3.4.3. Plasma glucose (mg dl^{-1}), lactate (mg dl^{-1}), sodium (mg dl^{-1}) and potassium (mg dl^{-1}) in females greater amberjack during experimental spawning period in 2016. Values are means ± SEM. (ANOVA, $P < 0.05$)

3.4.2 Spawning of F1 greater amberjack during 2017 spawning season

3.4.2.1 Experimental conditions

The experiments carried out in the present subtask were development during the 2017 spawning season. A group of 4 greater amberjack breeders (3 males and 1 female) born in captivity (average body weight 17.3±6.9 kg) were maintained in an outdoor covered circular tank of 50 m^3 with continuous water supply under natural photoperiod in the facilities of IEO in Tenerife, Canary Islands (Spain).

At the expected onset of the spawning season (May 2017), a passive egg collector was placed in the outflow of the spawning tank and checked daily, in order to collect the spawned eggs.

The fish were ready to be treated in the middle of June with the lowest dose of GnRHa proposed in the DOW (~25 µg GnRHa kg^{-1} body weight for females and ~60 µg GnRHa kg^{-1} body weight for males). However, a first spawning event was obtained before applying the GnRHa treatment and therefore the induction protocol planned for the spawning season in 2017 was not applied.

Eggs were collected every morning and their number (fecundity) was estimated by counting the total number of eggs. Fertilization success was evaluated by the presence of a viable embryo using a stereoscope. The diameter of eggs was measured using a binocular microscope. Each spawning was incubated in a 90 l tank with gentle aeration and a supply of filtered water.

3.4.2.2 Results

The F1 greater amberjack group (1 female and 3 males) stocked in a 50 m^3 circular outdoor tank carried out a total of 21 spontaneous spawning events during 125 days (**Fig. 3.4.4**).

The eggs were released each 5 or 10 days during the natural spawning season. The lowest number of eggs was collected in the period between mid-July and mid-August, coinciding with a longer period of time between successive spawn events.

Mean fertilization and hatching rates exhibited similar trends during the spawning season, reaching their highest values from mid-July to mid-October (**Fig. 3.4.4**).

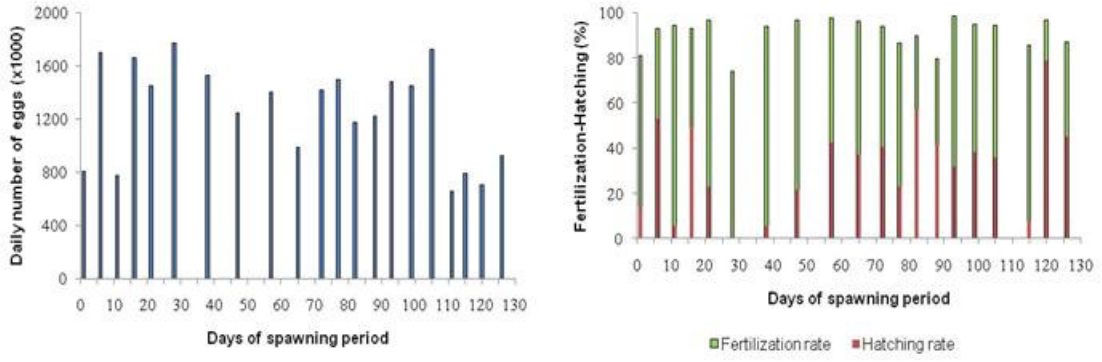

Figure 3.4.4. Daily number of eggs collected and fertilization and hatching rate registered during the experimental spawning period in 2017.

3.4.3 Conclusions

Hatchery produced greater amberjack (F1 generation) were able to finalize vitellogenesis and spermiation, and underwent repeated spawning for 4 months with a total production of almost 22 million eggs after treatment with GnRHa implants dose of ~75 micrograms. These results are better than those obtained with a lower dose (~50 micrograms).

In addition, during the spawning season 2017, 21 spontaneous spawning events were obtained from a single untreated female, with more than 25 million eggs produced.

The successful reproduction of F1 greater amberjack broodstock, is an important step towards the industrial aquaculture production of this valuable species.

References

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Task 3.5 Spawning induction of greater amberjack and egg collection in cages (led by HCMR, Constantinos Mylonas).

As was written in the 2nd Periodic Report, during the spawning season of 2016, efforts were made in HCMR and GMF to improve the egg collecting capacity of the cage, using a different setting. The egg collectors were mounted again to a depth of 5-m deep, as the previous year. However, this year the bottom of the cages was lifted even more, so that all the vertical sides of the cage were covered with the egg collector. At both the GMF and HCMR site, the bottom of the cage was covered with an extra fine mesh to reduce the possible currents that remove the eggs from the egg collector, and also prevent the eggs from passing through (**Fig. 3.5.1**). At HCMR, the fish were allowed to spawn spontaneously without any hormonal treatment, as we saw in the previous year that at any time we sampled the fish to induce them to spawn, some females contained oocytes at OM, suggesting that some spontaneous spawning does take place without any hormonal therapies. At GMF, the fish were induced to spawn and remained in the cage for spawning.

Figure 3.5.1. Underwater photo of the broodstock cage of HCMR, Souda Bay, at a depth of 5 m showing the new modified egg collection system. The blue tarpaulin (egg collector) is covering the side of the cage until 0.5 m from the bottom, while the green shading mesh is covering the bottom of the cage. A side section of about 0.5 m of the side of the cage is left unblocked to allow for water exchange in the cage.

As a result, at the GMF cage 200,000 eggs were collected on 18/6/2016 (**Table 3.5.1)**. At the same period (1/6-23/6/2016), eggs were also collected from the HCMR cage in Souda**.** The fish spawned 6 times spontaneously, but unfortunately only a very small and variable quantity of eggs (2-500 g) was collected. Reproductive evaluation of the fish was done only at the end of spawning season in order not to disturb the fish during their spontaneous spawning (please see above in section 3.2.2 for further details). Some of these eggs were used for the larval rearing trials of HCMR, with very satisfactory results, suggesting that it is possible for some eggs to be collected from the cages, without any hormonal stimulation. However, the very small amount that was collected suggests that (a) only a very small percentage of the females spawned and (b) only for a limited number of spawns, contrary to what has been achieved in response to a hormonal therapy with GnRHa.

For the 2017 spawning season, the GMF stock was not used for the purposes of this Task, since the fish were used for the timing of GnRHa treatment application experiment (See above in 3.2.6 for further details). On the other hand, in the HCMR sea cage we repeated the trial again leaving the fish to spawn spontaneously, without any hormonal treatment. However, instead of not examining the fish for maturity status until the end of the reproductive season in July, we planned to intervene in the middle of the season in June (see above Section 3.2.2 for further details). This was done in order to (a) document how many fish were spawning in the days before and (b) to induce all the fish to spawn so that we could determine what percentage of the spawned eggs we would collect. At the end, a very small number of eggs (a few 1000s) was collected at three times (**Table 3.5.1)** and when the fish were evaluated towards the end of June, females had mostly POs and AT oocytes, with two of them having signs of possible previous ovulations. So, unfortunately again we were not able to conclusively confirm that the egg collecting method used, with complete covering of the cage walls and extensive covering of the bottom of the cage is capable of being used for the large scale collection of fertilized eggs from greater amberjack, since we do not know (a) how many fish spawned and (b) how many times.

Table 3.5.1. Egg collection in sea cages after or without hormonal treatment with GnRHa implants of greater amberjack broodstock.

2016

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (number or g)
GMF	3	1	200.000
HCMR Souda	0	1	eggs*
	0	2	500 g
	0	3	3 g
	0	4	5 g
	0	5	10 g
	0	6	2 g

2017

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (number or g)
HCMR Souda	0	1	15 g
	0	2	10 g
	0	3	4 g

*eggs were present but not collected

Deviations from Annex I and their impact:

According to the original plan, relatively high quantities of recombinant leptin were produced and purified, which enabled the generation of specific polyclonal antibodies. These antibodies were found to be highly specific to the recombinant antigen, yet, failed to detect native greater amberjack leptin. While it could be interesting to compare circulating leptin profiles during the reproductive cycle in wild vs. captive reared greater amberjack, the impact on the deliverable is negligible, particularly as we were able to follow the expression levels of the leptin gene and highlight a time window during which it appears to play a significant role (D3.5, D3.6; Zupa et al., 2017).

Also, during the gonadal development there is an important mobilization of nutrients, mainly protein, lipids, with specific lipid classes and fatty acids, and carotenoids, from the liver and the muscle to the gonads and towards the eggs. Therefore, the analysis of the proximal composition (protein, fat content, moisture and ash), and also the complete profile of lipid classes and fatty acids and total contents of carotenoids of the available samples of gonads and eggs was prioritized. There was not enough sample left for the foreseen determinations of vitamins C and E.

WP 4 Reproduction & Genetics – pikeperch

WP No:	4	**WP Lead beneficiary:**		P1. HCMR
WP Title (from DOW):	Reproduction and Genetics – pikeperch			
Other beneficiaries (from DOW):	P1. HCMR	P9. UL		
Lead Scientist preparing the Report (WP leader):	Costas Tsigenopoulos			
Other Scientists participating:	Pascal Fontaine (P9)			

Objectives

1. Evaluate the genetic variability of captive broodstock in commercial RAS farms in Europe.
2. Compare this variability with the variability of wild individuals and define how a future genetic breeding program should be established for sustainable optimal performances through domestication of pikeperch.

Summary of work reported in the previous Reporting Period (1-12 Mo):

In the 1st Reporting Period, the evaluation of the genetic variation in captive pikeperch broodstocks (Task 4.1) has been completed and the *Deliverable 4.1 Genetic analysis of domesticated pikeperch broodstocks* was completed and submitted to the EU. We initially optimized two microsatellite multiplexes with seven and four loci and more than 400 breeders sampled from 6 countries were genetically screened. Genetic analysis of domesticated pikeperch broodstocks, provided a first assessment of the genetic diversity of captive pikeperch stocks and because there are only a few (around 10) commercial hatcheries that produce pikeperch in Europe, the genetic diversity was expected to be relatively lower compared to the genetic variability of natural populations (Saisa et al., 2010). In principle, each pikeperch farm uses its own stock, captured either from the wild or supplied by another farmer. Therefore, pikeperch populations differ from one farm to another depending upon the geographical origin of the captured wild populations, which were used as the starting base of the captive stocks.

The results have indicated that some broodstocks have adequate genetic variation and few of them originate from few fish and attention should be paid in the future to establish breeding programmes. In general, there was agreement with the stock origin and Finnish and Hungarian stocks from different companies are clustered together.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The objectives to evaluate the genetic variability of captive pikeperch broodstocks and make a comparison with wild individuals to define future breeding programs have been completed and the two associated deliverables have been submitted. A total of 21 populations / broodstocks were sampled and analysed, which included 13 captive broodstocks analysed in **Task 4.1 Evaluation of the genetic variation in available domesticated broodstocks of pikeperch**, and eight wild origin population analysed in **Task 4.2 Evaluation of the genetic variation in non-domesticated broodstocks of pikeperch**. The different stocks were grouped into three populations that were of Hungarian origin, Scandinavian origin and other origins (German, Polish and Tunisian). The different captive broodstock populations presented different levels of genetic variability that ranged from wide variability greater than observed in wild populations to broodstocks

that had reduced genetic variability that may have been the result of loss of variability through inbreeding. These broodstocks with reduced genetic variability should take measures to introduce greater variation into the base population for future breeding programs.

Summary of progress towards objectives (31-48 Mo):

No work has been carried out during this period, as all work has been completed during the previous reporting periods.

Details for each Task

Task 4.1 Evaluation of the genetic variation in available domesticated broodstocks of pikeperch (led by UL, Pascal Fontaine)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***D4.1 Genetic analysis of domesticated pikeperch broodstocks***.

Task 4.2 Evaluation of the genetic variation in non-domesticated broodstocks of pikeperch (led by HCMR, Costas Tsigenopoulos).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***D4.2 Population genetic analysis of wild and comparison with domesticated pikeperch populations to be applied in future breeding programs of the species***

Deviations from Annex I and their impact:

There were no deviations during the 3rd Reporting Period.

WP 5 Reproduction & Genetics – Atlantic halibut

WP No:	5	**WP Lead beneficiary:**		P7. IMR
WP Title (from DOW):	Reproduction and Genetics – Atlantic halibut			
Other beneficiaries (from DOW):	P1. HCMR	P17. NIFES	P22. SWH	
Lead Scientist preparing the Report (WP leader):	Birgitta Norberg			
Other Scientists participating:	Constantinos Mylonas (P1), Kristin Hamre (P17), Borre Erstad (P22), Joan Cerda (P3)			

Objectives

1. Improve fecundity and gamete quality in F1/F2 broodstock.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut

- Established wild caught broodstock had more regular ovulatory cycles and a higher fecundity than F1 broodstock.
- The F1 fish were first time spawners, which may have contributed to their poor performance.

Task 5.2 GnRHa implant therapy as a means to improve spawning performance

- A pilot study of GnRHa implantation in F1 breeders showed that 50 µg kg^{-1} GnRHa was sufficient to induce final maturation and ovulation.
- Most of the GnRHa implanted fish ovulated earlier and gave more eggs than sham-implanted (control) females. However, due to a low number of individuals (n value), results were not determined to be significant.

Task 5.3 Fecundity regulation

- Initial samples were taken by ovarian biopsy for analysis of fecundity regulation.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the second reporting period, advances were made in all tasks. In **Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut** there were few differences between fecundity, fertilisation, hatching, egg size and hormone content between eggs from wild-caught and farmed females. However, although there were few significant differences, wild-caught females appeared to be more predictable spawners and gave fewer but larger batches of eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified correctly and stripping was carried out close to ovulation –thus reducing or eliminating over-ripening. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the very considerable workload connected with spawning and egg collection in Atlantic halibut. Another approach explored in **Task 5.2 GnRHa implant therapy as a means**

to improve spawning performance would be to ensure (and regulate) ovulation using a GnRHa implant. The GnRHa implants did ensure and synchronize ovulations of the treated females and were found not to affect egg quality or quantity. During this reporting period no work was done in **Task 5.3 Fecundity regulation**. Samples were collected during the first reporting period and will be analysed during the third reporting period. This deviation from the DOW has been approved by the PC and is explained in the report below.

Summary of progress towards objectives (31-48 Mo):

While wild-caught females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, hatchery-produced F1/F2 females appear to suffer from a reproductive dysfunction, releasing small batches of eggs at irregular intervals. Consequently, reproductive performance of domesticated, wild-caught halibut and farmed (F1) females was compared in task 5.1. Our results showed no differences in fecundity between wild-caught and farmed females, but ovulatory intervals seemed more irregular in the farmed broodstock.

Fertilization and hatching rates were lower and egg diameter was slightly but significantly lower in farmed females. To investigate possible differences in endocrine regulation of maturation, in **Task 5.3 Fecundity regulation**, blood samples were taken at 3-5 week intervals from September 2016 to July 2017. The samples were analysed for the sex steroids estradiol-17ß and testosterone, and the gonadotropins Fsh and Lh. This is the first report of plasma concentrations of Fsh and Lh in Atlantic halibut.

Plasma profiles of sex steroids and gonadotropins were similar in farmed and wild-caught females, although average Fsh concentrations were higher during gametogenesis, and E2 and T appeared to reach peak concentrations earlier in wild-caught fish. The individual variation was large, however, making it difficult to conclude that any important differences were present between the farmed and wild-caught females.

Details for each Task

Task 5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut (led by IMR, Birgitta Norberg)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***D5.1 Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut***

Task 5.2 GnRH implant therapy as a means to improve spawning performance (led by HCMR, Constantinos Mylonas)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 5.2 An optimized GnRHa therapy protocol to improve spawning performance of F1/F2 Atlantic halibut, and to increase availability of eggs of stable and predictable quality.***

Task 5.3 Fecundity regulation (led by IMR, Birgitta Norberg).

Work done in task 5.1 showed some significant differences in spawning performance between wild-captured and farmed Atlantic halibut females. The Atlantic halibut is a group-synchronous, periodic spawner and in captivity wild-captured females will release 6-12 batches of eggs during a period of 2-4 weeks in the spawning season, which lasts from late February to late April in southwestern Norway. In order to obtain eggs with high viability, females have to be stripped according to their individual ovulatory rhythms, to prevent over-ripening and deterioration of the eggs (Norberg et al., 1991). While wild-caught females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, hatchery-produced F1/F2 females appear to suffer from a reproductive dysfunction, releasing small batches

of eggs at irregular intervals. Consequently, reproductive performance of domesticated, wild-caught halibut and farmed (F1) females was compared in task 5.1. Our results showed no differences in fecundity between wild-caught and farmed females, but ovulatory intervals seemed more irregular in the farmed broodstock (**Table 5.3.1**).

Table 5.3.1. Biometric and spawning performance data of domesticated and farmed halibut breeders at IMR, Austevoll (from Deliverable D5.1)

	Domesticated females	**Farmed (F1) females**
n	3 (4[1])	5
length (cm)	150.7 ± 6.2	113.4 ± 3.9*
weight (kg)	48 ± 5.7	19.2 ± 2.3*
number of batches ·female^{-1}	7.3 ± 0.6	9.4 ± 1.7
spawning interval (hours)	82.2 ± 8.4	72.4 ± 22.9
batch volume (mL)	2300 ± 900	700 ± 300*
total fecundity (mL·female^{-1})	16700 ± 420	6800 ± 130*
relative fecundity (mL·kg^{-1})	347 ± 70	349 ± 84
average fertilization (%)	89 ± 7	61 ± 29

[1] One domesticated female was left undisturbed for most of the season, due to a large skin lesion, and was not included in calculations.

*=significant difference

Fertilization and hatching rates were lower (**Fig. 5.3.1**) and egg diameter was slightly but significantly lower in farmed females (**Fig. 5.3.2**). To investigate possible differences in endocrine regulation of maturation, blood samples were taken at 3-5 week intervals from September 2016 to July 2017. The samples were analysed for the sex steroids estradiol-17ß and testosterone, and the gonadotropins Fsh and Lh. This is the first report of plasma concentrations of Fsh and Lh in Atlantic halibut.

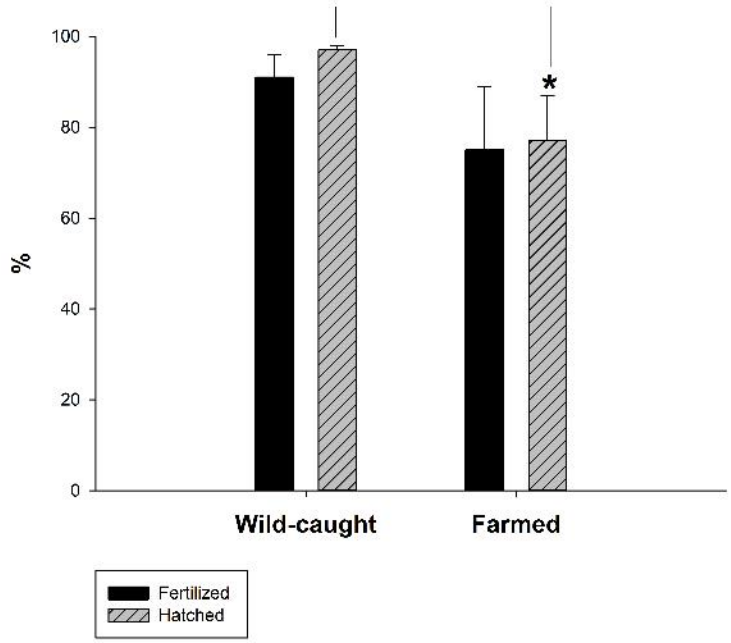

Figure 5.3.1 Fertilization and hatching rates in eggs from wild-caught and farmed female halibut broodstock.

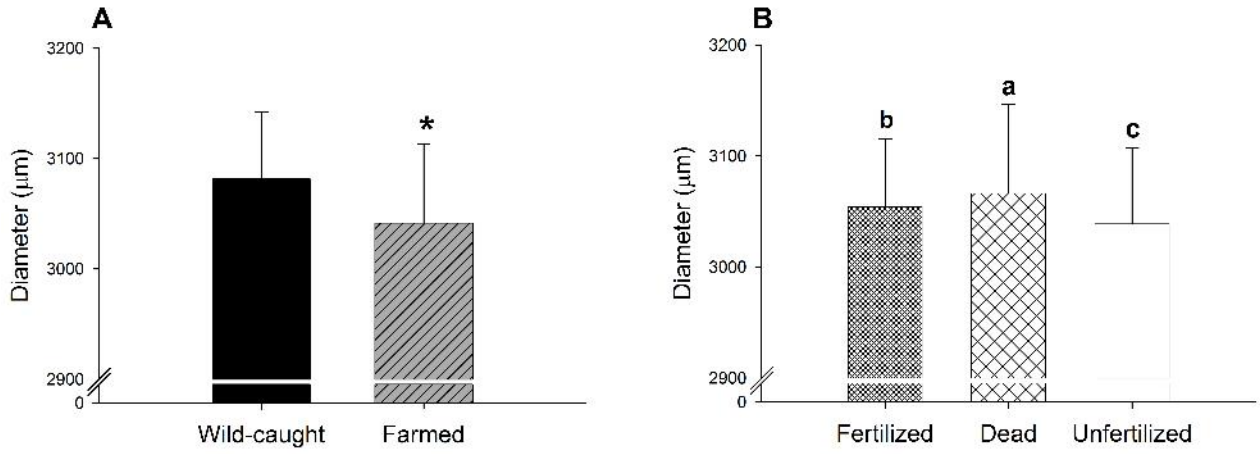

Figure 5.3.2 A. Egg diameter in wild-caught and farmed female halibut. **B**. Diameters of fertilized, dead and unfertilized halibut eggs.

Annual profiles of plasma hormone concentrations in wild-caught and farmed female Atlantic halibut

Methods

Blood samples were taken from the caudal vessels of 5 wild-caught and 5 farmed female Atlantic halibut at 3-6 week intervals, with the highest frequency during the spawning season. The sampled females were the

same individuals as in task 5.1. Blood samples were centrifuged for 5 minutes at 12000 rpm and 4°C. After centrifugation, plasma was divided in aliquots, frozen immediately on dry ice and stored at -80°C until analysis.

Analysis of sex steroids was carried out by ELISA, validated for halibut as described previously (Cuisset et al., 1994; Weltzien et al., 2002). Steroids were extracted from blood plasma by a method modified from Pankhurst and Carragher (1992). Briefly, plasma samples (100 µL) were mixed with 1 mL ethyl acetate, vortexed for 20 sec and centrifuged for 3 minutes at 1800 rpm and 4 ˚C. The organic phase was collected by a Pasteur pipette and the hydrophilic phase was extracted once more with 1 mL of ethyl acetate. The extracts were evaporated in a Speed Vac centrifuge (Savant 1000, USA), and dissolved in 1 mL buffer (phosphate 0.1 M pH 7.4, 0.4 M NaCl, 1 mM EDTA) by heating (60 ˚C for 10 min). The extracted and dissolved steroids were stored at -20 ˚C until analysis by an enzyme-linked immunosorbent assay (Cuisset et al., 1994. Extraction efficiency was >90% for all steroids. ED80 and ED20 were 0.004 ng ml-1 and 0.08 ng ml-1 for T and 0.006 ng ml-1 and 0.6 ng ml-1 for E2. Detection limits of the assays were 0.008 ng ml-1for T, 0.005 ng ml-1 for 11-KT and 0.015 ng ml-1 for E2, respectively. Internal standards were prepared from mature female Atlantic cod plasma extracted as described above. The accepted interassay coefficient of variation was 10% for all steroids; assays with higher deviation of the internal standard were re-run. The intra-assay coefficient of variation was 6.8 % for E2 (n=10) and 5.6 % for T (n=10). E2 and T antisera, acetylcholine esterase-labelled tracers and microplates precoated with monoclonal mouse antirabbit IgG were supplied by Cayman Chemicals (USA). Standard steroids were purchased from Sigma Aldrich (Sigma reference standards). Cross-reactivities for E2 and T antisera are described by the manufacturer.

Analyses of plasma Fsh and Lh concentrations were carried out by heterologous ELISAs, developed for Senegalese sole Fsh and Lh by Joan Cerdà's lab at IRTA, and validated for Atlantic halibut (Chauvigné et al., 2015, 2016). Plasma samples were hemolysis was detected were excluded from the analyses.

Results

Sex steroid concentrations

Mean plasma concentrations of E2 were 10-20 $ng{\cdot}ml^{-1}$ from October to December in both groups, increased from January to reach peak concentrations of 48.7±18 $ng{\cdot}ml^{-1}$ and 49.6±7.3 $ng{\cdot}ml^{-1}$ in February in wild-caught and farmed females, respectively. E2 remained high during the spawning period, decreased to basal levels <1 $ng{\cdot}ml^{-1}$ in May in all females and remained low for the remainder of the sampling period (Fig 5.3.3A).

Mean plasma T concentrations remained low, <4.5 $ng{\cdot}ml^{-1}$, in both groups until February when T reached 28.7±21.4 $ng{\cdot}ml^{-1}$ in wild-caught females, and March when T reached 32.9±12.5 $ng{\cdot}ml^{-1}$ in farmed females. Post-spawning, mean plasma T concentrations dropped to < 2.5 $ng{\cdot}ml^{-1}$ and remained low for the remaining sampling period (Fig 5.3.3B).

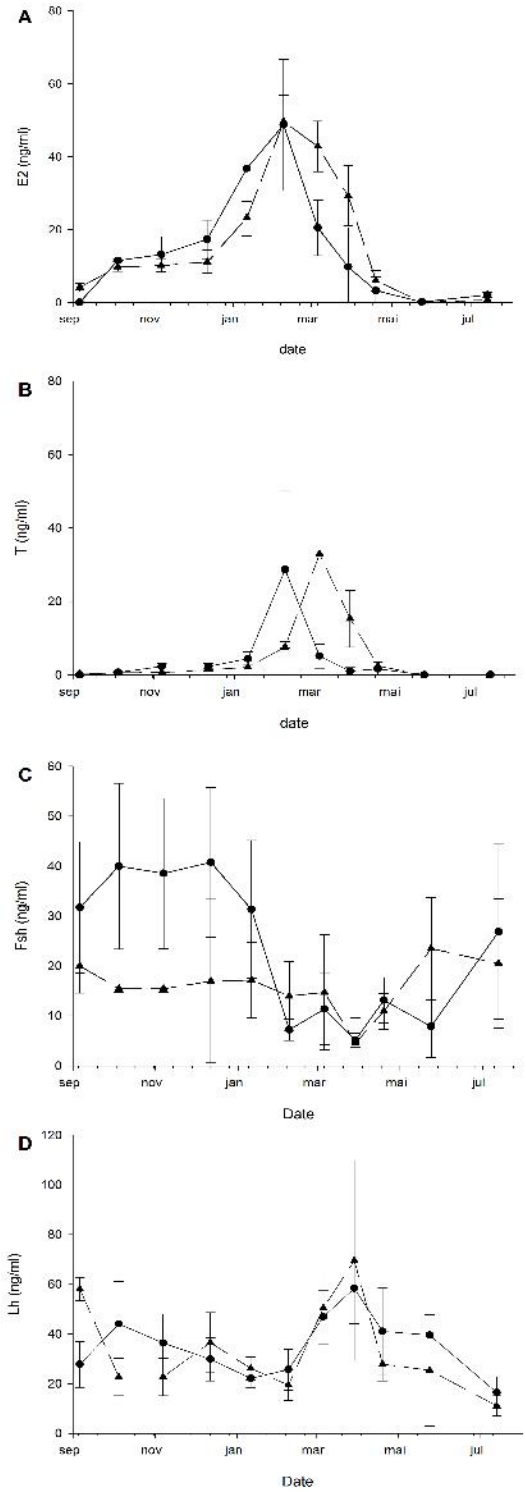

Figure 5.3.3. Annual plasma profiles of (A) Estradiol-17ß, (B) Testosterone, (C) Follicle-stimulating hormone and (D) Luteinising hormone in wild-caught (black circles, lines) and farmed (black triangles, stipled lines) halibut broodstock.

Plasma gonadotropin concentrations

During gametogenesis, from September to January, mean plasma Fsh concentrations were 30-40 $ng \cdot ml^{-1}$ in wild-caught, and 15-20 $ng \cdot ml^{-1}$ in farmed females. Individual variation was high, especially in wild-caught fish. Fsh decreased during spawning, this decrease was more pronounced in wild-caught than in farmed females. After spawning, from April onwards, mean plasma Fsh concentrations increased in both groups (Fig 5.3.3C).

Mean plasma Lh concentrations were relatively high, 20-60 $ng \cdot ml^{-1}$, from September to December in both groups. Before spawning, mean Lh concentrations appeared to decrease. Highest plasma Lh concentrations were seen during the spawning period, in March, with peak levels of 58.4±14.3 $ng \cdot ml^{-1}$ and 69.5±40.2 $ng \cdot ml^{-1}$ in wild-caught and farmed fish, respectively. After spawning, Lh concentrations decreased in both wild-caught and farmed females, and were <17 $ng \cdot ml^{-1}$ in July (Fig 5.3.3D)

Discussion

Plasma concentrations of sex steroids were similar to what has been reported previously in Atlantic halibut (Methven et al., 1992), with annual profiles following ovarian growth and maturation. Highest E2 levels were recorded just prior to spawning, in the beginning of February, while both E2 and T remained elevated through the spawning period. No differences in average concentrations were seen between wild-caught and farmed females, although the highest individual E2 concentrations (80.4 and 95.8 $ng \cdot ml^{-1}$) were detected in wild-caught females. The wild-caught females were larger that the farmed ones, had a higher total egg production and hence a larger total ovary weight. This would result in a higher total capacity for steroid production which may explain the higher plasma concentrations in some individuals.

Plasma concentrations of the gonadotropins, Fsh and Lh, were documented for the first time in Atlantic halibut. Mean Fsh concentrations were relatively stable during vitellogenesis, from October to early February, consistent with a constitutive release of Fsh from the pituitary. Fsh decreased to low levels during spawning but increased again after spawning was completed. This is consistent with previously reported results in other teleosts, including flatfish (cf. Levavi-Sivan et al., 2010; Chauvigné et al 2016). Mean Fsh concentrations were higher in wild-caught females than in farmed fish, but individual variations were high and further studies are needed to confirm if this result is consistent. Lh concentrations showed large individual variations through the reproductive cycle, but peak levels were apparent during spawning, in accordance with results in other teleost fish (Levavi-Sivan et al., 2010; Chauvigné et al 2016).

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Deviations from Annex I and their impact:

Due to unexpected problems with sampling, biopsy samples could not be collected. Instead, plasma concentrations of the gonadotropins Fsh and Lh were documented through the reproductive cycle for the first time in Atlantic halibut. In addition, fecundity analyses carried out in task 5.1 revealed no differences between farmed and female halibut. Therefore, and in view of the scarcity and high value of individual wild-caught halibut breeders, it was decided not to carry out potential fecundity analyses which would have necessitated sacrifice of females. The Deliverable D5.3 will be delivered before Month 52, when statistical analyses are completed.

WP 6 Reproduction & Genetics – wreckfish

WP No:	6	**WP Lead beneficiary:**		P8. IEO
WP Title (from DOW):	Reproduction and Genetics - wreckfish			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P14. IFREMER	P15. ULL
P19. CMRM	P32. MC2	P4. IOLR		
Lead Scientist preparing the Report (WP leader):	Blanca Alvarez (8)			
Other Scientists participating:	Constantinos Mylonas (P1), Ioannis Fakriadis (P1), Papadaki Maria (P1), Evaristo Pérez (8), Christian Fauvel (P14), Fatima Linares (P19), J. Luis Rodríguez (19), Antonio Villar (P32),			

Objectives

1. Increase the availability of wreckfish broodstocks in captivity,
2. Describe the reproductive cycle in captivity at the level of the pituitary and gonad,
3. Develop spawning induction procedures for *in vitro* fertilization, as well as spontaneous tank spawning,
4. Develop a CASA for evaluation of wreckfish sperm and establish cryopreservation protocols for use in *in vitro* fertilization applications.

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the 1st Reporting Period, work was completed in all of the proposed areas. Regarding **Task 6.1 Collect wild fish to establish new bloodstocks,** three wreckfish were captured. Morphometric measurements were performed and fin clip samples were taken for future genetic identification. For **Task. 6.2 Describe reproductive cycle**, bi-monthly (August-January) and monthly (February-July) samplings of gametes and blood were made from the 4 breeding stocks (P1. HCMR, P8. IEO, P32. MC2 and P19. CMRM). The samples, oocytes from females and sperm from males were described to provide a description of the annual changes in oogenesis and spermatogenesis for this species. Biometric, histology and biochemical samplings of 60 wild caught animals from the fish market allowed the calculation of weight/length relationship and other important parameters and biometric index, as a starting point for the culture of this species. Regarding **Task 6.3. Development of spawning induction procedures**, an induction trial with GnRHa implants was made using different doses and different developmental oocyte stages to obtain optimization of spawning. Natural and artificial spawning were also obtained from the two stocks (P1. HCMR and P14. IEO) with interesting results. Finally, in **Task 6.4. Evaluation of sperm characteristics and cryopreservation protocols,** experiments allowed establishing the assessment method for concentration and motility of sperm.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the 2nd Reporting Period, the work continued and advances were made with all tasks and objectives. **Task 6.1 Collect wild fish to establish new bloodstocks** has been complicated by the scarcity of wild

wreckfish. Despite of these problems, new contacts have been established to catch wreckfish and two juvenile wreckfish were captured, increasing the number of available fish for broodstock development. Continuing the work started in the first reporting period, a total of four broodstocks are being sampled for **Task. 6.2 Describe reproductive cycle.** The accumulation of data has shown that males exhibit good sperm quality with large amounts of expressible sperm during the reproductive period, and there is a proportion of males that spermiate throughout the year. The females increase oocyte size during the months March to July. In **Task 6.3. Development of spawning induction procedures** further trials to induce tank spawning with GnRHa were not successful and work began on combining GnRHa induced ovulation with *in vitro* fertilisation procedures. Initial work indicated that GnRHa is very effective in inducing oocyte maturation and ovulation consistently, and that stripped ova can be fertilised. All objectives in **Task 6.4. Evaluation of sperm characteristics and cryopreservation protocols** have been **completed** and Deliverables 6.1 and 6.2 have been submitted. The work in the second period demonstrated the feasibility of cryopreservation of wreckfish sperm, while chilled storage did not appear to be a good solution for the short-term management of sperm for artificial fertilization. The performance of frozen/thawed wreckfish sperm was half that of fresh sperm in terms of percentage of motile sperm and duration of swimming, while the velocity of sperm in modified Leibovitz was similar to that of fresh sperm.

Summary of progress towards objectives (31-48 Mo):

A summary of progress towards objectives, highlighting clearly significant results (max 0.5 page)

During the 3rd Reporting Period, the work continued and advances were made with all tasks and objectives. **Task 6.1 Collect wild fish to establish new bloodstocks** remains complicated due to the scarcity of wild wreckfish. On wreck fish was caught and died during the reporting period. Efforts will be increased further to obtain live wreckfish from fishermen. **Task. 6.2 Describe reproductive cycle.** All data has been obtained and analyzed to fully describe reproductive cycle. Wreckfish females can adapt to captivity, mature and produce eggs both under fluctuating natural and under constant low temperatures. Plasma sex steroid hormones in females correlate well with the maturity stages of females, except for 17,20β-P. However, some females exhibited reproductive dysfunctions with arrest before and during vitellogenesis. Males produce sperm of good quantity and quality, capable to fertilize the eggs produced. Moreover, wreckfish males can produce sperm all-year round, making it available to fish farmers for artificial fertilization whenever it is needed. Plasma sex steroid hormones in males rise when fish are fully spermiating, except for 17,20β-P. In some cases, although females spawned large numbers of eggs, these eggs were unfertilized, a fact that could be attributed to a failure in the male breeding behaviour. Further studies should look into the lack of maturation in the females and conduct experiments on the environmental conditions that the fish are held, in order to hopefully increase the number of females that can mature and spawn. In **Task 6.3. Development of spawning induction procedures** further trials to induce ovulation with *in vitro* fertilisation procedures were conducted. Promising advances were made and three females were induced to ovulate and eggs fertilized. Ovulation was induced with both GnRHa implants and injections. This work will be continued to standardize induction procedures.

Details for each Task

Task 6.1 Collect wild fish to establish new broodstocks (led by CMRM, Fatima Linares).

As previously reported, the decline in wreckfish catches in Galicia makes it difficult to obtain fish to increase the wreckfish broodstocks. Despite of the effort made to contact Galician fishermen, in this period only one fish was caught on 9/07/2017 (body weight 4 Kg and size 55cm). The fish was punctured to empty the air from the coelomic cavity and it was introduced into a quarantine tank (0_2 95%, Salinity = 35 ppt, pH = 7.84 and Tª = 17.2ºC) in the Aquarium A Coruña facilities (AF). Anti-inflammatory treatment was made for shock and the fish presented and improvement for a few days but then quickly, its physical condition deteriorated losing buoyancy. On the 20/7/2017 the fish died and the necropsia showed numerous skin alterations, especially on the left side of the body that had a deep corneal ulcer.

Loss of scales, haemorrhages from the middle part of the body towards caudal, ventral and in jaw and fraying fins was observed. Gills have normal coloration without parasites.

6.1.1. Details of the necropsy of wild wreckfish caught in 2017.

The juvenile specimens captured during 2014 and 2015 have been maintained as follows. Four were held at the IEO (one of them died) and the fifth at the Acuario de O Grove facilities. Weight and total length were 6.05 kg and 73 cm, 11.25 kg and 79 cm, 10.58 kg and 77 cm, 2 kg and 46 cm (died) of the four fish in the IEO and 3.99 Kg and 83 cm in Acuario de O Grove.

Furthermore, wreckfish from Acuario O Grove were monitored until April 2017, 7 fish: 2 female, 3 males and 1 undetermined and 1 immature. The average weight of the 6 mature fish was 13.39 ± 1.40 Kg, the standard and total length were 76.33±3.67 cm and 88.17±4.17 cm. The immature fish had 3.99 Kg and 73 and 83 cm of standard and total length respectively. On 10/7/2017, 29/8/2017 and 19/09/ 2017, 3 fish died in Acuario O Grove : 2 females (weight 12.5 and 11.6 Kg) and 1 male (weight 8.8 Kg). All of them have a big amount of perivisceral fat (%) particularly around the visceras. Some samples of liver, kidney, brain and spleen were sent to Ictiopathology Department of University of Santiago to perform bacteriological and virological analysis. No virus infection was found and related to bacterial infection, the results were positives in Vibrio sp. but they were not clearly associated to the death of fish.

Some samples were taken out of liver, muscle and gonad to perform biochemical analysis (proteins, lipids and fatty acids).

Because of the big amount of fat found in these specimens we thought they could have a problem with the food supplied and we recommended a change of the diet.

Next year the contacts with fishermen from different parts of the Galician coast will be increased hoping to get some wild fish to enhance the wreckfish broodstock.

Task 6.2 Describe reproductive cycle (led by IEO, Blanca Alvarez).

Introduction

The wreckfish is a globally distributed, anti-tropical species that inhabits continental coasts and oceanic islands at depths of 100-1000 m (Roberts, 1989), forming three genetically distinct stocks, in the North Atlantic and the Mediterranean Sea, in Brazil and in the South Pacific (Ball et al., 2000). It is a gonochoristic species with no sexual dimorphism and spawns at the continental slope at depths of 300-500 m, with the formation of spawning aggregations (Peres & Klippel, 2003). Its long life and late maturation, good adaptation to captivity and fast growth (Machias et al., 2003; Papandroulakis et al., 2004), together with its high flesh quality and market value, make the wreckfish an excellent candidate for the diversification of aquaculture production. Viable eggs and larvae have been already obtained from wild broodstocks of the southern hemisphere congener of the wreckfish, the hapuku *Polyprion oxygeneios*, in New Zealand (Anderson et al., 2012). More recently, juvenile production by F1 generation hapuku has also been achieved (Symonds et al., 2014). In the wreckfish, however, although attempts have been made, spawning is inconsistent, produced eggs exhibit low fertilization (Fauvel et al., 2008) and larvae do not survive more than 25 days after hatching (DIVERSIFY, WP 18).

The development of methods for the control of spawning and the production of good quality eggs are essential for the culture of any animal species. However, there are a number of reproductive dysfunctions observed in fish maintained in captivity, especially females, which result in lack of spawning. Examples of reproductive dysfunctions observed in fish held in captivity are lack of gametogenesis in the European eel *Anguilla anguilla* (Perez et al., 2011), lack of maturation or ovulation in meagre *Argyrosomus regius* (Mylonas et al., 2013) and greater amberjack *Seriola dumerili* (Mylonas et al., 2004b; Zupa et al., 2017) and lack of spawning of the F1 generation of the sharpsnout seabream *Diplodus puntazzo* (Micale et al., 1996; Papadaki et al., 2017) and the Senegalese sole *Solea senegalensis* (Guzmán et al., 2008). In males, reproductive dysfunctions involve limited or no sperm production and lack of synchronization of sperm production and ovulation (Mylonas et al., 2017), with the most pronounced example being the European and the Japanese eel (*Anguilla japonica*), which remain immature and fail to produce sperm in captivity (Peñaranda et al., 2010). The description of the reproductive cycle, except for allowing for the identification of the spawning period and spawning preferences of each species (temperature and photoperiod), enables the recognition of possible reproductive dysfunctions and leads to the development of protocols for spawning induction and production of viable eggs (Mylonas et al., 2013).

The aim of the present Task was to describe the reproductive cycle of wreckfish, using fish from fisheries landings and wild-caught fish maintained in captivity. Wild fish were either found in the fish market (2014) or were fished (2014 and 2015) and their biometric parameters were measured. Captive wreckfish maintained in different facilities in Europe were monitored for 2 years, following oocyte growth and maturation stage, sperm quality variations and the associated sex steroid plasma concentrations in four different broodstocks maintained in captivity in Spain and Greece.

Materials and methods

Capture of wild wreckfish

A total of 60 wild fish were sampled between January and October of 2014 in the fish market in order to obtain information on this species. For each animal sampled, total length (cm), total and eviscerated weight (kg), peri-visceral fat (%), as well as the gonadosomatic ((gonad weight/body weight) x 100) and hepatosomatic index ((liver weight/body weight) x 100) was determined. Samples from the stomach, liver, gonads, muscle and fins were taken for biochemical (P19. CMRM, P15. ULL) and histological studies.

During 2015 (on 7 and 14 of August) two wreckfish were captured using a hand net in a fishing area located 5 miles West of Corrubedo Cape, A Coruña (**Fig. 6.2.1**). Fish were transported by sea on a ship with flow-through water until the "Acuario de O Grove" facilities, where the fish were transferred to a quarantine tank. A sample from the fin was also taken for genetic analysis. These fish were transported to P8. IEO facilities in Vigo in March 2016. These two juveniles (4.86 and 0.94 kg in body weight) were maintained separated from the existent stock at the P8. IEO, until they became adults. Simultaneously, the growth and

development were followed of the three juvenile specimens captured during 2014, two held at the P8. IEO, and the third at the Acuario de O Grove. Furthermore, the development was monitored of the Acuario O Grove wreckfish broodstock, which constituted of 7 fish: 2 females, 3 males and 2 undetermined with an average weight of 11.57±1.86 Kg.

Figure 6.2.1. *Wreckfish* captured in 2015 in the fishing area 5 miles to the West of Corrubedo Cape, La Coruña.

Although increasing effort has been made to contact Galician fishermen, it is important to note that the decline in catches of wreckfish in Galicia made it difficult to obtain specimens of wild wreckfish to establish new wreckfish broodstocks.

Captive wreckfish maintenance

Four different broodstocks at different research institutes in Greece and Spain were used, at the Hellenic Center for Marine Research (HCMR, n=3) in Heraklion, Crete, Greece, the Instituto Español de Oceanografía (IEO, n=13) in Vigo, Spain, the Aquarium Finisterrae (AF, n=21) in A Coruña, Spain and the Conselleria do Medio Rural e Mariño (CMRM, n=11) in Pontevedra, Spain. One female and 2 males of mean weight ± S.D. 13.1 and 9.08±2.66 kg, respectively, were kept at HCMR in a 15-m^3 tank, under simulated natural photoperiod and constant temperature of around 16°C. Fish were fed twice per week with frozen fish. On June 12, 2016 one of the males died, leaving the broodstock of HCMR with one female and one male fish. Ten females and 3 males of 16.24 ± 4.05 and 11.58±1.59 kg, respectively, were kept at IEO in two 110-m^3 tanks under natural photoperiod and water temperature. Twelve females and 9 males of 23.14±6.79 and 17.36±3.25 kg, respectively, were kept at MC2 in a large exhibition tank of 3500 m^3 and in the breeders' tank of 33 m^3 under simulated natural photoperiod and natural water temperature. Finally, 8 females and 3 males of 14.43±2.8 and 13.29±0.55 kg, respectively, were kept at CMRM in two tanks of 120 and 180 m^3 under natural photoperiod and water temperature. On August 15, 2016 one female fish died from the CMRM broodstock. The temperature conditions at the four different locations are shown in **Fig. 6.2.2**.

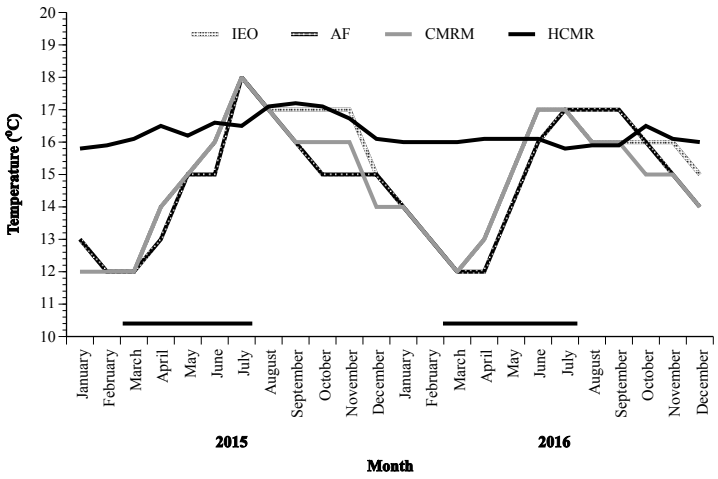

Figure 6.2.2. Tank water temperature (°C) of wreckfish broodstocks at four different sites in Greece (Hellenic Center for Marine Research, HCMR) and Spain (Instituto Español de Oceanografía (IEO), Aquarium Finisterrae (AF) and the Conselleria do Medio Rural e Mariño (CMRM) from January 2015 until December 2016.

For egg collection, a passive egg collector was placed in the outflow of the tank, in order to verify the occurrence of any spawning and collect the spawned eggs. At HCMR, eggs were collected into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 ml, after vigorous agitation. Fertilization success was evaluated at the same time by calculating the number of viable eggs in respect to the total number of eggs spawned. At IEO, CMRM and MC2, eggs were collected in a graduated cylinder and the volume (ml) of the total number of eggs spawned and of floating eggs was determined (**Fig. 6.2.3**). Then, fertilization success was estimated in a sub-sample of the floating eggs using a stereoscope. The total number of eggs spawned was estimated by multiplying the observed egg volume by 150, which is the number of wreckfish eggs found in 1 ml of seawater.

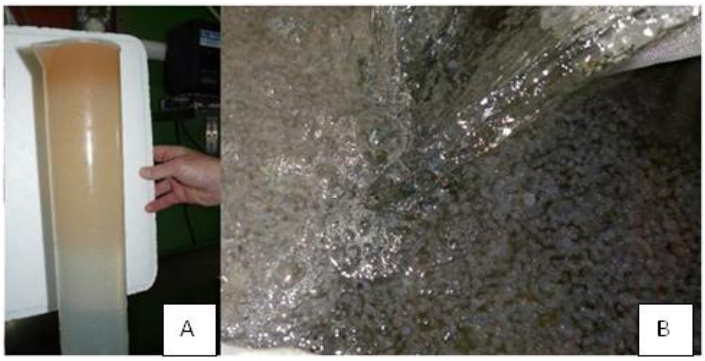

Figure 6.2.3. Spontaneous spawn (A) counted volumetrically from the egg collector and (B) in the egg collector of tank S-2 at the P14. IEO.

Samplings and histology

Fish reproductive cycle was monitored from March 2015 to October 2016. Samplings were conducted monthly from February until June, and bimonthly from July until January **(Fig. 6.2.4)**. To determine the sex of the specimens from which a biopsy could not be obtained, as the gonopore was completely closed, ultrasound was used (**Fig. 6.2.5**).

Figure 6.2.4. Broodstock sampling at P14. IEO, P19. CMRM and P32. MC2.

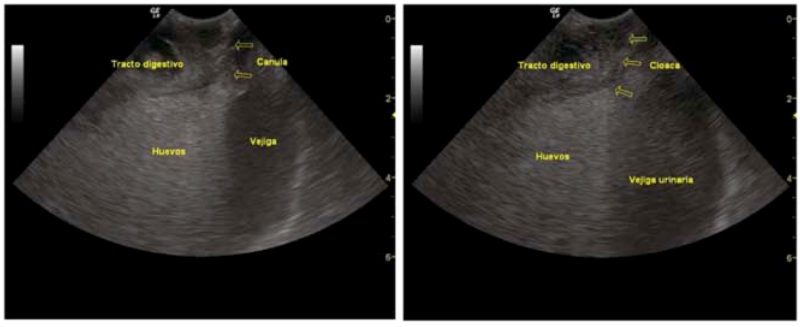

Figure 6.2.5. Gonad echography pictures of two wreckfish, to identify sex of breeders held at P32. MC2 and P19. CMRM .

At each sampling, the weight of the fish was measured and biopsies were collected from female fish. Fish were tranquilized initially in their tank with the use of clove oil (0.01 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of clove oil (0.03 ml l^{-1}) (Mylonas et al., 2005). Ovarian biopsies for the evaluation of oocyte development were obtained with the use of a Pipelle de Cornier catheter. A wet mount of the biopsy was examined under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced oocytes (n = 10). A portion of the biopsy was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Females that did not show any sign of oocyte maturation were excluded from the analysis. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. Sperm was collected after rinsing the fish with clean seawater and blot drying the genital pore. Small volumes of sperm (50–100 µl) were collected in order to avoid influencing the quantity and/or quality of sperm during subsequent collections. The collected sperm was stored on ice and then transferred to a 4 °C refrigerator until evaluation. Spermiation index was evaluated based on the presence and ease of milt release upon the application of gentle abdominal pressure (Mylonas et al., 2003). Spermiation index was reported on a subjective scale from 0 to 2, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. Sperm quality parameters that were evaluated included sperm concentration (number of spermatozoa ml^{-1} of milt), percentage of spermatozoa showing forward motility immediately after activation (initial sperm motility, %) and duration of forward sperm motility of at least 10% of the spermatozoa in the field of view (motility duration, min). Sperm concentration was estimated after a 2121-fold dilution with seawater using a Neubauer haemocytometer under 200x magnification (in duplicate) in a compound light microscope (Nikon, Eclipse 50i). Sperm motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 µl of sperm with a drop of about 50 µl of saltwater (in duplicate). Males that did not show any sign of maturation (i.e. did not produce sperm for the duration of the study) were excluded from the analysis. Blood was collected from all fish at each sampling, in order to measure sex steroid hormone concentrations. Blood was centrifuged at 6000 rpm for 15 min and plasma was collected and stored at −80 °C until analysis. Due to a mistake during the March 2015 sampling in IEO, CMRM and MC2, samples of this month were excluded from the plasma sex steroid analysis.

Histological processing

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70–96%). Serial sections of 3 µm were obtained with a microtome (Reichert Jung, Biocut 2035, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to (Bennett et al., 1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

Hormone measurements

For the quantification of T, E2, 11-KT and 17, 20β-P in the plasma, already established and well described enzyme-linked immunoassays (ELISA) were used (Cuisset et al., 1994; Nash et al., 2000; Rodríguez et al., 2000) with some modifications, and using reagents from SpiBio (France). For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After decanting of the organic phase, drying of the supernatant was done under a stream of nitrogen (Reacti-vap III, Pierce, USA). Samples were reconstituted in 250 µl of reaction buffer for running in the ELISA.

Statistical analysis

Differences in mean oocyte diameter, sperm motility duration, motility percentage, density and survival within months, were assessed with the use of Student's t test, at a minimum significance of $P<0.05$. Differences in oocyte diameter and steroid hormone concentrations in relation to oocyte stage and spermiation index were assessed with the use of one-way ANOVA, followed by Tukey's HSD test, at a minimum significance of $P<0.05$. Unless otherwise mentioned, results are presented as mean ± SEM. Statistical analyses were performed using the statistical software JMP (SAS Institute Inc., Cary, NC).

Results

Biometric parameters of wild wreckfish captured in 2014 are shown in **Table 6.2.1**. Total weight of wild wreckfish varied between 3.6 and 18 kg, and total length varied between 56 and 98 cm (**Fig. 6.2.6**). A relation between weight and length was established, both for males and females (**Fig. 6.2.7**).

Table 6.2.1. Biometric parameters and indexes of the 60 animals sampled in the Azores fisheries.

BIOMETRIC PARAMETER (60 WILD WRECKFISH)	MEAN	STD
TOTAL LENGHT	76,09	6,788
ST LENGHT	66,38	7,629
PERÍMETER	55,68	5,986
WEIGHT (Kg)	7,52	2,169
EVIS. WEIGHT (Kg)	6,99	1,967
GONAD WEIGHT (g)	17,10	20,831
LIVER WEIGHT (g)	95,70	71,671
FAT PERIVIS. WEIGHT (g)	76,25	72,233
STOMACH WEIGHT (g)	125,90	56,183
INTESTINE LENGHT (cm)	94,53	15,555
INTESTINE WEIGHT (g)	99,27	62,688
GSI FEMALES	0,30	0,184
GSI MALES	0,13	0,126
SHI	1,21	0,497
VSI	10,31	17,233

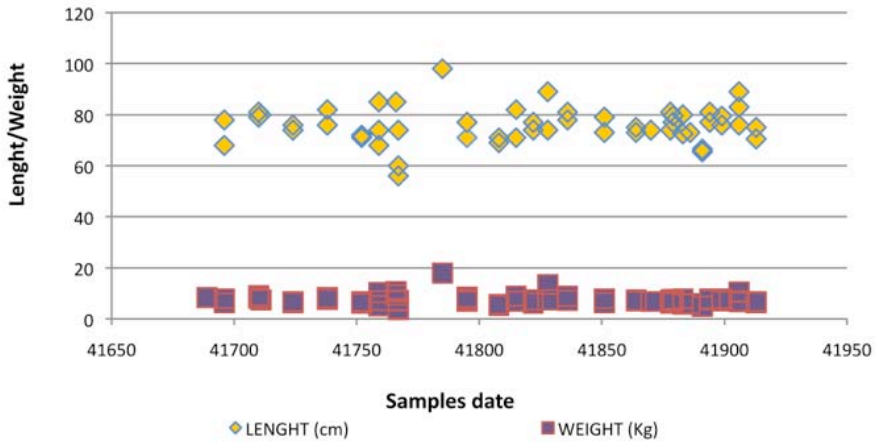

Fig. 6.2.6. Total length and weight of the 60 animals sampled in the Azores fisheries.

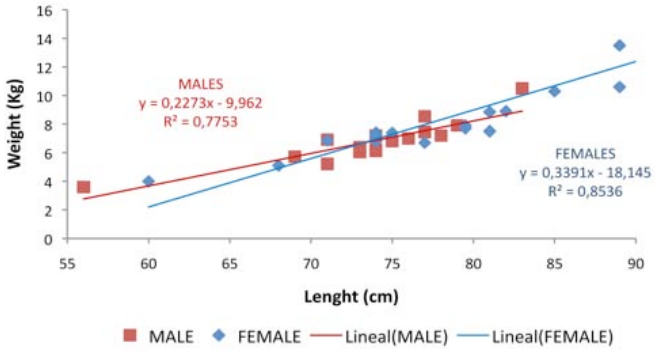

Figure 6.2.7. Weight/length relationship from males and females sampled in the Azores fisheries.

No relation was observed between weight, sex and perivisceral fat from the 60 fish sampled in the fish market (**Fig. 6.2.8**). No relation was observed between perivisceral fat % and date of capture from the 60 dead wild fish sampled (**Fig. 6.2.9**). This information would be important for nutrition studies and future elaboration of artificial feeds for this species (WP12 Nutrition - wreckfish).

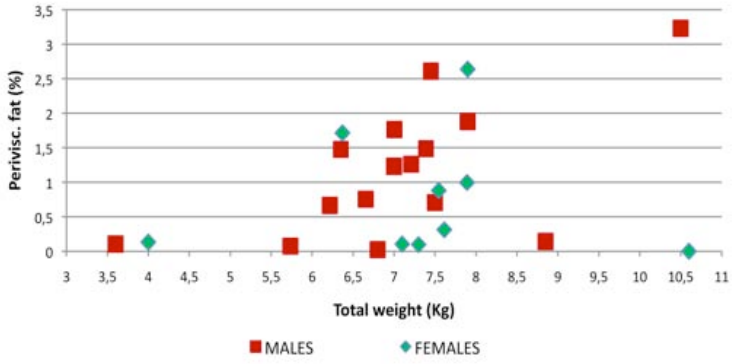

Figure 6.2.8. Relation between weight, sex and perivisceral fat (%) of the 60 animals sampled from the Azores fisheries.

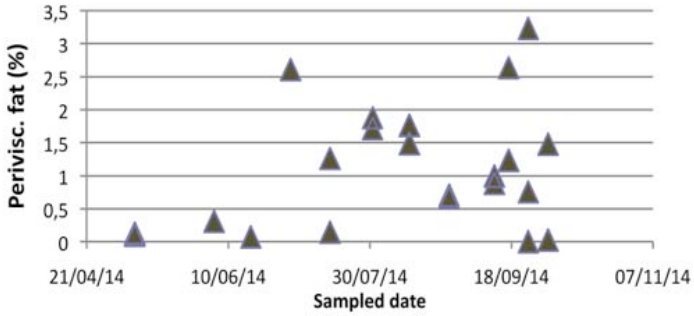

Figure 6.2.9. Relation between perivisceral fat % and time of capture of the 60 animals sampled in the Azores fisheries.

Wreckfish reproductive period begun with oocytes reaching vitellogenesis (**Fig. 6.2.10A, 6.2.11A**) and proceeded with oocyte maturation after lipid droplets coalescence (**Fig. 6.2.10B, 6.2.11B**). During final oocyte maturation, yolk coalescence was completed and the germinal vesicle was located in the periphery of the oocyte **(Fig. 6.2.10C, 6.2.11C)**, whereas after ovulation small primary, lipid vesicle and atretic oocytes could be observed in female fish gonads, together with post-ovulatory follicles if spawning had occurred (**Fig. 6.2.10D, 6.2.11D**).

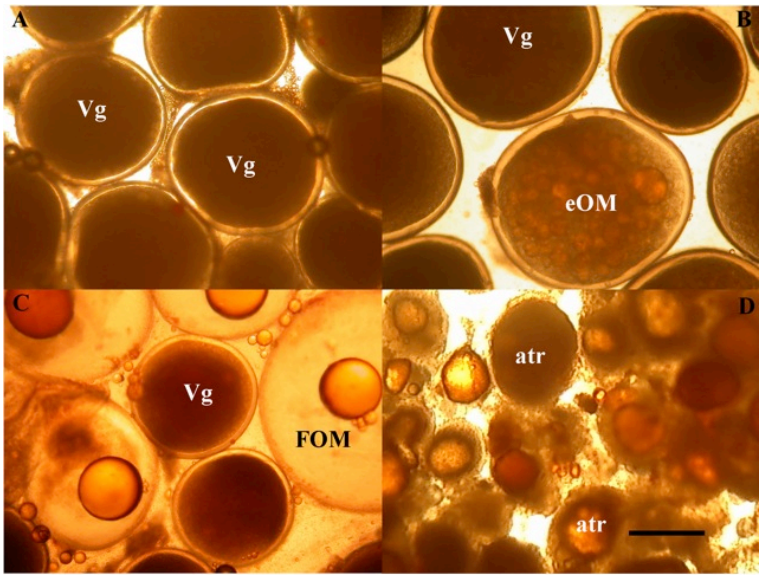

Figure 6.2.10. Photomicrographs of wreckfish biopsies, showing oocytes at successive stages of development: vitellogenesis (Vg, A), early oocyte maturation (eOM) with lipid droplet coalescence (B), final oocyte maturation (FOM, C) and atresia at the end of the reproductive season (D). The bar represents 500 µm.

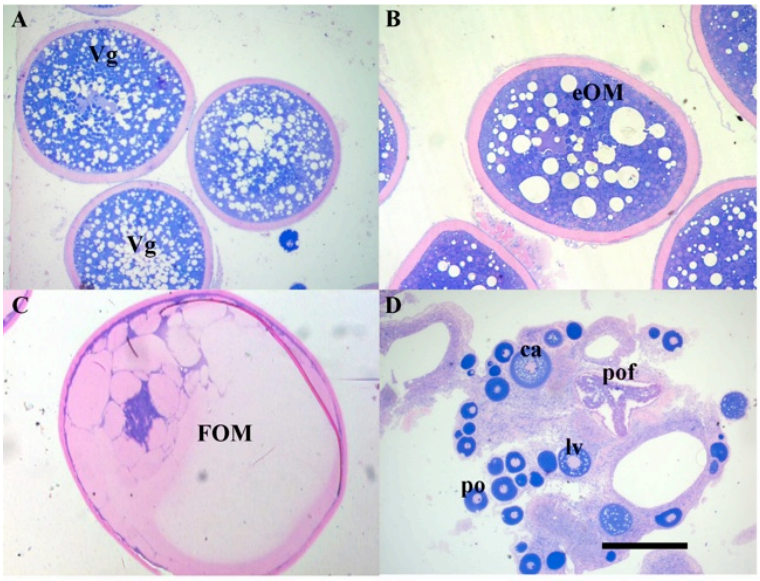

Figure 6.2.11. Photomicrographs of histological sections of wreckfish biopsies, showing vitellogenic oocytes (Vg, A), early maturing oocytes with lipid droplet coalescence (eOM, B), final oocyte maturation (FOM, C) and gonad at the end of the reproductive season with primary oocytes (po), lipid vesicle oocytes (lv), cortical alveoli oocytes (ca) and post-ovulatory follicles (pof). The bar represents 500 µm.

Oocyte development of some of the wreckfish females did not seem to advance beyond the cortical alveoli stage, and their oocytes did not reach more than 350 μm in diameter during the whole year. When excluding these fish (4 females from IEO and 2 females from CMRM), wreckfish oocyte diameter was found to reach its highest values from March until June or July and its lowest values from September until December during both years of the study (**Fig. 6.2.12**).

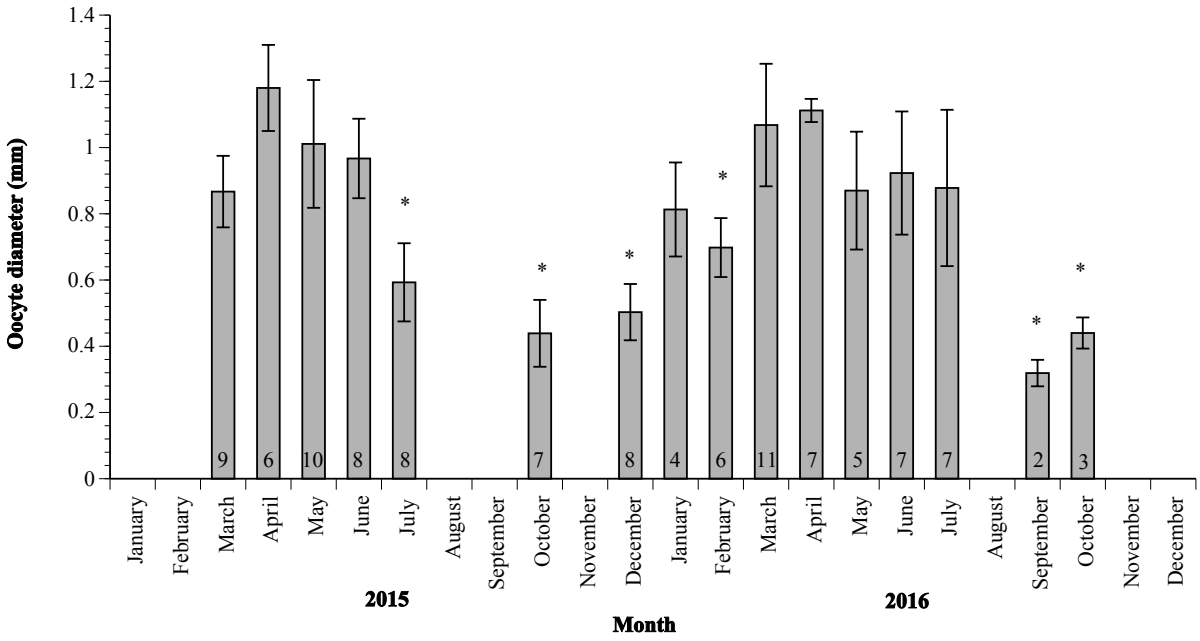

Figure 6.2.12. Mean (±SEM) oocyte diameter of wreckfish broodstocks at four different sites in Greece and Spain during the annual reproductive cycles from March 2015 until October 2016. The numbers inside the bars indicate the number of females biopsied at each month. Asterisks (*) denote significantly lower values than maximums observed (April 2015 and 2016).

The first significant increase of oocyte diameter was observed at vitellogenesis, with oocytes reaching around 1 mm diameter. Oocytes grew significantly during oocyte maturation, and remained at the same size at ovulation, at a mean diameter of around 1.4 mm (**Fig. 6.2.13A**). Due to the differences in the environmental conditions under which the fish were held at the different locations, sex steroid hormone levels were expressed in the present study not versus time, but versus the reproductive stage of both females and males. Moreover, due to the high variation between samples, female wreckfish E2 concentration did not show statistically significant changes with oocyte stage, although a trend of increasing values until vitellogenesis and decreasing values thereafter was visible (**Fig. 6.2.13B**). As far as 17,20-P concentrations are concerned, its values did not seem to change in respect to oocyte stage (**Fig. 6.2.13C**), whereas T remained at low levels at the lipid vesicle and cortical alveoli stage and increased during vitellogenesis. A second increase was observed during oocyte maturation and a significant decrease at ovulation (**Fig. 6.2.13D**).

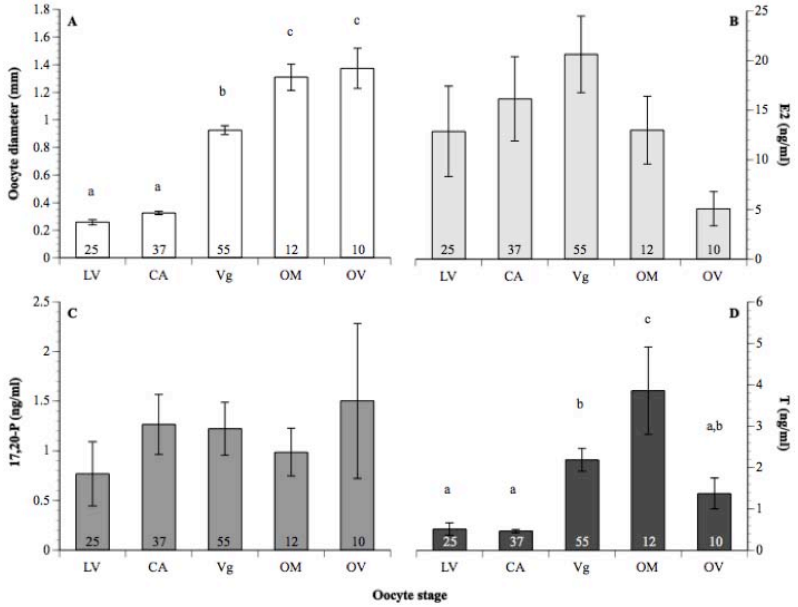

Figure 6.2.13. Mean (±SEM) oocyte diameter (A), concentrations of 17β-estradiol (E2, B), 17, 20-dihydroxy-pregnenolone (17,20-P, C) and testosterone (T, D) at different stages of oocyte development (LV: lipid vesicle, CA: cortical alveoli, Vg: vitellogenesis, OM: oocyte maturation, OV: ovulation). Different letter superscripts indicate statistically significant differences in oocyte diameter and T between different oocyte stages. The numbers inside the bars indicate the number of samples at each oocyte stage.

Spermiating males could be found all-year round, with the percentages of S0, S1, S2 and S3 stage fish varying between months (**Fig. 6.2.14**). The highest percentage of non-spermiating fish (S0 spermiation index) was found from September until December, whereas high percentages of spermiating fish (S2 and S3 spermiation index) were found just before and during the reproductive season of females, from January until July, during both years of the study.

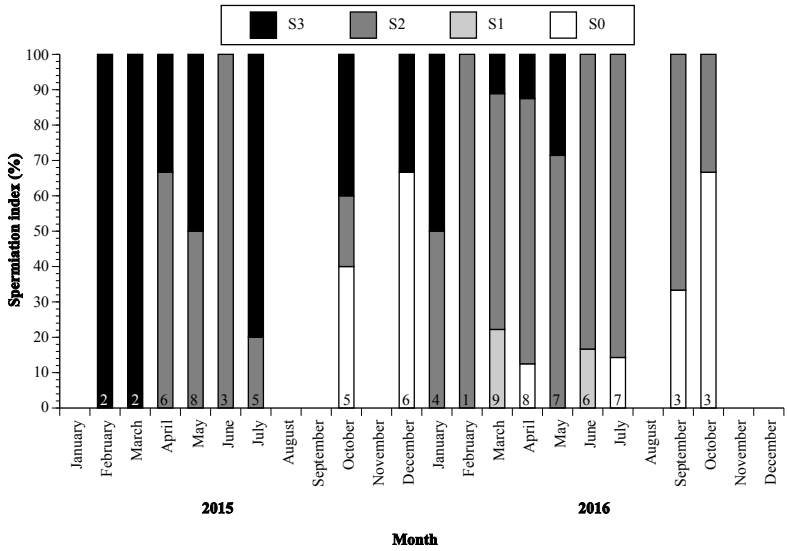

Figure 6.2.14. Percentage of male wreckfish at different spermiation index stages, in respect to month from February 2015 until October 2016. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. The numbers inside the bars indicate the number of wreckfish males examined each month.

Sperm motility duration and motility percentage exhibited high and almost unchanged values during both years of the study (**Fig. 6.2.15A and B**), whereas sperm density exhibited high values during the whole year, with the highest values observed in March of both years (**Fig. 6.2.16A**). As far as sperm survival at 4°C is concerned, it exhibited constant values during the whole year, with lower values only in March, June and September 2016 (**Fig. 6.2.16B**).

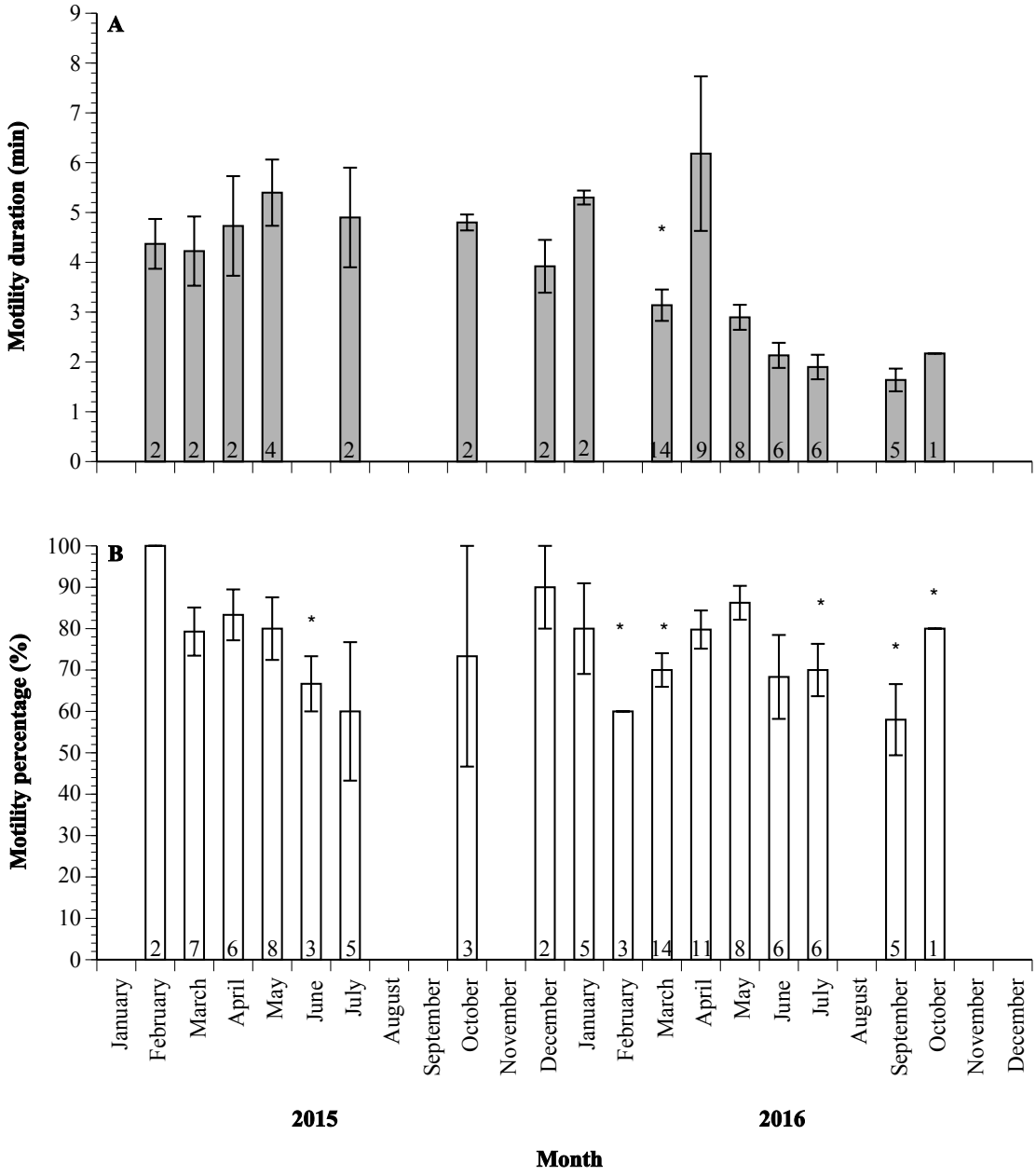

Figure 6.2.15. Mean (±SEM) sperm motility duration (A) and sperm motility percentage (B) of sperm collected by wreckfish of four different broodstocks in Greece and Spain at different months of the year from February 2015 until October 2016. Asterisks indicate statistically significant differences from the maximums (April 2016 for motility duration and February 2015 for motility percentage). Different numbers inside the bars indicate the number of sperm samples used for each sperm quality parameter.

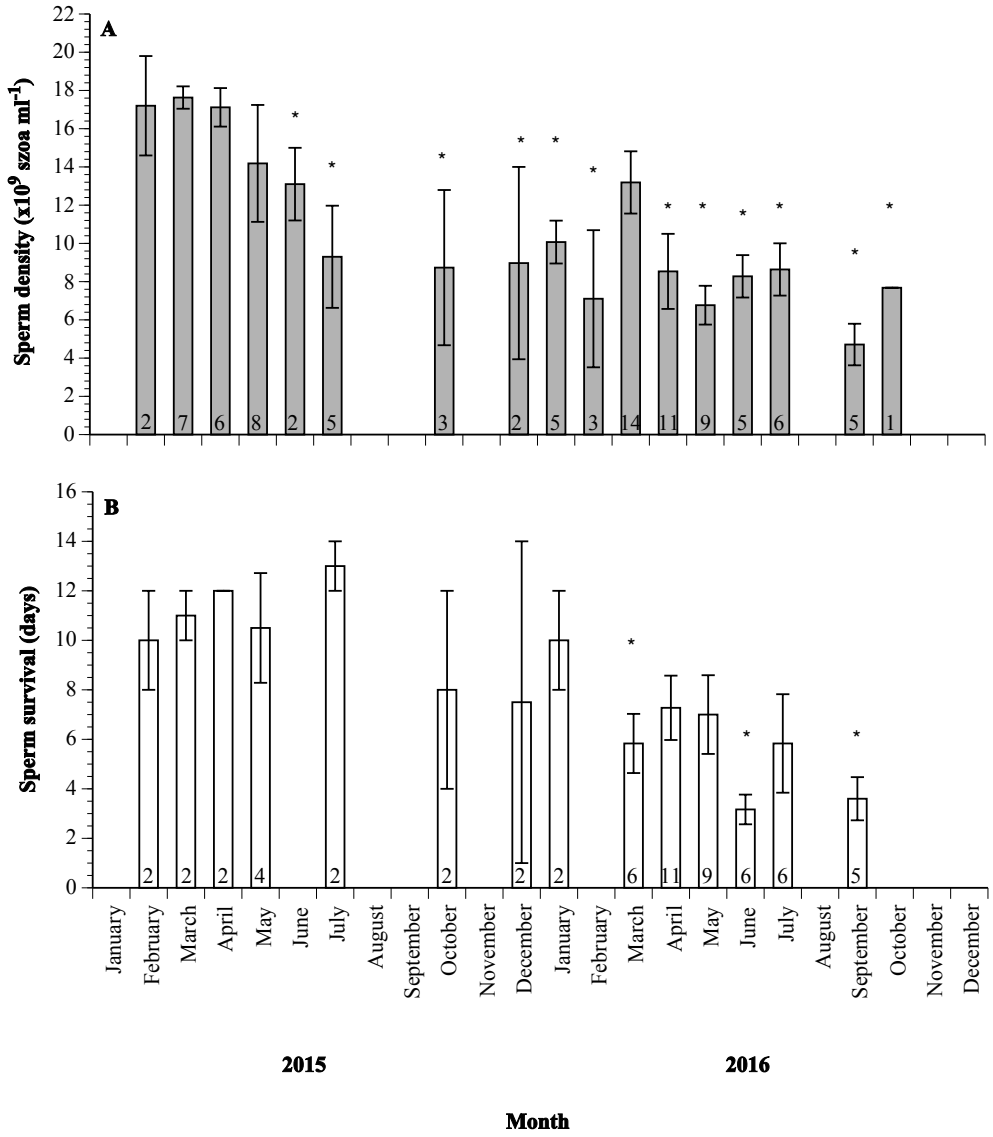

Figure 6.2.16. Mean (±SEM) sperm density (A) and survival at 4°C (B) of sperm collected by wreckfish of four different broostocks in Greece and Spain at different months of the year from February 2015 until October 2016. Asterisks indicate statistically significant differences from the maximums (March 2015 for sperm density and July 2015 for survival). Different numbers inside the bars indicate the number of sperm samples used for each sperm quality parameter.

Testosterone (**Fig. 6.2.17A**) and 11-KT (**Fig. 6.2.17C**) had low values at the S0 and reached their highest values at the S3 spermiation stage, whereas 17, 20-P did not change significantly with fish spermiation index (**Fig. 6.2.17B**).

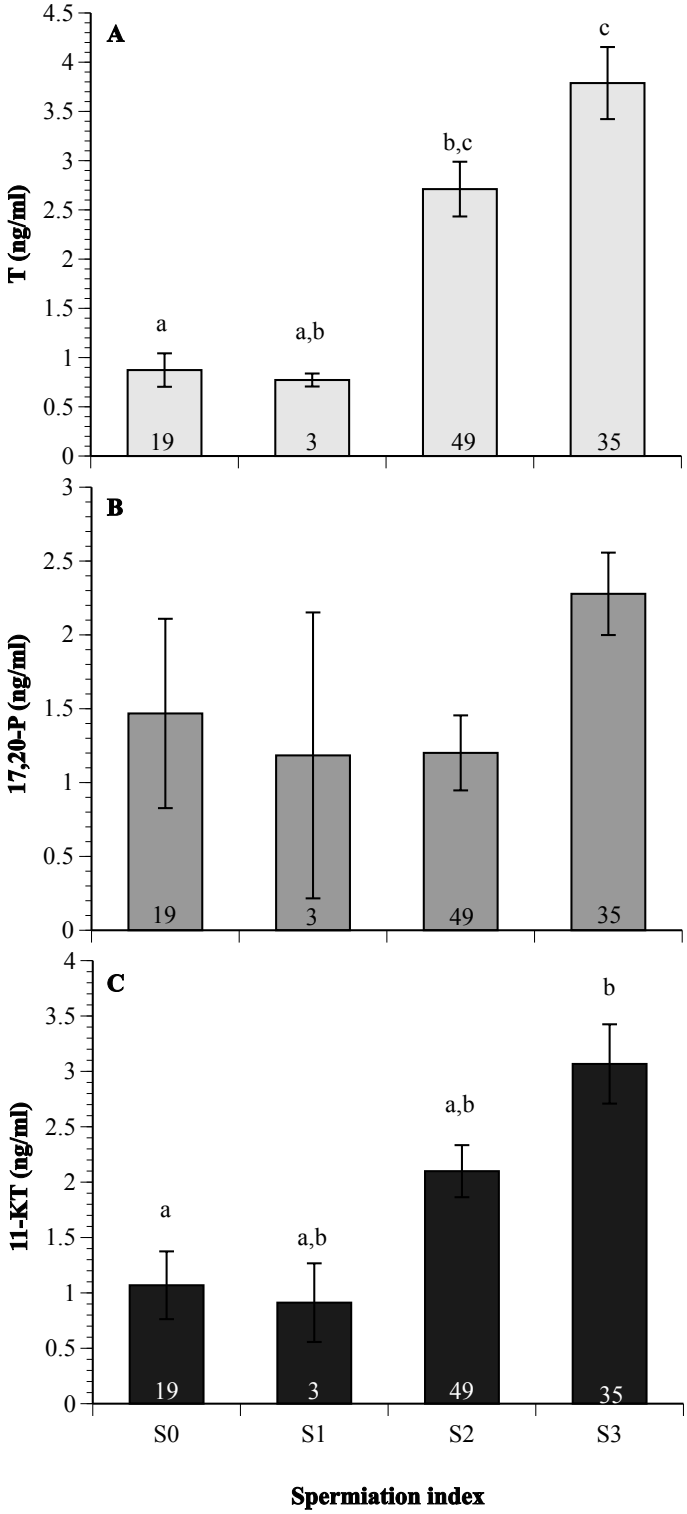

Figure 6.2.17. Mean (±SEM) plasma concentrations of testosterone (A), 17, 20β-P (B) and 11-KT (C) of four different broodstocks of wreckfish in Greece and Spain at different spermiation index stages. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. Different letter superscripts indicate statistically significant differences in T and 11-KT at different spermiation index stages, whereas numbers inside the bars indicate the number of male wreckfish found at each spermiation index stage.

Spontaneous spawns of captive wreckfish broodstocks were observed during 2015, 2016 and 2017 **(Fig. 6.2.18)**. In 2015 and 2016 spawning lasted from March until July, with most of the spawns coming from the IEO and the MC2 broodstocks. In 2017, spawning lasted from March until May and spontaneous spawns were also achieved at the CMRM broodstock, showing very high fertilization percentages (**Fig. 6.2.18C**). Relative fecundity and fertilization varied a lot, with fertilization percentage exhibiting values from 0% until 100% (**Fig. 6.2.18**). Mostly in 2015 and 2016, even when fecundity values were high, fertilization percentage was often zero or close to zero. Individual female wreckfish were observed to spawn every 3-4 days (not shown).

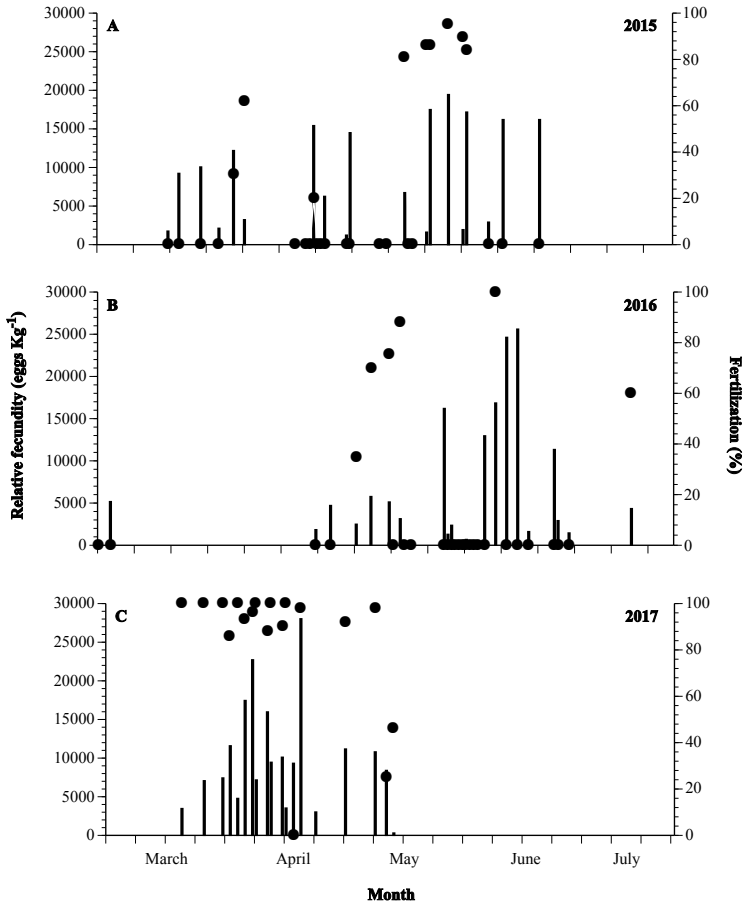

Figure 6.2.18. Mean relative fecundity and fertilization percentage (%) of the spawning events of the four different wreckfish broodstocks during 2015 (A), 2016 (B) and 2017 (C).

Discussion

The spawning period of the southern hemisphere wreckfish occurs in the austral winter, from July until October (Peres & Klippel, 2003), and coincides with the spawning period of the hapuku (Wakefield et al., 2010). In the present study, increased oocyte diameter of wreckfish oocytes was found already in January, with peak values between March and July, defining this to be the reproductive period, in accordance with former studies, where attempts were made for spawning induction in May (Papandroulakis et al., 2008; Peleteiro et al., 2011). Oocytes were small during the lipid vesicle and the cortical alveoli stage and increased in size at vitellogenesis, when they reached 1 mm in size (Martínez-Vázquez et al., 2016), whereas spawned eggs were around 2 mm in diameter and had from one to multiple oil globules, as has been also

shown for the hapuku (Anderson et al., 2012). A large egg size is considered essential for demersal fishes, as it is related to higher individual survival in a relatively constant environment, in contrast to pelagic small eggs that have to face a changing environment, where survival is more difficult (Duarte & Alcaraz, 1989). For comparison, in pelagic fishes, mean egg diameter is 1.02 mm in the red porgy *Pagrus pagrus* (Mylonas et al., 2004a), 1.03 mm in greater amberjack (Mylonas et al., 2004b) and 1.15 mm in European sea bass *Dicentrarchus labrax* (Cerdá et al., 1994). On the contrary, in demersal flatfishes egg diameter ranges between 1.84-2 mm in the plaice *Pleuronectes platessa* (Kennedy et al., 2007) and 2.9-3.3 mm in the Atlantic halibut *Hipoglossus hipoglossus* (Brown et al., 2006).

However, although some of the females managed to proceed with oocyte development and reach maturation, 4 females from IEO and 2 females from CMRM did not progress beyond the cortical alveoli stage and their oocytes did not increase in diameter more than 350 μm. This fact occurred during both years of the study in the same females. Normally, the cortical alveoli stage defines the early developing ovary (Lowerre-Barbieri et al., 2011). However, in some fish species, ovaries can maintain the cortical alveoli oocyte developmental stage for up to 9 years before maturation and first spawning, such as the common and the spotted wolffish *Anarhichas minorin* and *Anarhichas lupus* (Gunnarsson et al., 2006; 2008). Other species may be able to have cortical alveoli oocytes in their ovaries when immature, such as the Greenland halibut *Reinhardtius hippoglossoides* (Rideout et al., 1999). The specific wreckfish females of the present study were all captured in 2009, so it cannot be certain whether they were able to mature yet, as wreckfish first maturation is achieved at 10 years for females and 9 years for males in nature (Peres & Klippel, 2003). However, of a total of 31 females, these are the only ones that ceased oocyte development at the cortical alveoli stage and remained at this stage for almost two years. Considering the above, it can be inferred that the specific females exhibited a kind of reproductive dysfunction that led to arrest of oocyte development at a very early stage of vitellogenesis. Another option is that the specific females skipped spawning by arresting oocyte development at the cortical alveoli stage, a phenomenon which has received increased attention in recent years and has been demonstrated in a number of species (Rideout et al., 2005; Skjæraasen et al., 2012). Skipped spawning in nature has been mainly attributed to poor nutrition during the spawning period and it can occur mostly in smaller females that do not migrate to the spawning grounds but stay at the feeding grounds for energy reservation (Skjæraasen et al., 2012; Zupa et al., 2009).

Reproductive dysfunctions at later stages of vitellogenesis due to dysfunctional release of luteinizing hormone (LH) from the pituitary at the end of vitellogenesis are common in a number of species (Mylonas et al., 2010), a phenomenon that probably occurred also in a large number of females of the present study. In particular, 20 fish in the present study reached vitellogenesis, but did not seem to proceed to maturation. This reproductive dysfunction could be attributed to the environmental conditions of the tanks where the fish were kept. However, constant temperature of 16°C did not seem to have a significant effect on the reproductive development of fish, since fish held at this temperature exhibited the same reproductive performance as fish under naturally fluctuating temperature, e.g. they developed vitellogenic oocytes and managed to give some sporadic spawning events, suggesting that environmental parameters other than photoperiod and temperature may affect wreckfish reproductive performance. It is known that low temperature is preferred by the wreckfish, as loss of appetite and growth cessation were observed at temperatures >20°C (Papandroulakis et al., 2004); the same holds true for the hapuku, as it was shown that fish over 1 kg grow better at 18 than at 22°C (Tromp et al., 2016). Moreover, hapuku broodstocks in New Zealand are maintained at fluctuating temperatures from 10-19°C during the non-reproductive season and at 10 or 13.5°C during the reproductive season (Symonds et al., 2014).

Endocrine control of fish oocyte maturation has been described as follows: during vitellogenesis, T is produced in the theca cells and converted to E2 in the granulosa cells (Nagahama, 1994); E2 then promotes vitellogenin synthesis in the liver. After vitellogenesis, E2 drops and T increases during germinal vesicle migration; at that time, 17,20β-P or 17,20β, 21-trihydroxy-pregnenolone (20β-S) is produced as the maturation inducing steroid (MIS) in order to induce final oocyte maturation (Nagahama, 1994). The pattern of sex steroid changes in relation to the stage of oocyte development in the present study followed the above-mentioned biochemical cascade. In particular, T was low until vitellogenesis and increased as maturation proceeded but decreased at ovulation; at the same time, E2 showed a pattern of increase until vitellogenesis and decrease thereafter, although not statistically significant. On the other hand, 17, 20β-P seemed to

maintain low and unchanged levels during oocyte development. Recent studies in different fish species support the involvement of T in oocyte maturation and the low levels of both T and E2 at ovulation. For example, T induced oocyte maturation, in terms of germinal vesicle breakdown, but not ovulation when administered in vivo to zebrafish *Danio rerio* oocytes (Tokumoto et al., 2011). Moreover, estrogen-synthesizing genes were down regulated in the preovulatory period of the rainbow trout *Oncorhynchus mykiss* (Bobe et al., 2006). On the other hand, 17,20β-P remained relatively stable at maturation both in Atlantic cod *Gadus morhua* (Kjesbu et al., 1996) and in greater amberjack (Zupa et al., 2017). Low and relatively stable levels of 17,20β-P may reflect rapid catabolism of the hormone or transformation to conjugated forms (Zupa et al., 2017) or may indicate that this steroid is not the actual MIS for the specific fish species, as has been suggested for the Atlantic cod and the yellowfin porgy *Acanthopagrus latus* (Jeng et al., 2012; Kjesbu et al., 1996).

Spermiating wreckfish males were present in the captive broodstocks studied all-year round, even at small numbers from September until December. This fact can be very useful for aquaculturists, as wreckfish sperm seems to be available most of the year for artificial insemination practices. Spermiating males all –year round have been also found in the Senegalese sole, a species able to reproduce at least at two different periods during the year (García-López et al., 2006a). Most spermiating males could be found from March until July, covering the reproductive period of females.

Sperm quality parameters maintained high levels throughout the year. For example, mean sperm density ranged between 4.5-11.5 spermatozoa x 10^9 ml^{-1}, sperm motility was always higher than 60%, motility duration ranged between 1.5 and 6 min and survival of sperm at 4°C ranged between 3 and 10 days. To our knowledge, this is the first study reporting on sperm quality of wreckfish and no study has been conducted on its congener, the hapuku. However, the mean values observed fall within the values already reported for other marine fish species, for example mean density has been found to be 8.6 - 23.7 spermatozoa x 10^9 ml^{-1} in the red porgy (Mylonas et al., 2003), 12-27.1 spermatozoa x 10^9 ml^{-1} in the sharpsnout seabream (Papadaki et al., 2008), 18.9-31.5 spermatozoa x 10^9 ml^{-1} in the meagre (Mylonas et al., 2013) and 38 spermatozoa x 10^9 ml^{-1} in the Atlantic bluefin tuna *Thunnus thynnus* (Suquet et al., 2010). Similarly, motility duration ranged between 2-4 min in the red porgy (Mylonas et al., 2003), 2-6 min in the sharpsnout seabream (Papadaki et al., 2008), 0.78-1.27 in the meagre (Mylonas et al., 2013) and 10-11 min in the Atlantic bluefin tuna (Suquet et al., 2010). In this sense, sperm quality of wreckfish males can be considered good, with no noticeable dysfunction involved, neither in the quality nor in the quantity of sperm produced.

In male fish, spermatogenesis is controlled by the two gonadotrophic hormones, follicle–stimulating hormone (FSH) and LH. Follicle-stimulating hormone acts on the Leydig cells to stimulate androgen production, whereas LH acts on the Sertoli cells to support germ cell survival and development (Schulz & Miura, 2002). Androgens then act on the gonads; T has been associated with spermatogenesis and 11-KT with spermiation, with 11-KT being the most effective androgen in teleosts (Borg, 1994). In the present study, both T and 11-KT were found to reach their highest values at late stages of spermiation (S3 spermiation index), like in cod, where higher T and 11-KT levels were observed concomitantly with the presence of free spermatozoa in the testis lumen (Dahle et al., 2003), but unlike the Senegalese sole, which was found to exhibit the highest T and 11-KT values at late spermatogenesis and not at functional maturation (García-López et al., 2006b). On the other hand, 17,20β-P seemed to keep constant low values at all stages of spermiation. These low and unchanged levels of 17,20β-P can be attributed to the reasons discussed above for females. Another possible explanation for the unchanged values of 17,20β-P may be that this hormone is produced constantly by fish that are able to produce sperm all-year round, as happens also with the Senegalese sole, which shows relatively constant 17,20β-P values, as well (García-López et al., 2006b). Moreover, 20β-S has been suggested to be the MIS in most males in marine fishes, in contrast to freshwater species, for which 17,20β-P is the effective MIS (Schulz et al., 2010).

In conclusion, the results of the present study show that wreckfish females can adapt to captivity, mature and produce eggs both under fluctuating natural and under constant low temperatures. Plasma sex steroid hormones in females correlate well with the maturity stages of females, except for 17,20β-P. Some females, however, cease oocyte development at the cortical alveoli stage and their oocytes did not appear to grow larger than 350 μm, or at the vitellogenesis stage and cannot reach maturation. Males, on the other hand, produce sperm of good quantity and quality, capable to fertilize the eggs produced. Moreover, wreckfish

males can produce sperm all-year round, making it available to fish farmers for artificial fertilization whenever it is needed. Plasma sex steroid hormones in males rise when fish are fully spermiating, suggesting that except for 17,20β-P, they correlate well with the maturity stages of males. One possible reproductive dysfunction that could be attributed to male wreckfish is the very low fertilization percentage found in a big number of spontaneous spawns. In some cases, although females spawned large numbers of eggs, these eggs were unfertilized, a fact that could be attributed to a failure in the male breeding behaviour. Further studies should look into the lack of maturation in the females and conduct experiments on the environmental conditions that the fish are held, in order to hopefully increase the number of females that can mature and spawn. Moreover, hormonal induction of oocyte maturation and spawning, for a better control of spawning and egg production, as well as in vitro fertilization trials, could overcome the breeding behavioral problem of males and increase the current observed fertilization percentage.

Task 6.3 Development of spawning induction procedures (led by IEO, Blanca Alvarez).

HCMR stock: As described in the 2nd Periodic Report, month 30 (the final month of the 2nd period) was in the middle of the reproductive season of 2016. As a result, a part of the data regarding the experiments of that spawning season are presented here in the 3rd Periodic Report, beginning from where the report was left in the 2nd Periodic Report. At that time, an earlier trial of induction of spawning was just starting, compared to the 2015 spawning season. After the first spawning (9/5/2016, data in 2nd Periodic Report), fish were handled again on 23/5/2016, when the female was in post ovulation stage, having at the same time some oocytes in early OM of 1500 μm diameter (**Fig. 6.3.1**). The female was given a GnRHa implant of 1000 mg to promote the maturation and male an implant of 500 μg to ensure sperm production. Three days later (26/5/2016) the female was stripped of the eggs (5,000 eggs, **Fig. 6.3.2**), which were inseminated artificially. The female also contained Vg, OM and overripe oocytes, of 1225, 1550 and 1750 μm diameter, respectively (**Fig. 6.3.1**). On 28/5/2016, a batch of 1,293 g of eggs (approximately 400,000 eggs) was stripped off the female (**Fig. 6.3.3 A,B**) and again artificial inseminated with sperm. However, both artificial insemination trials were not successful, since no viable eggs were observed the following days.

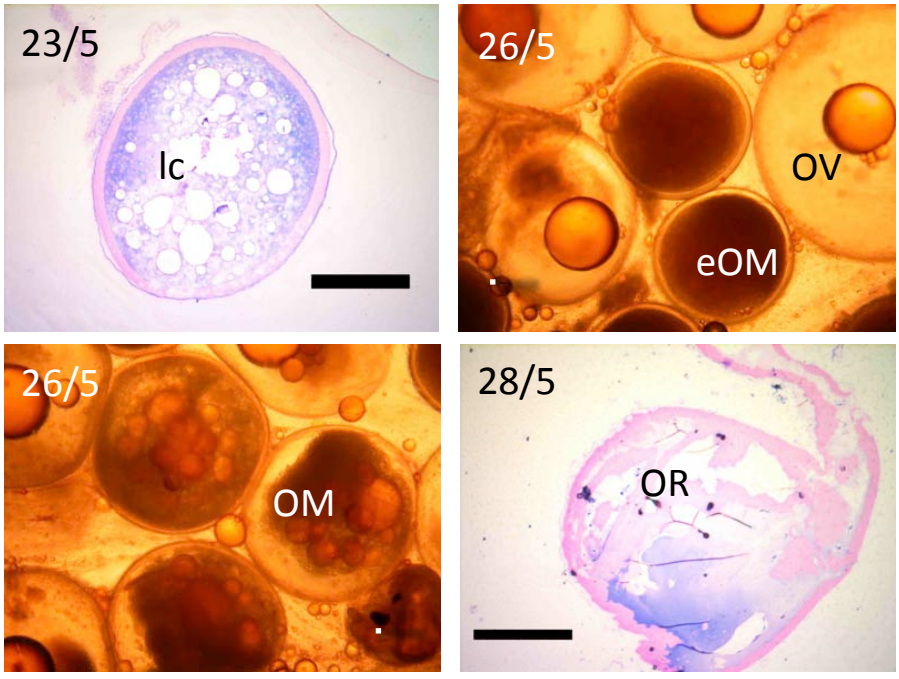

Figure 6.3.1. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2016 reproductive season (dates on each photo). lc = lipid coalescence, eOM = early Oocyte Maturation, OM = Oocyte Maturation, OV = Ovulated, OR = Overripe. Bar = 500 μm

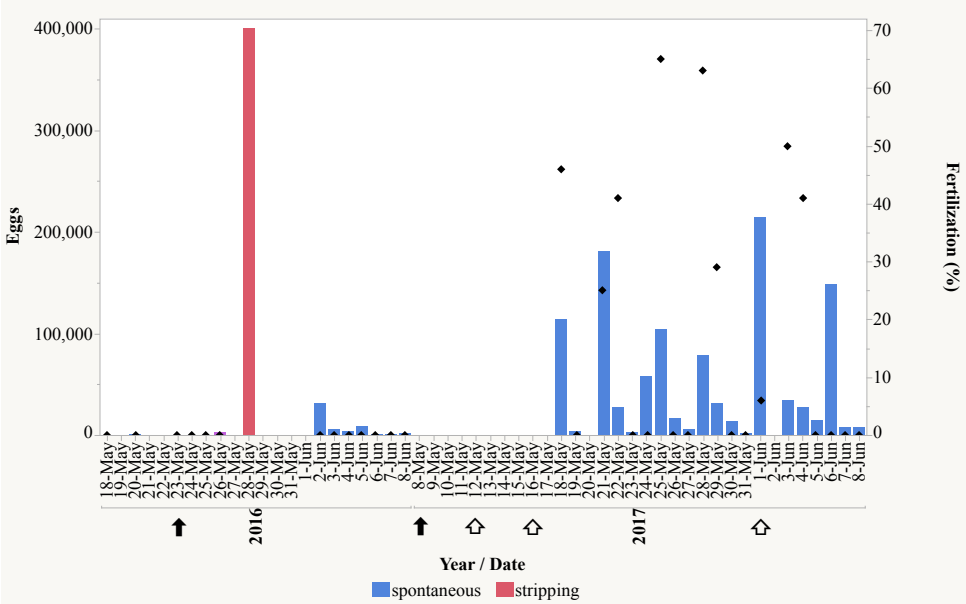

Figure 6.3.2. Fecundity (eggs, bars) and fertilization (%, marks) of induced spawning of wreckfish with GnRHa treatment in 2016 and 2017 spawning periods. Spontaneous spawns are indicated with blue bars while stripped spawns are indicated with red bars. Filled arrows indicate induction with GnRHa implants while empty arrows indicate induction with GnRHa injections.

In the 2017 spawning season, the female was treated with a 600 μg GnRHa implant on 8/5/2017 and the male with 400 μg. At that time, the female was at the stage of vitellogenesis with oocytes of 1225 μm in diameter (**Fig. 6.3.4**), while the male was producing fluent sperm. Four days later (12/5/2017) the female had progressed since oocytes at early OM of 1500 μm, concomitantly with Vg oocytes of 1250-1300 μm in diameter were present, and a liquid GnRHa injection of 20 μg/kg was given. Two days later (14/5/2017) the female was at the same situation as before, in terms of ovarian maturation condition. On 16/5/2017 at noon, the female had oocytes in OM of 1750 μm in diameter, and after 11 hours, it had oocytes at pre-ovulation stage of 2125 μm (**Fig. 6.3.4**). We again injected the fish with the same dose to induce the maturation of the second batch of oocytes (1500 μm) and let the fish to spawn spontaneously. Two days later, 114,000 eggs were collected and 46% were fertilized (**Fig. 6.3.2, Fig. 6.3.3 C,D,E**). The next day (19/5/2017), oocytes at the OM stage of 1550 μm were present in the ovary of the female, with some overripe eggs. Spawning events of variable fecundity and fertilization took place the next days (**Fig. 6.3.2**). The fish were checked again on 1/6/2017 and the female had early OM oocytes of 1375 μm in diameter. At the same time, a 3rd GnRHa injection was given to the female, while a GnRHa implant was given to the male. The last treatment provoked six spawns the next days, however with lower fecundity and fertilization. Sperm, at the time of each evaluation, was of good quality both for 2016 and 2017 reproductive season (**Fig. 6.3.5**).

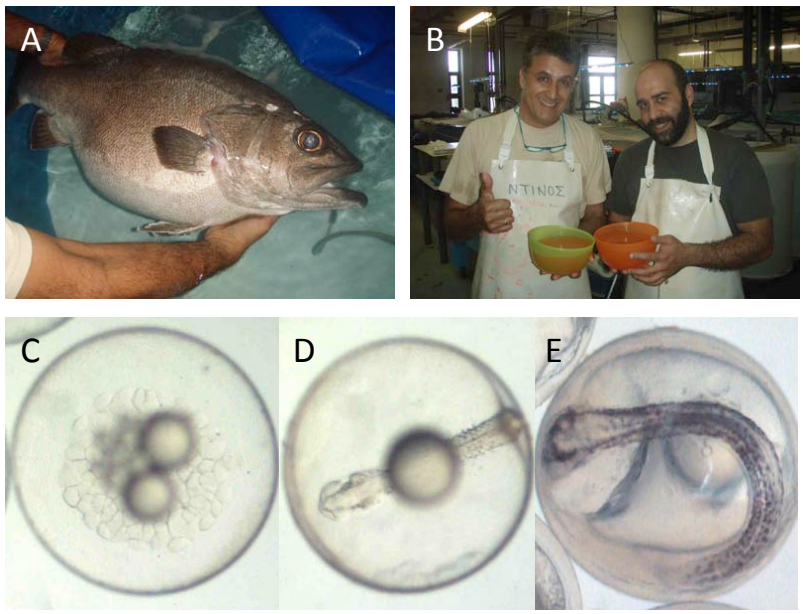

Figure 6.3.3. A) Female wreckfish at HCMR just before stripping and an *in vitro* fertilization attempt, B) Eggs after artificial insemination, C,D,E) Fertilized egg of wreckfish.

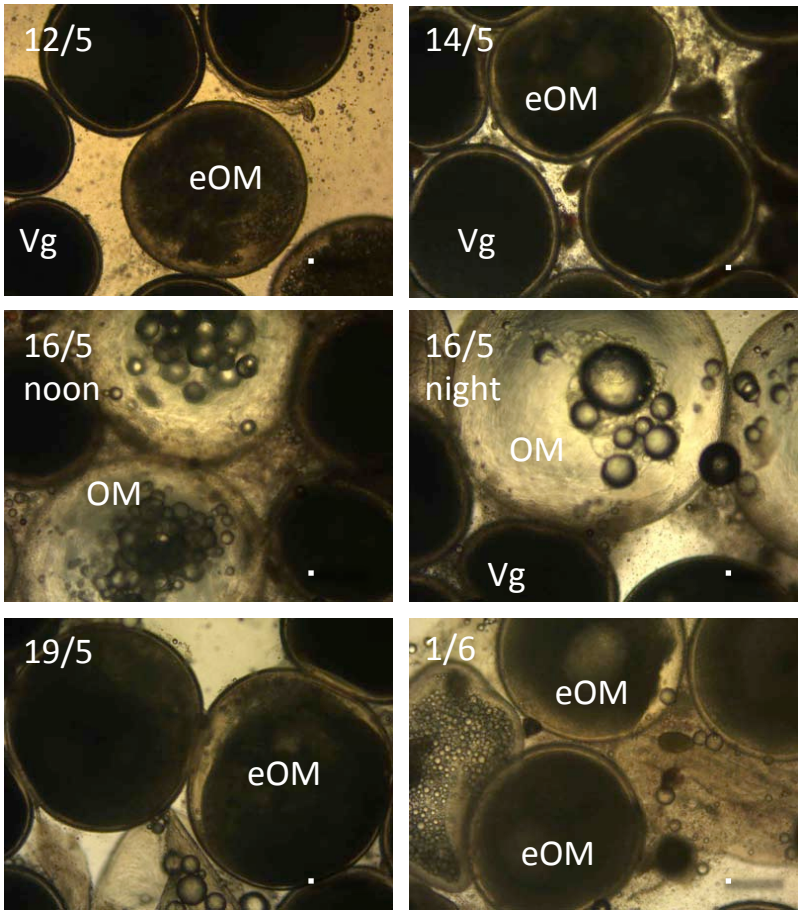

Figure 6.3.4. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2017 reproductive season (dates on each photo). Vg = vittelogenic oocyte, eOM = early Oocyte Maturation, OM = Oocyte Maturation. Bar = 500 μm

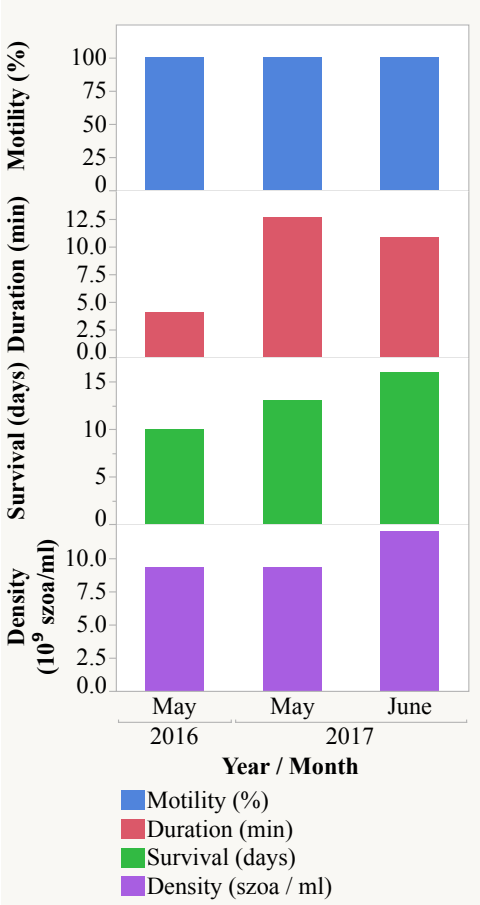

Figure 6.3.5. Sperm quality parameters of the wreckfish at P1. HCMR during the 2016-2017 spawning induction experiments.

IEO, CM2 and CMRM STOCKS

Respect to spawning induction in these stocks, the experiments were continued in order to achieve the objectives. Some fishes of the two stocks, both at the CMRM (2016) and the CM2 facilities were selected to test the different GnRHa hormone dose to induce spawning (**Table 6.3.1**).

The females response to the GnRHa implants varied, with no response (2015), with a spontaneous or stripping spawns with non viable eggs (2016) or with good results in the fecundation but not in hatching (2017). There was only one spawn that was successfully cultured until 25 day post hatching (2016).

YEAR	STOCK	FISH	WEIGHT (KG)	DATE IMPLANT	OOCYTES SIZE	Imp. GnRH (µg)	Dosis (µg/kg)	SPAWNING DATE	Total eggs (ml)	FECUNDATION(%)	OBSERV.
2015	IEO		16,5	09/06/2015	0,95	500	30,3				NO SPAWNS
	MC2		27,35	11/06/2015	1,121	500	18,3				
			33,15		1,092	500	15,1				
2016	MC2	5853	18,2	23/06/2016	1,197	1000	54,9	29/06, 3/07, 8/07, 12/07, 16/07 y 20/07	2200, 325, 180, 980, 730 y 670	86, 78, 49, 85, 75 y 60	ALL SPONTANEOUS. LARVAE ALIVE UNTIL 25 DPH
		5544	16		1,119	750	46,9				NO SPAWNS
	CMRM	3FF2	14,3	28/06/2016	1,388	750	52,4	11/07 y 12/07	100 y 150		SPONTANEOUS AND STRIPPING. NON VIABLE EGGS
		7B19	13,5	28/06/2016	1,255	750	55,6				NO SPAWNS
			13,5	12/07/2016	1,36	750	55,6	18/07 .	750	47	STRIPPING. NON VIABLE EGGS
			13,7	26/07/2016	2,23	750	54,7				NO SPAWNS
2017	MC2	7B78	23	1 ST:07/06/2017	1,161	1750	76,1	20/06/2017	3500	0	STRIPPING. DIED BECAUSE IT DOESN'T STRIPPING IN ADECUATE TIME
				2ND:15/06/2017	1,161	2000	87,0				
		2B47	18.5	1ST: 11/05/2017	1,135	1000	54,0	27/05/2017	240	65,6	NON HATCHING
				2ND:18/05/2017	1,035	500	27,0	30/05/2017	700	3,5	LARVAE ALIVE UNTIL 25 DPH
		5853	21,3	1ST:30/06/2017	1,2	1750	82,2	16/07/2017	900	0	SPONTANEOUS. NON VIABLE EGGS
				2ND:04/08/2017	1,8	1500	70,4	09/08/2017	3750	0	SPONTANEOUS. NON VIABLE EGGS

Table 6.3.1. GnRHa implant data of the different trials during 2015, 2016 and 2017 in some females of the three broodstocks in Galicia: IEO, MC2 and CMRM.

During 2017 trials with a female from CMRM facilities was done. On the 30th of May a GnRHa implant of 69.4 µg kg^{-1} was administered to a female with a 1.29 mm of oocyte size. As no response was obtained, on the 16th of June, with a oocyte size of the female of 1.74 mm, first and only one injection of 20 GnRHa µg kg^{-1} was administered. A spontaneous spawn was obtained on the 19th of June, but all eggs was overrriped (**Fig 6.3.6**).

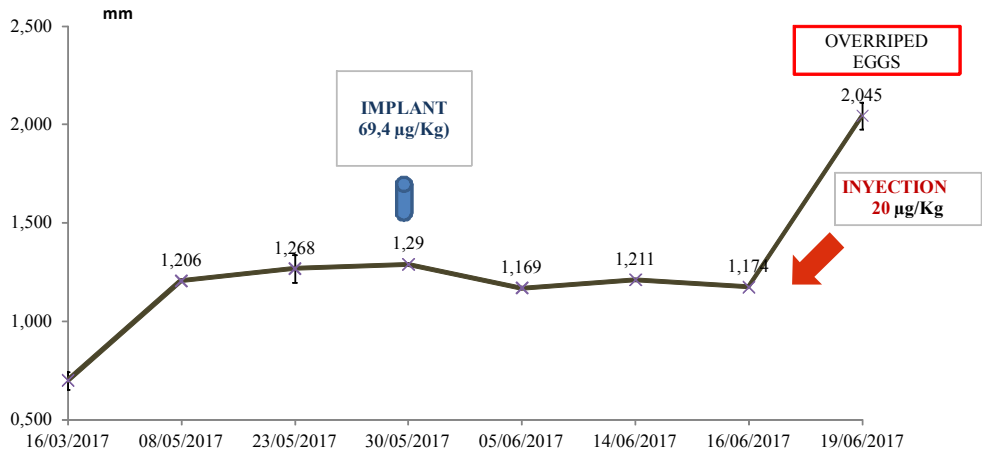

Figure 6.3.6. Trial with a female from CMRM facilities. The 30th of May a implant with a 69,4 µg of GnRHa/kg of female with a 1,29 mm of oocyte size was administrated. The 16th of June, with a oocyte size of the female of 1,74 mm, first and only one injection was boosted.

The necessity of more information on this subject required more females. Therefore, we used the stock from Isidro de la Cal Company, and worked with two of their females, based on an agreement made for that purpose. The experiments were carried out during the months of June and July of 2017.

On the 8/06/2017, a 16 Kg female with an oocytes size of 1.35mm was implanted at first with a 62.5 µg kg^{-1} of GnRHa, with no response. On the 2nd of June the first GnRHa liquid intramuscular 20 µg kg^{-1} injection og GnRHa was administrated (**Fig. 6.3.7**). The female was checked every 12 h after injection. Three days after, there were no changes in the oocytes development, and a second injection was administrated with a 20 µg kg^{-1} of GnRHa. As there were no changes, on June 15th a third 20 µg kg^{-1} GnRHa injection was given. Two days later, a little quantity of egg it was obtained by stripping. Every 12 h the female was checked and stripped, and a few overriping eggs were obtained. Six days after the third injection, 950 ml of eggs were obtained by stripping. From these, 750 ml of floating eggs were fertilized. One day after that, a spontaneous spawn of 110 ml was obtaiend, with only 20 ml of floating eggs. On the 26th of June, a forth injection with the same doses of GnRHa (20 µg kg^{-1}) was given. During the successive days, the female was checked and a little amount of eggs were obtained by stripping until the 2nd of July with a little spontaneous spawn, as well as a high volume obtained by stripping (525 ml). From the floating eggs (375 ml), only 10% were fertilized and all died before hatching.

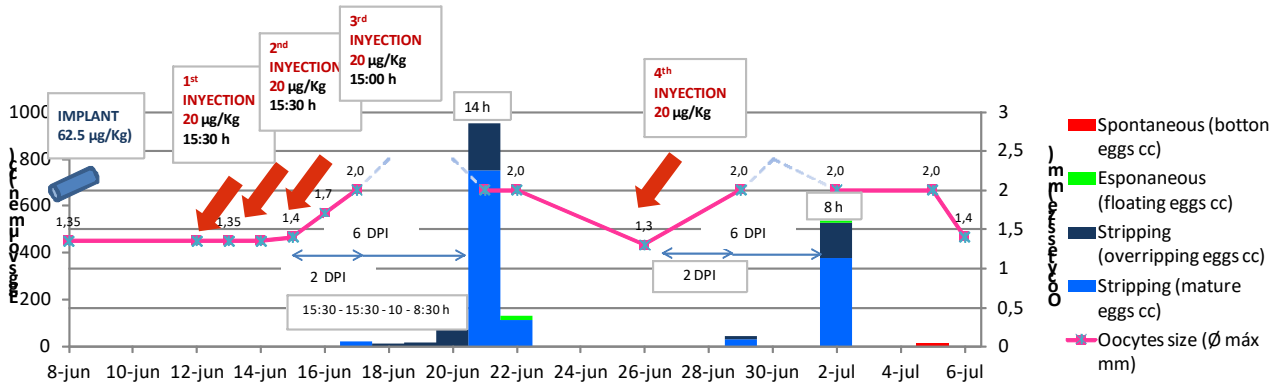

Fig. 6.3.7. Trial of hormonal induction (GnRHa) with a 16 Kg female from Isidro de la Cal facilities.

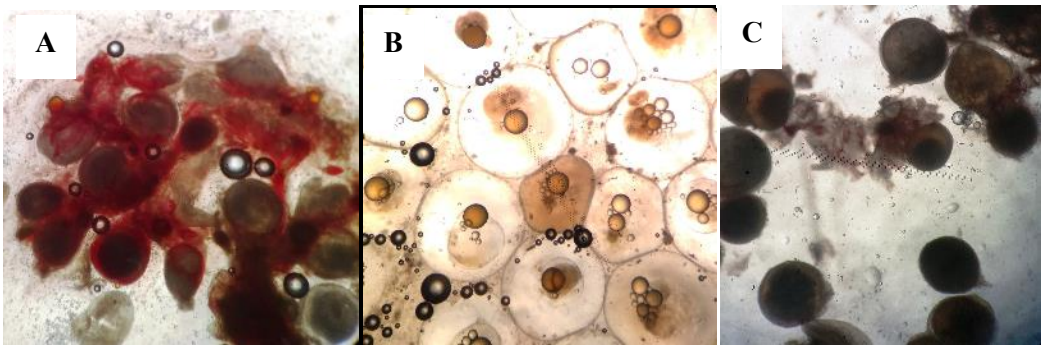

Figure 6.3.8. Gonadal biopsies of the 16 Kg female, the 17th (A), 18th (B) and 19th (C) of June 2017.

Another Isidro de la Cal's female was treated with a GnRHa injection with the same protocol (**Fig 6.3.8**). On the 23rd of June the first dose was administrated with oocytes size of 1.3 mm. Three days later, the second injection was given and one day after the female was very swollen and a plug was extracted. The next day, 315 ml of floating eggs were obtained and were fertilized. Every three days after the stripping, batches of overripped eggs were obtained.

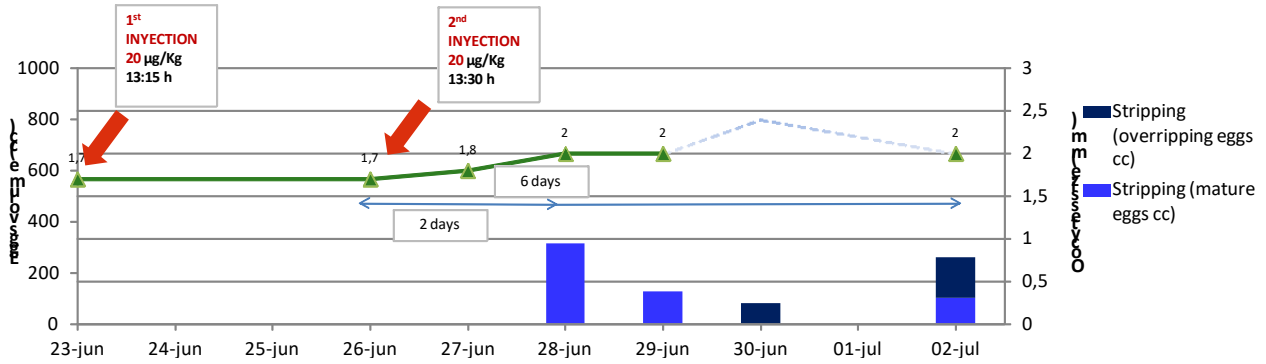

Fig. 6.3.9. Trial of hormonal induction (Injection with GnRHa) with a 18 Kg female from Iidro de la Cal facilities.

As a result of these trials, we obtained information on the ovulation time after induction with the GnRHa hormone. Results suggest that the injections are more effective than the implants, with a faster response. The problem is the risk of gonadal plugs if the hormonal dose is not adequate. Results show a time of response of about six days after the injection. Also, at second day after injection, the ovulation reaction seems to start.

Task 6.4 Evaluation of sperm characteristics and cryopreservation protocols (led by IFREMER, Christian Fauvel).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D6.1 Computer Assisted Sperm Analysis (CASA) for wreckfish sperm*** and ***Deliverable D6.2 Cryopreservation method for wreckfish***.

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Deviations from Annex I and their impact:

The major obstacle in finish all the activities included in T**ask 6.2. Describe reproductive cycle**, is the lack of wreckfish pituitaries, which are crucial to validate and optimize the available heterologous ELISAs for this species. This is a prerequisite before analyzing plasma samples that we have already collected. Since we are depending on episodically dead fish and can`t plan in advance a sampling campaign. We are trying to obtain wreckfish from the fish market to get pituitaries and validate the ELISAs procedures to finish this task. Respect to the nutritional status of the wild fish, the proximal composition and fatty acids profile of wild wreckfish are finished, but the carotenoids and C and D vitamins could not be measure due to a problem with the transport of the tissues samples. This problem could be solve during the next months.

WP 7 Reproduction & Genetics – grey mullet

WP No:	7	**WP Lead beneficiary:**		P4. IOLR
WP Title (from DOW):	Reproduction and Genetics – grey mullet			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P13. UNIBA	P14. IFREMER
P15. ULL	P25. DOR			
Lead Scientist preparing the Report (WP leader):	Hanna Rosenfeld (P4)			
Other Scientists participating:	Constantinos Mylonas (P1), Neil Duncan, Sandra Ramos, Wendy Gonzalez, Elvira Fatsini, Joan Cerda, Francois Chauvigne, Ignacio Gimenez (Raraavis) (P3), Aldo Corriero (P13), Christian Fauvel (P14), Covadonga Rodriguez (P15), Hagay Sarusi (P25)			

Objectives

2. Evaluate the effectiveness of hormone-based treatments on synchronizing gonadal development and improving gamete (eggs and sperm) quality in mature grey mullet,
3. Develop hormone-based treatments for induced spawning of grey mullet,
4. Optimize a scaled-up breeding of grey mullet in captivity under natural and manipulated photo-thermal regimes,
5. Assess the effects of captivity on first sexual maturity and reproductive potential of captive-reared and hatchery-produced grey mullet broodstocks.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Lacking the natural spawning environment, captive grey mullet fail to reproduce spontaneously, largely due to a failure to undergo complete gametogenesis. Therefore, **Task 7.1 Evaluated the effectiveness of hormone-based treatments on synchronizing gonadal development.** A combined treatment consisting of follicle stimulating hormone (FSH) and dopamine antagonist (metoclopramide) on spermatogenesis in males and follicle growth and maturation in females was tested. The methylotrophic yeast (*Pichia pastoris*) expression system was used to produce large quantities of bioactive recombinant single-chain FSH, which was used in a series of *in vivo* assays. Unlike the controls, the hormonally treated groups (injected with rFSH and metoclopramide during the onset of the reproductive season) demonstrated synchronized gonadal development within and between sexes, with higher rates, over time, of spermiating males and post-vitellogenic females. In **Task 7.2 Development of hormone-based treatments for inducing spawning**, spawning induction trials that timed the administration of GnRHa and metoclopramide with advanced stages of gamete maturation were relatively successful producing tens of millions of fertilized eggs. Nevertheless, our results highlight two major problems: (i) female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) episodic fertilization rate ranging between 0 to 98%, implicating the need to further fine tune and optimize the hormone-based breeding protocol for captive grey mullet.

Summary of work reported in the previous Reporting Period (13-30 Mo):

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development. The yeast expression system was used to produce large quantities of bioactive recombinant single-chain FSH (r-FSH), which was used in a series of *in vivo* assays. According to the original workplan, several hormonal treatments were tested in order to advance gametogenesis in captive grey mullet males and females. Treatment consisting of r-FSH and dopamine antagonist (metoclopramide) performed best giving rise to enhanced spermiation in males and follicle growth and maturation in females was tested. Unlike the controls, the hormonally treated groups demonstrated synchronized gonadal development within and between sexes, with higher rates, over time, of spermiating males and post-vitellogenic females. In **Task 7.2 Development of hormone-based treatments for inducing spawning,** spawning induction trials that timed the administration of GnRHa and metoclopramide with advanced stages of gamete maturation were relatively successful producing tens of millions of fertilized eggs during natural (September-November 2014, 2015) and shifted (January-February 2016) reproductive season. Nevertheless, our results highlight two major problems: (i) female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) episodic fertilization rate ranging between 0 to 98%, implicating the need to further fine tune and optimize the hormone-based breeding protocol for captive grey mullet. In **Task 7.5 Establish a shipping protocol for grey mullet eggs,** a previously developed protocol available at the IOLR was found to be applicable to shipping grey mullet eggs. Yet, further fine-tuning of the latter protocol will be carried out during the forthcoming grey mullet natural spawning season.

Summary of progress towards objectives (31-48 Mo):

Task 7.3 Optimization and scale-up of a breeding protocol for grey mullet in captivity. The established breeding protocol for captive grey mullet can be effectively applied during natural as well as artificially shifted spawning seasons. During 2016 and 2017, tens millions of quality eggs were produced giving rise to mass production of robust fingerlings. The basic breeding units consisting of a single female and three males, seems to improved synchronization and increase the fertilization rate. Broodstock diet containing fish oil (FO), which is relatively rich in n-3 LCPUFA, positively affected hatching success and larvae survival. In **Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish.** The size of age 6 hatchery-produced specimens is equal to that of wild individuals of the same age class. All the age 2 mullets analysed were still sexually immature. The biometric and histological analyses showed that body growth and gonad development of age 2 hatchery-produced mullets proceed in a slightly faster way compared with wild-caught specimens. The 3 year old grey mullet exhibit sex related growth and gonadal development patterns. The 3 year old hatchery produced mullet females and males exhibited enhanced gonadal maturation than that in the wild-caught captive-reared fish, probably the outcome of domestication. In **Task 7.5 Establish a shipping protocol for grey mullet eggs,** short term (≤ 11 h) shipping of gastrula stage, grey mullet eggs can be carried out with a relatively high egg density. For the long term (26 h) shipments protocol available at the IOLR can be successfully applied to mullet eggs, with a maximal stocking density of 15,000 eggs l^{-1} and a total sea water volume of 10 l.

Details for each Task

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development (led by IOLR, Hanna Rosenfeld).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D7.1 Analysis of sperm motility: General protocol and propositions for mullet sperm quality assessment***; ***Deliverable 7.2 Production of recombinant bioactive LH and FSH assay for grey mullet***; and ***Deliverable 7.3 Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet***

Task 7.2 Development of hormone-based treatments for inducing spawning (led by IOLR, Hanna Rosenfeld).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 7.3 Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet***

Task 7.3 Optimization and scale-up of a breeding protocol for grey mullet in captivity (led by IOLR, Hanna Rosenfeld).

Aiming to optimize and scale-up the breeding protocol for captive grey mullet experiments that were carried out during 2016 and 2017 by P4. IOLR have focused on: (i) defining the best performing breeding unite, (ii) expanding the spawning season through the use of manipulated photo-thermal regime, and (iii) improving broodstock diet. These experiments employed the hormonal-therapy protocol that was calibrated at the early phase of the project and summarized in ***Deliverable 7.3***.

Grey mullet breeders, consisted of P4. IOLR hatchery-produced (G1) fish that were individually tagged and maintained in 4 m^3 tanks supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40 ‰ salinity and subjected to a natural or manipulated photo-thermal regime (a 4 month shift). Food was provided at the rate of 1-1.5% of their body weight.

To accelerate gonadal development mullet females and males were injected at the onset of gametogenesis (natural season: mid-July; shifted season: mid-October) with metoclopramide (15mg/KgBW) combined with r-FSH (5 µg per kg BW). One month later the males received MT-EVAc implant (5 mg/kgBW). Gonadal biopsies were timed with the advanced stages of gametogenesis (natural season: September-October; shifted season: December-January). The relative abundance of fully mature females, and spermiating males were recorded. Females were considered fully mature once their oocytes reached an average diameter greater than 550 µm and more than 50% of sampled oocytes exhibit germinal vesicle migration. Sperm quality was classified into one of four categories based on its quantity, fluidity and ability to spread in the water.

Spawning induction trial were carried out during 2016 and 2017. Once identified, a reproductively mature female was stocked with either two or three spermiating males (unless specified otherwise) in a 1-m^3 tank supplied with seawater at 24-27°C. The selected fish were treated with GnRHa combined with Metoc. Each treatment consisted of priming (GnRHa 10 µg/kg; Metoc 15mg/kg) and resolving injections (GnRHa 20 µg/kg; Metoc 15 mg/kg) given 22.5 h apart.

Breeding unit effects on spawning success

In previous spawning induction trials (P4. IOLR; 2014, 2015) fertilization rates were inconsistent and ranged between 0 to 98%, pointing to impaired breeding behaviour, either in terms of courtship or spawning synchronization between the sexes. Therefore, during the natural spawning season (2016) three breeding units varying in ratios of female to male and tank size, were tested and the rates of spawns per induction trails, attaining fertilized eggs per spawning event and hatching success were recorded and summarized in **Table 7.3.1.** While the bigger breeding unit, consisting of 3 females and 6 males in 3 m^3 tank, was found to be successful in terms of spawning events (100% of the induction trials), the fertilization rate was relatively low (50%). In contrast, the small breeding unit, consisting of a single female and 3 males in 1 m^3, demonstrated reduced spawning events (50% of the induction trials) yet, with higher rates of fertilization and hatching successes (**Table 7.3.1**).

Table 7.3.1. Variable breeding units of captive grey mullet and their outputs in terms of spawning, fertilization and hatching success

Breeding unit	A (n=10)	B (n=5)	C (n=2)
Spawning rate (%)	50	80	100
Fertilization rate (%)	80	75	75
Hatching success (%)	92.6 ± 3.5	72.9 ± 11.6	73.4 ± 7.3

A= 1 female X 3 males, 1 m^3 tank; **B**= 2 females X 3 males, 1 m^3 tank; **C**= 3 females X 6 males, 3 m^3 tank

Captive grey mullet reproductive performance during a shifted spawning season

Four year old grey mullet females (n=18; av. BW= 1868 ± 74 g) and males (n=18; av. BW= 1154 ± 46 g) were acclimatized to 4-month shifted photo-thermal regime. Fish were conditioned for spawning and their reproductive performance was monitored as described above. Results demonstrate the fish responsiveness to the shifted reproductive season. During December 2016 through January 2017, females (45%) gradually reached the post-vitellogenic stage with oocyte diameter exceeding 500 µm (**Fig. 7.3.1**), whereas males (40-50%) were steadily spermiating (**Fig. 7.3.2**). Following hormonal treatment spawning has occurred in 3 out of 4 trials (**Table 7.3.2**). Fecundity in the shifted (2.22 ± 0.19 million eggs / kg BW) and the natural (2.5 ± 0.1 million eggs / kg BW) spawning season are comparable.

Table 7.3.2. Summary of the spawning data obtained from hormonally induced females during a shifted spawning season (December 2016-January 2017).

Date	Female BW (g)	Floating eggs (ml)	Sinking eggs (ml)	Fecundity (10^6 eggs/Kg BW)
20.12.2016	1660	272	710	2.1296
15.1.2017	2310	1070	180	1.9481
15.1.2017	2470			
23.1.2017	1930	1310	70	2.5741

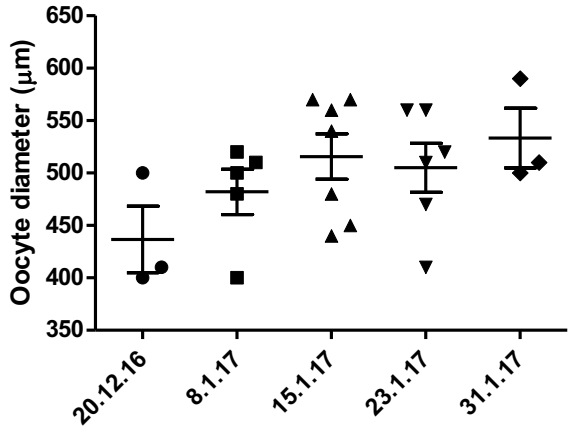

Figure 7.3.1. Oocyte growth in hormonally-treated grey mullet females subjected to shifted photo-thermal regime.

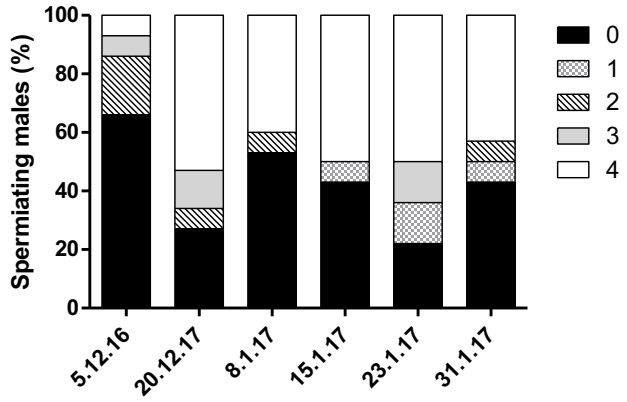

Figure 7.3.2. The abundance of spermiating males during a shifted spawning season (December 2016-January 2017). 0- no milt, 1- traces of viscous milt, 2-relatively small amounts of white milt, 3- fluid milt 4-flowing fluid milt, easily spread in the water.

Broodstock diet

Based on the results of proximal and main fatty acid (FA) composition in gonads from wild and domesticated mullets (Task 13.4), a new broodstock diet containing fish oil (FO), which is relatively rich in n-3 LCPUFA, was tested in comparison to the P4. IOLR grow out diet containing soybean oil (VO). During the onset of the reproductive season (early July 2017), 6-year old captive grey mullet broodstocks were divided into two groups that were fed with either FO or VO diets (run in triplicates). Fish were conditioned for spawning as described above and their reproductive outputs are summarized in **Table 7.3.3**.

Results indicate no apparent diet effects on reproductive performance of the grey mullet broodstocks. Nonetheless, the FO diet seems to affect egg color (**Fig. 7.3.3**) and to increase the hatching success and survival rate of the larvae (see Task 13.4).

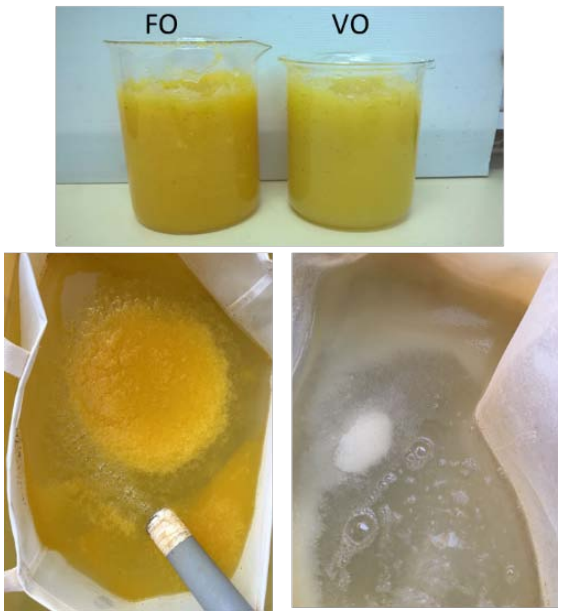

Figure 7.3.3. Effects of diets containing fish (FO) and soybean (VO) oil on grey mullet egg color.

Table 7.3.3. Comparison of spawning data obtained from grey mullet broodstocks fed diets differing in their oil source: fish oil (FO) vs. soybean oil (VO).

	DIETS	
	FO	VO
Females BW (g)	1660.36 ± 67.8	1753.67 ± 75.5
Males BW (g)	987.86 ± 46.06	905.77 ± 48.11
Abundance of post vitellogenic females (>500 µm)	71.86 ± 5.9	69.86 ± 9.4
Abundance of spermiating males - September, 2017	41.67 ± 4.8	46.30 ± 13.0
Abundance of spermiating males - October, 2017	28.97 ± 16.8	13.09 ± 7.2
Fertilization rate (%)	50	66
Fecundity (million eggs/kg BW)	2.12 ± 0.1	2.89 ± 0.9
% hatching 0 DPH	37.25	32.2
% Survival 0 DPH	60.25	51.25

Spawning induction trials with wild-caught captive-reared grey mullet broodstock

During the months of November 2016 through to February 2017 a total of 85 grey mullet ranging from 0.3 to 4 kg were caught and transferred to the facilities in IRTA. Quarantine and parasite treatments were applied and the wild fish were adapted to captivity. During the adaptation period that extended from March through to July feeding was a problem. A wide range of diets, different pellets of different sizes, pastes, algae, polychaetes and mussels were presented to the mullet. A mixed response was obtained ranging from fish that feed well and increased in weight to fish that lost weight.

During the same period, *Mugil cephalus* recombinant FSH (rFSH) was produced using the mammalian CHO (Chinese Hamster Ovary) expression system. Briefly, five grey mullet were injected with GnRHa and Metoclopramide to increase the production of gonadotropins in the pituitary. For each animal, the pituitary was dissected, frozen immediately in liquid nitrogen and stored at -80ºC. Total RNA was extracted from the pituitaries with lysis solution and 2-Mercaptoethanol (GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich Co.) Complementary DNA (cDNA) was obtained by reverse transcription. To clone *fshβ* and *α* subunits, a PCR was made adding MgCl2, dNTP mix, DTT and primers designed on *Solea senegalensis* F1 to cDNA. The PCR products were subcloned in pGEM-T Easy Vector and transformed. Recombinant colonies were identified by showing a specific colour and were incubated. Recombinant plasmid DNA was extracted using GenElute Plasmid Miniprep Kit (Sigma-Aldrich Co.) by restriction enzymes. The recombinant plasmid DNA was sequenced and the selection of *α* chain was determined upon comparison with sequences from other species. The sequences were sent to Rara Avis Biotec S.L. and single-chain rFSH was produced in mammalian CHO (Chinese Hamster Ovary) cells.

On Aug 1, the wild mullet held in captivity were sampled for maturity status and all fish were immature. The possible males had no sperm and the females had an oocyte diameter of 96 ± 26 µm. On Aug 8, an experiment was initiated with the aim to evaluate the effect of rFSH on gametogenesis. Two groups of 12 mullet (8 female and 4 male, mean weight 990 ± 212g) were set up and received weekly intramuscular injections either of CHO cells medium (Control fish) or rFSH at a dose of 15 µg/kg. Females with oocytes greater than 350 µm were also injected on week 12th with 5 µg/kg of *Solea senegalensis* rLH, on week 13th

with 10 µg/kg of rLH and on week 15th with 1000 IU/kg of hCG (human chorionic gonadotropin). Females with oocyte diameter greater than 550 µm (final stages of vitellogenesis) were induced to complete maturation and ovulate following the protocol with GnRHa and Metoclopramide used in Diversify. Ovarian biopsies and blood samples were collected throughout the experiment. Ovarian biopsies were fixed in Bouin's solution for histology and examined as a fresh sample to measure the diameter of the 20 largest and most advanced vitellogenic oocytes. Plasma was taken from blood samples for the analysis of steroids and gonadotropins. The presence of flowing milt was assessed and motility measured. The experiment has continued for four months and has not been completed.

Initial changes in oocyte diameter and levels of estradiol in the group treated with rFSH and compared to the control group indicated that the rFSH has induced gametogenesis (**Fig. 7.3.4**). Oocyte diameter increased significantly ($P<0.05$) from 96.25 ± 26.31 µm at the start of the experiment to 306.75 ± 116.81 µm on week 10 in the group treated with rFSH compared to the control group that did not register any change in oocyte diameter (**Fig. 7.3.4a**). Similarly, plasma levels of estradiol increased significantly ($P<0.05$) from 37.90 ± 29.47 pg/mL at the start of the experiment to 332.36 ± 182.89 pg/mL on week 3 in the group treated with rFSH, whilst no change was observed in estradiol levels in the control group (**Fig. 7.3.4b**). Work continues with the fish to obtain the completion of vitellogenesis and oocyte maturation as well as to complete the analysis of the plasma and oocyte samples.

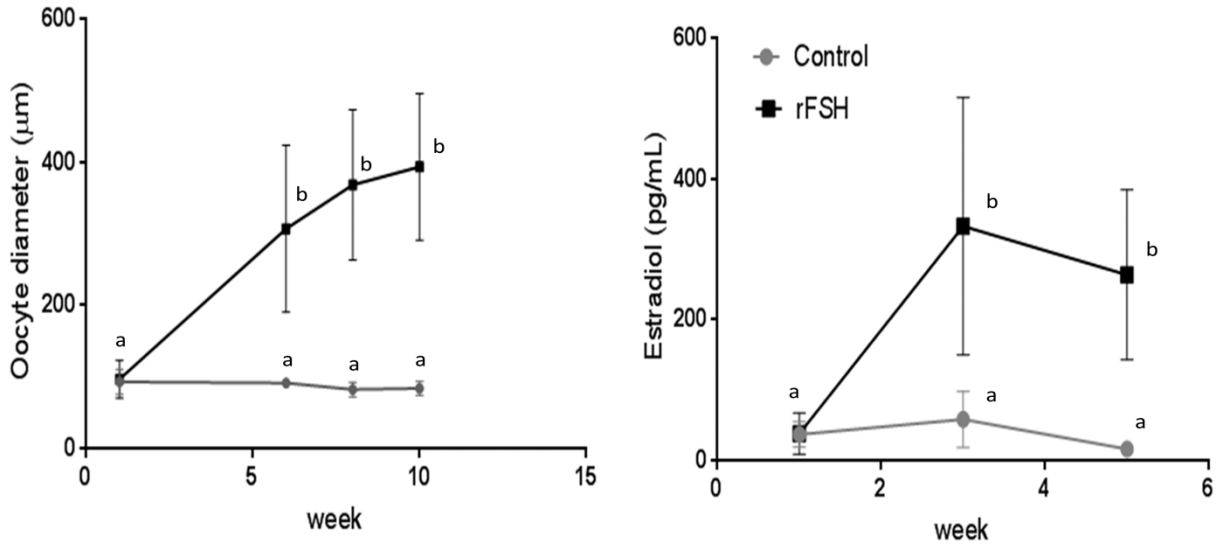

Figure 7.3.4. (a) Oocyte diameter (µm) and (b) estradiol plasma levels (pg/ml) in control (grey circle) and rFSH-treated (black square) groups of grey mullet (*Mugil cephalus*) over the initial weeks of experiment. Different letters indicate significant differences ($P<0.05$).

Conclusions

1) The established breeding protocol for captive grey mullet can be applied effectively during natural as well as shifted spawning seasons. During 2016 and 2017, tens millions of quality eggs were produced giving rise to mass production of robust fingerlings.

2) The basic breeding units consisting of a single female and three males, seems to improved synchronization and increase the fertilization rate.

3) Shifted spawning season can be easily achieved via photo-thermal manipulation.

4) Broodstock diet containing fish oil (FO), which is relatively rich in n-3 LCPUFA, positively affected hatching success and larvae survival.

Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish (led by IOLR, Hanna Rosenfeld)

INTRODUCTION

Farming conditions often have opposing effects on the fish growth and sexual maturity. In some species, the improved food availability, promote sexual maturation at reduced age and size, whereas in others sexual maturity is being blocked due to lack of appropriate environmental stimuli. Advanced and spontaneous sexual maturity under captive conditions can facilitate grey mullet roe production (bottarga) as a high valued product. Therefore, the current task aims to characterize pubertal development in wild-caught vs. hatchery produced grey mullet subjected to captive conditions.

MATERIALS AND METHODS

Fish holding and sampling procedure

Experimental fish consisted of hatchery-produced (P7. IOLR spawning date: 31st October 2014) and wild-caught grey mullet of the equivalent cohort. The latter were originally caught in the Ebro River delta and imported from Spain to Israel by Madan (Kibuz Ma'agan Michael, Israel). Following quarantine and acclimatization of the wild caught mullet, fish (a mean weight 6 g) of each group were stocked (March 2015), in 19 m^3 tanks at densities of 90 and 45 fish/ m^2 (high [H] and low [L], respectively). Fish were subjected to ambient seawater salinity (40 ppt; Gulf of Eilat, Red Sea) and photo-thermal regime and fed P4. IOLR grow out diet ad libitum.

At age 2 (early-November, 2016) and age 3 (mid-September), grey mullet juveniles of the L and H hatchery-produced and wild-caught captive-reared groups, were sampled by P4. IOLR. In addition, in order to obtain reference values on body growth and gonad development (including ovary texture and colour) from adult grey mullets, five captive-reared specimens belonging to a 6-year old stock were sampled by P4. IOLR in early November 2016 and seventeen wild specimens, caught by traditional trap nets (lavoriera) in the Schiapparo Channel (Apulia, Italy) during their migration from the Lesina Lagoon to the South Adriatic Sea, were sampled by P13. UNIBA in early September 2016.

From each fish, biometric data (Total Length, TL, in cm; Body Mass, BM, in g; Liver Mass, LM, in g) were recorded and gonads, blood, muscle, liver, brain and pituitary were sampled. From all the wild specimens sampled in Italy, scales were also taken for age determination. Gonado-somatic and hepato-somatic indices were calculated as GSI = 100xGM/BM and HIS= 100xLM/BM, respectively.

A list of all the sampled fish, with sex, biometric data and age is provided in **Tables 7.4.1, 7.4.2** and **7.4.3**.

Age determination, growth and analysis of biometric data

Age and growth

The age estimate of wild grey mullets was carried out through the analysis of the scales. From each specimen a variable number of scales were treated according to the following protocol reported by McCurdy et al. (2002). Briefly, scales were removed from the skin taken from a body area between the pectoral and first dorsal fin, rinsed in tap water and in 70% ethanol and finally placed between two microscope slides. Subsequently, they were observed with a binocular lens microscope Wild M3C (Leitz, Heerbrugg, Switzerland) under transmitted light, connected through a digital camera DC 300 (Leica, Wetzlar, Germany) to the image analyser Quantiment 500 W (Leica, Wetzlar, Germany).

Scales show typical dense concentric growth rings (circuli) whose arrangement displays periodical (seasonal) variations: circuli density increases and circuli crowd during the slow growth season (winter) when they tend to form a solid line or annulus. The age of the fish was estimated based on the number of annuli counted on their scales (Meunier, 2002) (**Fig. 7.4.1**).

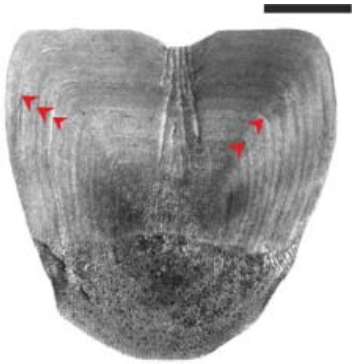

Figure 7.4.1. Scale from a 5-year old wild grey mullet. Bar = 3 mm. Arrowheads point to annuli.

Table 7.4.1. List of wild and captive-reared grey mullet adults sampled in Italy and at IOLR.

Fish Origin	Sampling area	Sampling Date	Sex	Total Length (TL, cm)	Body mass (BM, g)	Gonad mass (GM, g)	Liver mass (LM, g)	Age (year)
Wild	Schiapparo channel	Early September 2016	f	38.0	574.9	111.3	6.1	4
			f	40.0	602.5	96.8	10.0	4
			f	40.0	595.0	108.0	9.7	4
			f	42.0	864.9	9.3	13.9	5
			f	43.0	807.2	166.7	7.5	5
			f	45.0	798.0	154.9	9.1	5
			f	46.0	843.0	150.0	11.0	5
			f	47.0	1071.5	232.6	14.2	6
			f	49.0	1132.0	245.0	15.8	6
			f	50.0	1332.0	280.4	12.3	6
			m	37.0	477.0	20.3	10.0	4
			m	38.0	473.0	14.0	6.0	4
			m	38.0	528.0	22.8	4.5	4
			m	41.0	632.9	17.4	13.8	5
			m	41.0	640.0	29.2	8.2	5
			m	45.0	942.0	27.5	20.4	5
Captive-reared	P7. IOLR	03/11/2016	f	47.1	1240.0	128.2	21.8	6
			f	50.3	1480.0	8.1	32.7	6
			f	54.4	1970.0	34.4	34.6	6
			m	41.2	750.0	0.6	5.9	6
			m	45.2	1130.0	2.8	20.3	6

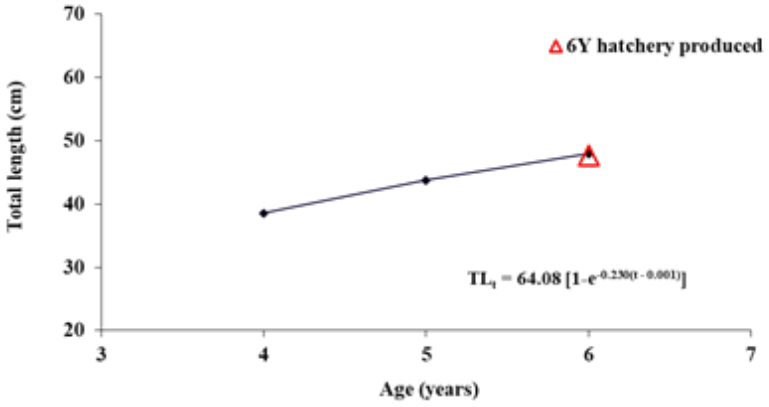

Figure 7.4.2. Von Bertalanffy growth curve of wild grey mullet. TL_t = predicted total length at age t. The red triangle refers to age 6 hatchery produced specimens.

Table 7.4.2. List of age 2 grey mullet juveniles sampled at IOLR.

Fish Origin	Rearing density	Sampling Date	Sex	Total Length (TL, cm)	Body mass (BM, g)	Gonad mass (GM, g)	Liver mass (LM, g)
captive-reared	low	03/11/2016	f	22.2	100	0.07	0.60
			f	23.3	120	0.11	0.91
			f	25.3	150	0.13	1.05
			f	36.2	520	1.20	4.94
			m	18.3	60	0.00	0.49
			m	22.7	120	0.06	1.03
			m	23.6	130	0.00	0.93
			m	30.7	270	3.04	2.98
			m	33.2	390	0.08	4.13
			m	37.0	510	0.10	5.74
captive-reared	high	03/11/2016	f	23.0	120	0.23	1.15
			m	21.7	90	0.00	0.86
			m	22.8	110	0.00	1.24
			m	23.0	120	0.09	1.25
			m	23.1	120	0.00	1.11
			m	24.7	130	0.10	1.60
			m	26.5	180	0.00	1.70
			m	28.0	200	0.11	2.10
			m	28.1	220	0.04	2.49
			m	28.2	200	0.11	2.25
hatchery produced	low	03/11/2016	f	29.1	240	0.41	2.08
			f	29.5	270	0.64	3.09
			f	31.0	270	0.40	2.10
			f	31.0	270	0.70	2.39
			f	32.5	310	0.56	2.54
			f	32.6	340	0.90	3.08
			f	32.9	340	0.88	4.01
			f	32.9	330	0.67	2.92
			m	30.6	270	0.03	2.69
			m	30.8	270	0.05	3.12
hatchery produced	high	03/11/2016	f	24.3	120	0.15	1.59
			f	25.0	150	0.42	1.07
			f	25.2	150	0.28	1.18
			f	26.6	170	0.33	1.29
			f	26.9	160	0.27	1.23
			f	34.6	370	0.97	4.16
			f	36.4	430	1.13	3.94
			f	39.1	510	3.25	5.83
			m	24.0	130	0.03	1.24
			m	37.2	450	0.17	5.70

Table 7.4.3. List of age 3 grey mullet juveniles sampled at IOLR.

Fish Origin	Rearing Density	Sampling Date	Sex	Total Length (TL, cm)	Body Mass (BM, g)	Gonad Mass (GM,g)	Liver Mass (LM, g)
Captive -reared	low	14/09/2017	F	50.2	1330	21.30	6.85
			F	36.7	650	11.73	9.77
			F	32.1	330	2.32	3.35
			M	34.6	370	0.87	2.80
			M	33.1	310	0.27	2.72
			M	35.4	410	1.08	4.65
			M	42.3	820	0.21	8.68
			M	33.8	340	1.42	2.89
			M	34.5	450	1.20	4.58
			M	30.1	270	0.64	2.56
Captive -reared	high	14/09/2017	F	41.4	750	2.70	7.80
			F	43.2	1030	64.10	7.54
			F	37.0	500	1.21	3.90
			M	43.0	900	1.15	12.75
			M	30.4	250	0.55	3.04
			M	33.8	330	0.43	4.09
			M	34.5	370	1.86	3.53
			M	32.5	270	0.34	3.19
			M	33.0	300	0.69	2.00
			M	33.4	290	0.75	3.07
Hetchery - produced	low	14/09/2017	F	29.6	240	0.55	2.56
			F	34.7	390	1.00	5.83
			F	34.9	470	1.55	6.15
			F	36.5	580	102.00	4.68
			F	45.7	1100	169.29	10.15
			F	43.5	780	122.28	6.84
			F	38.5	525	51.43	9.56
			F	36.5	480	1.27	6.03
			M	32.9	340	0.15	4.76
			M	40.1	580	15.96	5.40
			M	36.4	440	7.87	6.10
			M	25.4	240	0.01	2.15
Hetchery - produced	high	14/09/2017	F	48.2	1143	114	11.865
			F	29.3	180	0.445	1.495
			F	33.7	370	1.075	4.01
			F	35.1	360	0.67	3.17
			F	42.1	780	159.22	4.59
			F	37.2	530	69.485	5.67
			M	40.8	670	9.85	17.96
			M	29.3	230	0.025	1.92
			M	35.6	430	3.16	5.19
			M	24	10	0.01	0.895

Among the 16 adult wild grey mullet used for age determination, 6 were 4 years old, 7 were 5 years old and 3 were 6 years old (**Table 7.4.1**).

Estimate of wild grey mullet theoretical growth in length was obtained by fitting the von Bertalanffy growth model (Bertalanffy von, 1938) to the mean lengths at estimated age. The obtained von Bertalanffy parameters were: TL_∞ = 64.08 cm; k = -0.230; t_0 = -0.001 (**Fig. 7.4.2**).

Interestingly, as shown in **Figure 7.4.2**, the mean length at age 6 hatchery-produced grey mullets reared at IOLR perfectly overlapped that of wild specimens of the same age sampled in Italy, indicating that rearing in captivity allowed a body growth similar to that occurring in the wild.

Biometric data analysis

Hatchery produced 2 year old grey mullets appeared to be larger and heavier than cognate captive-reared individuals (**Fig. 7.4. 3**). No statistically significant effect of rearing density on fish size and weight was found.

Hatchery-produced specimens, particularly those reared in high density conditions, showed a higher GSI than captive-reared fish (**Fig. 7.4.4**). No difference was observed in HIS among the different experimental groups (**Fig. 7.4.4**).

Fulton's condition factor, which is indicative of the overall fish nutritional state, was similar between captive-reared and hatchery-produced fish stocked at low density but, in high density rearing conditions, it was significantly higher in captive-reared than in hatchery-produced fish (**Fig. 7.4.5**). Hatchery-produced fish reared at high density had a significantly higher condition factor compared with the same group reared at low density (**Fig. 7.4.5**).

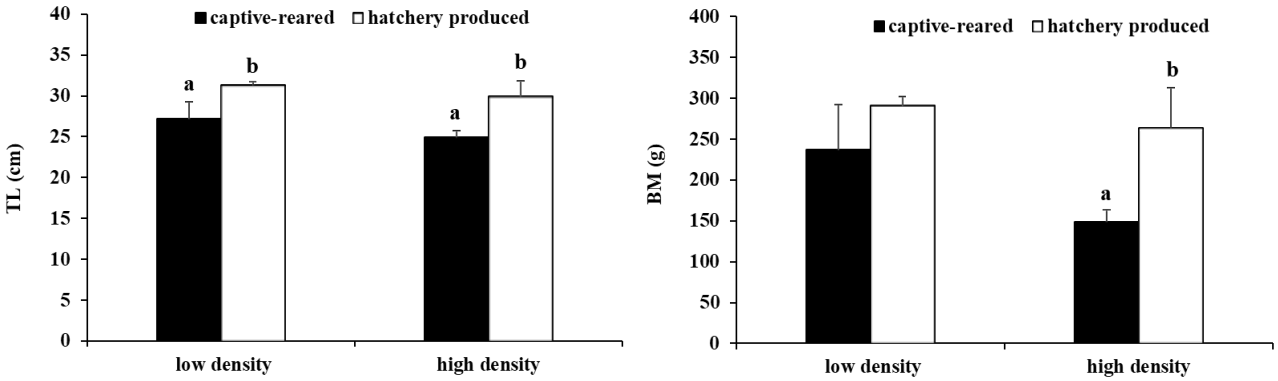

Figure 7.4.3. Total length (TL) and body mass (BM) of 2 year old hatchery-produced and captive-reared grey mullets. Different letters above bars indicate statistically significant differences between captive-reared and hatchery-produced fish. No statistically significant effect of rearing density on TL and BM was observed.

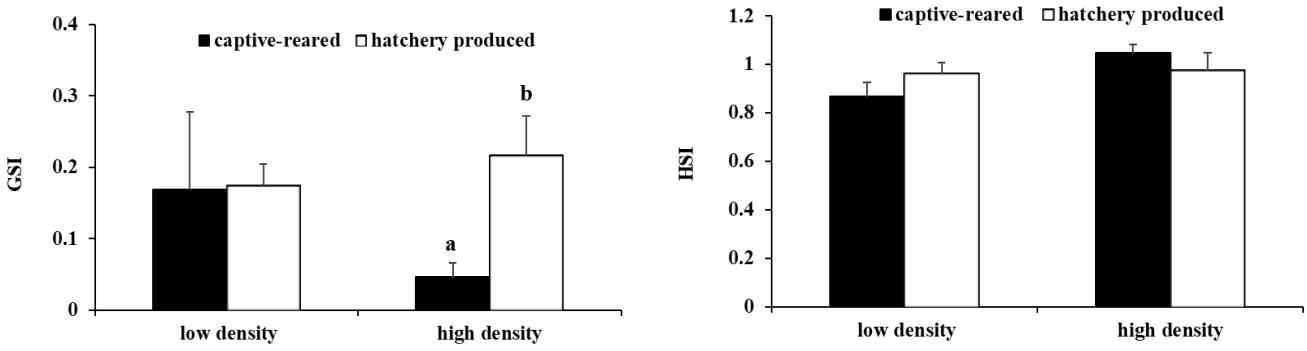

Figure 7.4.4. Gonado-somatic (GSI) and hepato-somatic (HSI) indices of 2 year old hatchery-produced and captive-reared grey mullets. Different letters above bars indicate statistically significant differences between captive-reared and hatchery-produced fish. No statistically significant effect of rearing density on HIS was observed.

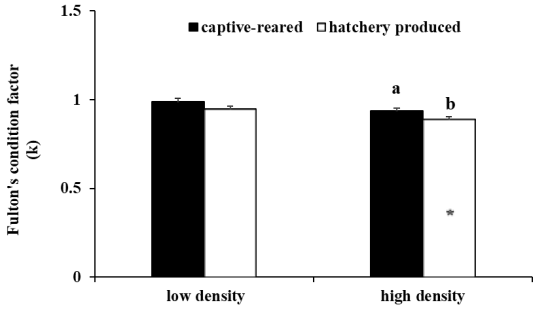

Figure 7.4.5. Fulton's condition index in 2 year old captive-reared and hatchery-produced grey mullets stocked at different density. Different letters above bars indicate statistically significant differences between captive-reared and hatchery-produced fish. Asterisk indicate significant difference between hatchery-produced specimens reared at low and high density.

No significant ($P > 0.05$) density effects could be observed among the 3 year old captive-reared and hatchery-produced grey mullets. Therefore, to increase the statistical power we combined data sets collected for the H and L sub-groups. Gender-wise analyses indicate that captive-reared females are larger and heavier than cognate males (**Fig. 7.4.6**). Although not significant ($P > 0.05$), the hatchery-produced grey mullets appear to exhibit a similar trend (**Fig. 7.4.6**).

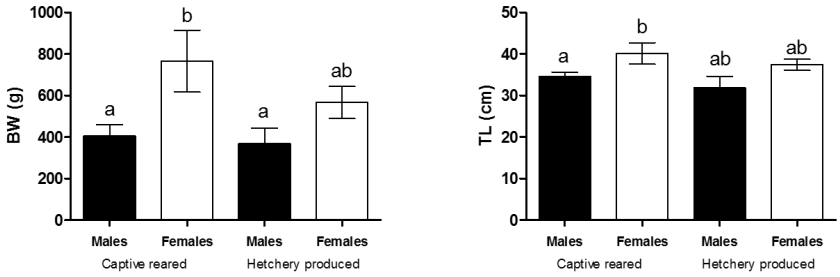

Figure 7.4.6. Body weight (BW) and Total length (TL) of 3 year old hatchery-produced and captive-reared grey mullets. Different letters above bars indicate statistically significant differences between groups.

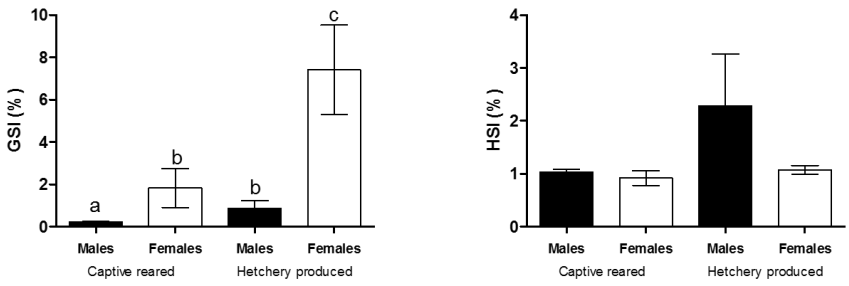

Figure 7.4.7. Gonado-somatic (GSI) and hepato-somatic (HSI) indices of 3 year old hatchery-produced and captive-reared grey mullets. Different letters above bars indicate statistically significant differences between groups. No statistically significant effect of rearing density on HIS was observed.

Hatchery-produced and captive-reared females, showed significantly ($P < 0.05$) higher GSI values compared to those in cognate males (**Fig. 7.4.7**). Interestingly, approximately 50% of the hatchery-produced females GSI exhibited markedly developed gonads with GSI values ranging between 10-20% while all the others were far lagging behind showing values between 0.2 to 0.3 %. The GSI values in captive reared females were significantly ($P < 0.05$) lower than those of the hatchery produced females. No difference was observed in HIS among the different experimental groups (**Fig. 7.4.7**).

Histological analysis of grey mullet gonads

Females

In the examined samples, oogonia along with the following oocyte developmental stages were observed, perinucleolar, lipid/cortical alveoli, early vitellogenesis, late vitellogenesis (**Fig. 7.4.8**). Neither hydrated oocytes nor postovulatory follicles were found in any specimen. Different oocyte development stages were identified in the ovaries of captive-reared female greater amberjack.

Oogonia (**Fig. 7.4.8a**) (diameter 8-10 μm), often found in small clusters, were rounded cells with a large central euchromatic nucleus containing sparse eterochromatic patches.

Perinucleolar stage oocytes (**Fig. 7.4.8a, b, c**) (diameter 15-100 μm) were characterized by the presence of one or two large nucleoli centrally or eccentrically located in the nucleus and a variable number of small nucleoli adjoining the nuclear envelope. Early perinucleolar oocytes were rounded in shape and showed a strong ooplasm basophily. Late perinucleolar stage oocytes showed a reduced ooplams basophily and a variable polyedric shape. Flat follicular cells surrounded oocytes at this stage.

Oocytes at lipid/cortical alveoli stage (**Fig. 7.4.8d**) (diameter 100-150 μm) showed a further reduction of ooplasm basophily, numerous cortical alveoli in the peripheral ooplasm, many small lipid droplets in the inner ooplasm and the appearance of a thin zona radiata.

Secondary growth oocytes. Early vitellogenic oocytes (**Fig. 7.4.8d**) (diameter 150-300 μm) were characterized by the appearance of small eosinophilic yolk globules in the peripheral ooplasm and a further increase of the zona radiata thickness. Follicular cells surrounding oocytes at this stage became cubic. Late vitellogenic oocytes (**Fig. 7.4.8e**) (diameter 300-500 μm) showed an increase of the quantity and size of yolk granules, numerous lipid droplets amongst yolk globules and a ticker zona radiata.

Alpha atretic vitellogenic follicles displayed zona radiata fragmentation, coalescence of yolk globule and nucleus disintegration; in beta atretic follicles zona radiata and yolk globules were completely reabsorbed (**Fig. 7.4.8f**).

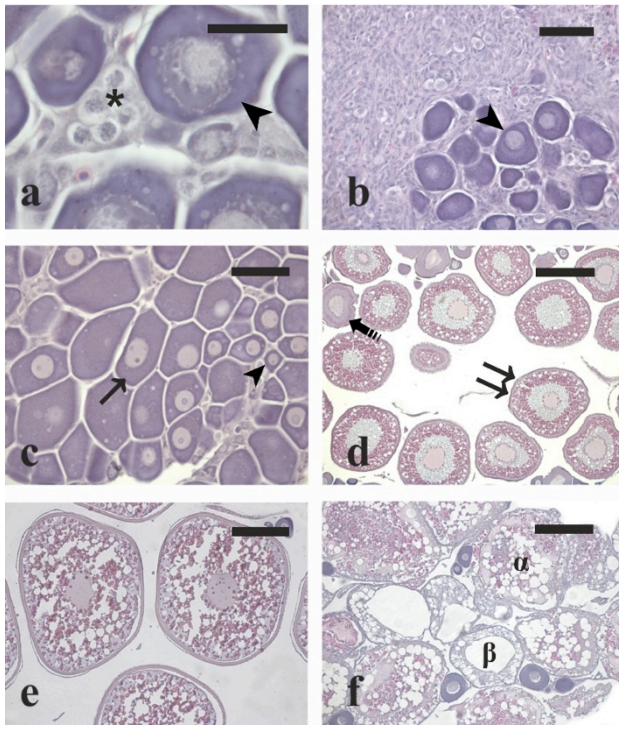

Figure 7.4.8. Micrographs of grey mullet ovary sections showing oogonia and oocytes in different developmental stages. a) and b) Oogonia (asterisk) and early perinucleolar stage oocytes (arrowhead). c) Early (arrowhead) and late (arrow) perinucleolar stage oocytes. d) Cortical alveoli stage (dashed arrow) and early vitellogenic (double arrow) oocytes. e) Late vitellogenic oocytes. f) α and β atretic vitellogenic follicles. Haematoxylin-eosin staining. Magnification bars = 20 μm in (a), 50 μm in (b)-(c), 200 μm in (d)-(e)-(f).

All the fish of the age class 2, both wild-caught and hatchery-produced, showed immature gonads. Apparently, grey mullet ovaries finalize their structure during the second year of life: the majority of age 2 grey mullet had ovaries with late perinucleolar stage oocytes as the most advanced oocyte stage (**Fig. 7.4.8c**), however, some of these fish had still ovaries constituted mainly by oogonia intermingled by small groups of early perinucleolar stage oocytes (**Fig. 7.4.8b**). Histologically, no clear difference could be observed among the different experimental groups (captive-reared *vs* hatchery-produced; high density *vs* low density) and no difference in oocyte diameter was found between fish stocked at different density. However, hatchery-produced grey mullets had significantly larger oocytes than captive-reared specimens (**Fig. 7.4.9**).

Adult wild grey mullets (estimated age 4-6) sampled during their migration towards the spawning ground of the Adriatic Sea showed large ripe ovaries pale yellow (**Fig. 7.4.10a**) to pale orange (**Fig.7.4.10b**) in colour. Histologically, none of the ovaries showed signs of imminent (oocytes in final maturation) or recent (post-ovulatory follicles) spawning and all of them showed late vitellogenic oocytes as the most advanced oocyte stage (**Fig. 7.4.8e**). These finding seems to confirm that in wild grey mullet the ovary ripening process (i.e. vitellogenesis) occurs in estuarine/brackish waters and then the fish move to sea waters where oocyte maturation and spawning occur.

The ovaries of the three age 6 captive-reared female grey mullets showed extensive alfa and beta atresia of vitellogenic follicles (**Fig. 7.4.8f**), sign of cessation of the reproductive activity.

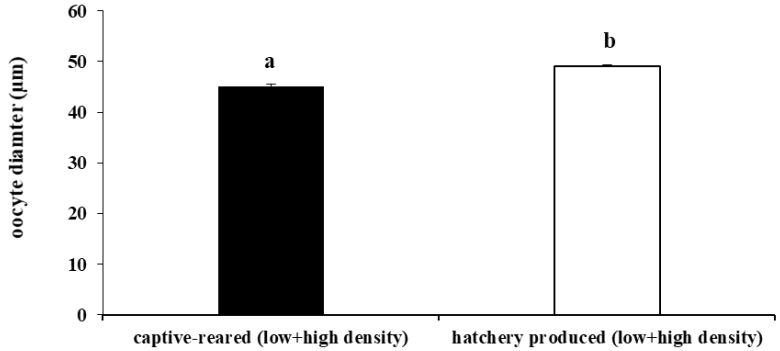

Figure 7.4.9. Oocyte diameter of age 2 hatchery-produced and captive-reared grey mullets. Different letters above bars indicate statistically significant difference.

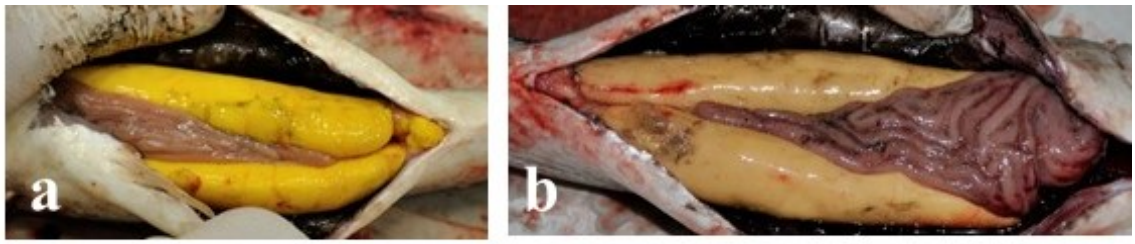

Figure 7.4.10. Ripe ovaries from wild grey mullets showing differences in shape and colour.

Males

All the fish of the age class 2, both wild-caught and hatchery-produced, showed immature testes. As for ovaries, grey mullet testes finalize their structural organization during the second year of life, because only

some of the examined testes showed well-structured seminiferous lobules. The seminiferous lobules of these testes, however, did not show active spermatogenesis and were constituted only by spermatogonia and somatic (Sertoli) cells (**Fig. 7.4.11a**). One of the age 2 specimens, macroscopically classified as male, showed an intersex gonad, with seminiferous lobules containing an active male germinal epithelium (all stages of spermatogenesis and luminal spermatozoa) along with primary growth oocytes (**Fig. 7.4.11b**).

All the adult wild grey mullets (estimated age 4-6) sampled during their migration towards the spawning ground of the Adriatic Sea had mature testes showing the lumen of seminiferous lobules filled with spermatozoa (**Fig. 7.4.11c**). Apparently, these fish had not yet started to spawn.

The two age 6 hatchery-produced males showed spent testis with residual luminal spermatozoa and no active spermatogenesis (**Fig. 7.4.11d**).

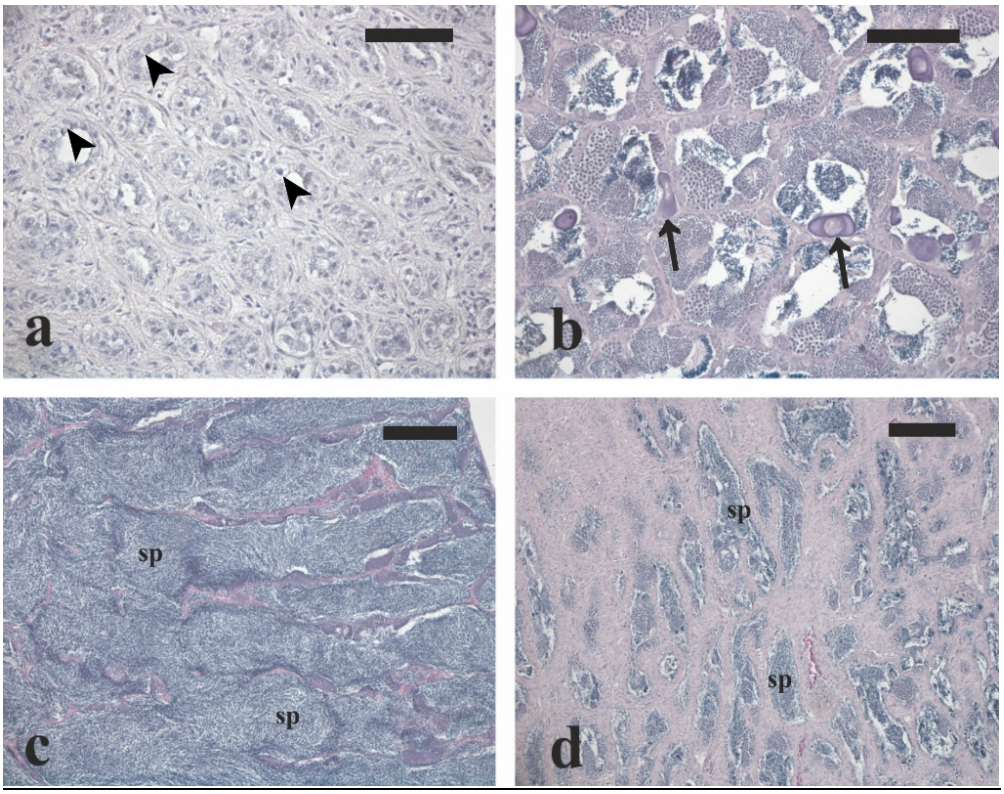

Fig. 7.4.11. Micrographs of grey mullet testis sections. a) Testis from an immature age 2 hatchery-produced specimen showing small seminiferous lobules. Only spermatogonia, along with somatic cells, are visible. (b) Gonad section from an age 2 hatchery-produced intersex showing the presence of all stages of spermatogenesis. Scattered perinucleolar stage oocytes are visible. c) Testis section from a mature wild specimen sampled during migration from the Lesina Lagoon to the spawning ground of the Adriatic Sea. Seminiferous lobule lumina are filled with spermatozoa. d) Spent testis from a hatchery-produced, age 6 grey mullet showing residual spermatozoa in the lumen of seminiferous lobules. Haematoxylin-eosin staining. Magnification bars = 50 μm in (a), 100 μm in (b), 150 μm in (c)-(d). Arrowhead, spermatogonia; arrow, perinucleolar stage oocytes in an intersex gonad; sp: spermatozoa in the lumen of seminiferous lobules.

During the fourth reporting period, the study of attainment of sexual maturity in captive grey mullet the will be extended to the age class 3 specimens and will also include the analyses of gonadotropins and vitellogenin at the gene expression level.

Conclusions

1) The size of age 6 hatchery-produced specimens is equal to that of wild individuals of the same age class.
2) All the age 2 mullets analysed were still sexually immature. The biometric and histological analyses showed that body growth and gonad development of age 2 hatchery-produced mullets proceed in a slightly faster way compared with wild-caught specimens.
3) The 3 year old grey mullet exhibit sex related growth and gonadal development patterns.
4) The 3 year old hatchery produced mullet females and males exhibited enhanced gonadal maturation than that in the wild-caught captive-reared fish, probably as a result of domestication.

Task 7.5 Establish a shipping protocol for grey mullet eggs (led by P25. DOR)

The development of a protocol for the transferring of grey mullet eggs to the various partners in the DIVERSIFY project was based on methodology developed earlier by **P4. IOLR** for shipping Atlantic bluefin tuna (BFT) eggs to different Mediterranean partners (Greece, Spain, Malta, Italy) in the EU 7th framework projects SELFDOTT (212797) and TRANSDOTT (311904) (De la Gándara 2012, Bridges, 2014). In brief, this protocol recommends the stocking of 10 l of filtered (10 µm) seawater with 10-15,000 gastrula-stage bluefin tuna eggs l^{-1} in a 20 l cubitainer, which is placed in a Styrofoam container. Pure oxygen is added to supersaturate the container seawater as well as flushing the air layer in the upper part of the cubitainer. One to two ice packs, wrapped in cardboard, are placed adjacent to the air-oxygen layer (not against the water layer) and the package closed. Total transit time from egg collection at sea to arrival at the Eilat facility ranged between 26-29 h. The egg shipments consistently arrived at the Eilat institute in excellent condition where oxygen (>200%), pH (6.5-7.0) and temperature (ca. 22-23°C) were within acceptable limits.

MATERIALS AND METHODS

In 2015, the long term egg transport trial simulation was attempted but the level of egg fertilization was very low and the results are not presented here. This study was successfully repeated in 2016 and is described below. On the other hand, short term transports of grey mullet eggs within Israel were carried out from partner **P4. IOLR** to **P25. DOR** and one shipment was made from the kibbutz Ma'agan Michael, which is south of Haifa, to the **P4. IOLR.** Results of these experiments (**Table 7.5.1**) indicate that higher amounts of eggs and egg stocking densities can be carried out provided that transport times are markedly shorter.

Table 7.5.1. Shipping conditions as well as percent oxygen and hatching results of grey mullet eggs sent from **P4. IOLR** to **P25. DOR** in 2014 using the BFT egg shiping protocol, as well as eggs sent from Ma'agan Michael (approximately the same transit time as shipments sent to DOR, ~10 h), which were sent in plastic bags in Styrofoam boxes with no ice packs.

Spawning date	Box no.	Egg vol. sent (ml)	Egg number	Total Vol. sent (l)	Eggs/l	% O_2 *	Time in transit (h)	% hatching*
	Eggs sent from IOLR to DOR							
16.8.14	1	350	1.26 x10^6	15	84000	272	9.0	85
16.8.14	2	250	0.825x10^6	15	55000	280	9.0	85
3.10.14	1	250	1.6x10^6	15	55000	330	10.5	90
3.10.14	2	250	1.6x10^6	15	55000	330	10.5	90
16.10.14	1	350	1.26 x10^6	15	84000	265	9.0	85
	Eggs sent from Kibbutz Ma'agan Michael to IOLR							
14.8.14	1	500	1.65 x10^6	15	110000	270	11	96
14.8.14	2	300	99000	15	66000	265	11	97

*Measured at destination

2016-long term transport simulation

The eggs used in this study were spawned at 05:00 on November 2, 2016, with a diameter of 796.3 ±24 µm (n=34) in 40 ‰ seawater at 25.2°C. The eggs were stocked at gastrula 1(G1) in 12 translucent rectangular shaped cubitainers in order to test two egg densities (10,000 and 15000 eggs l^{-1}) in two cubitainer volumes (10 and 15 l) in triplicate replicates and is described in **Table 7.5.2**. To each container pure oxygen was added and a data logger was placed in one of the cubitainers. All cubitainers were placed in a Styrofoam boxes and two icepacks were placed on the upper part of the cubitainer where there is air and no water.

Table 7.5.2. Egg simulated transport treatments testing two densities and two water volumes.

Treatments	Eggs (x10,000)/l	Water Volume*	Total eggs
1	10	10	100,000
2	15	15	225,000
3	10	15	150,000
4	15	10	150,000

*40 ‰ filtered, ambient sea water.

All boxes were then placed in a non-air conditioned room (25°C) and were gently and periodically shaken (every 90 min during the day). After 26 h the containers were opened and the ammonia, oxygen, temperature and pH were measured. In addition, from each cubitainer, eggs were taken for (1) examination under a microscope, (2) stocking in 5 ml well plates (1-2 eggs $well^{-1}$; n=3), which were placed for 17 h in an incubator at 22.4°C in order to determine percent (%) hatching and survival at the end of 0 dph (**Fig.7.5.1**).

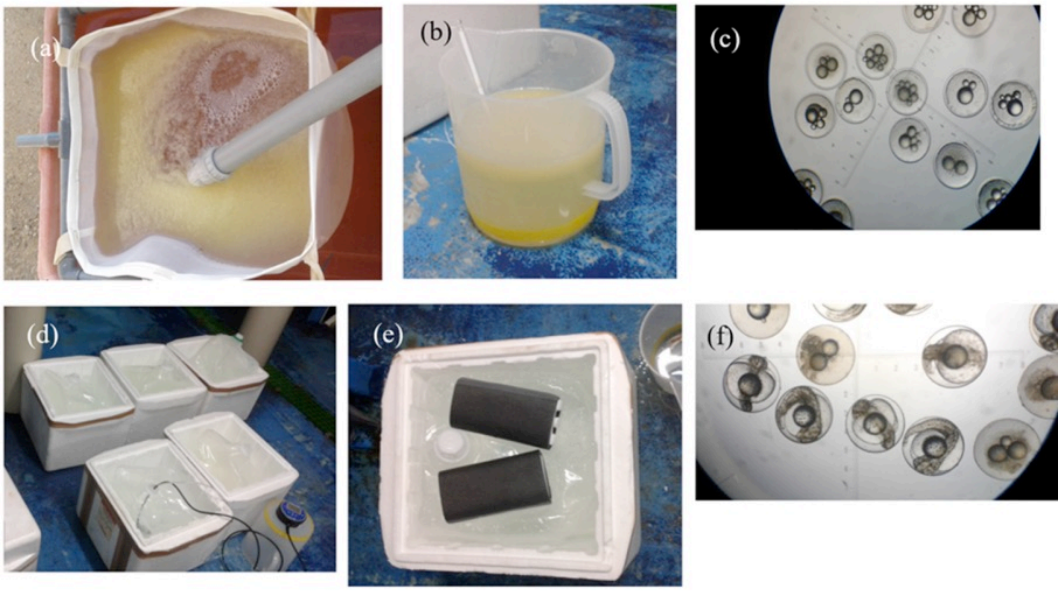

Figure 7.5.1. Grey mullet eggs (a) in a collector beside spawning tank, (b) separated into viable and non-viable eggs, (c) stocking at G1 stage in (d) cubitainers with (e) ice packs and (f) after 26 h of simulated transport.

RESULTS AND DISCUSSION

The egg stocking and water parameters of the cubitainers of the different treatments at the end of 26 h of transport simulation or 34 h after spawning are shown in **Table 7.5.3**. The results largely support the application of the bluefin tuna egg transport protocol for the long term shipment of grey mullet fertilized eggs. The ice packs maintained the cubitainer temperature, without any major fluctuations, at ca. 22.5°C in a 25°C room after 26 h, which is a minor decrease from the stocking temperature of 23.4°C. The requirement for packaging that insulates and reduces temperature fluctuation during transport is critical. This is because egg shipments will be exposed to a range of temperatures in the plane's cargo bay and airport holding facilities during and between flights as well as when transporting them by land to the end user. Temperature increase during egg incubation can be source of stress on the eggs and affect mortality (Wagner et al. 2009; Thépot and Jerry, 2015), hatching patterns (Laurel and Blood, 2011, Thépot and Jerry, 2015) and larval development later on (Jobling, 1997; Laurel and Blood, 2011. The egg treatments in the study not only showed relatively consistent temperature during simulation but demonstrated at the end of the trial saturated oxygen levels (>295%) and a moderate reduction of approximately 1pH unit (7.03± 0.1) from ambient sea water (8.0) in **Table 7.5.3**. Nevertheless, treatment 2, which tested the effect of the high egg density and seawater volume (15,000 egg l^{-1} in 15 l) and consequently the greatest number of eggs (225,000 eggs $cubitainer^{-1}$), showed significantly (P=0.0001) higher total ammonia nitrogen (TAN) compared to the other treatments (**Fig. 7.5.2A**). TAN is a measurement of the end product of protein catabolism and is comprised primarily of both ionized (NH_4+) and non-ionized ammonia (NH_3) (Wajsbrot et al.1993). The ionized form is more permeable to biological membranes and is many times more toxic than the ionized form. Although there are reports that non-ionized NH_3 is more detrimental to first feeding larvae than yolk-sac larvae and eggs (Rice and Stokes, 1973), the results of the present study suggest a mullet egg sensitivity to this metabolite. The excess unionized ammonia (NH_3) of treatment 2, although not directly measured, was responsible for the marked (P=0.0001) reduction of viable eggs (**Fig. 7.5.2B**), hatching success (**Fig. 7.5.2C**), and larval survival to the end of 0 dph (**Fig. 7.5.2D**). Elevated concentrations of ammonia affected hatching success as well as larval deformity and survival in Nile tilapia (*Oreochromis niloticus*; El-greisy et al. 2016) and red drum (*Sciaenops ocellatus*; Holt and Arnold, 1983). It is conceivable that the ammonia was produced from water borne bacteria but a high proportion of viable eggs, good hatchability and survival was also shown in the treatment with 10,000 eggs l^{-1} in a cubitainer volume of 15 l, consequently, it was concluded that the excess ammonia was egg produced, particularly during later development toward hatching (Rønnestad et al., 1994)

Taken altogether, the results suggest that the bluefin tuna protocol for egg transport can be successfully applied to the shipment of live grey mullet eggs (gastrula 1), provided that cubitainers stocked with a maximum of 15,000 eggs l^{-1} should not exceed a total sea water volume of 10 l (total of 150,000 eggs). This procedure will likely ensure, after a 26 h transit time, a high proportion of viable eggs with a good hatching rate and survival to the end of 0 dph.

Table 7.5.3. Water parameters at the end of 26 h in the cubitainers from each of the treatments and 34 h after spawning

Treatment (eggs/l in final volume	Cubitainer no.	Temperature (^{0} C)	pH	Oxygen
10,000 eggs/l in 10 l	1	22.3	7.03	235
	2	22.5	6.95	195
	3	22.2	7.01	199
15,000 eggs/l in 15 l	4	22	7.0	200
	5	21.9	6.99	220
	6	22.5	7.01	290
10,000 eggs/l in 15 l	7	22.3	7.03	181
	8	22.2	6.8	200

	9	22.3	7.17	149
15,000 eggs/l in 10 l	10	22.5	7.05	195
	11	22.1	7.2	196
	12	22.2	7.15	203

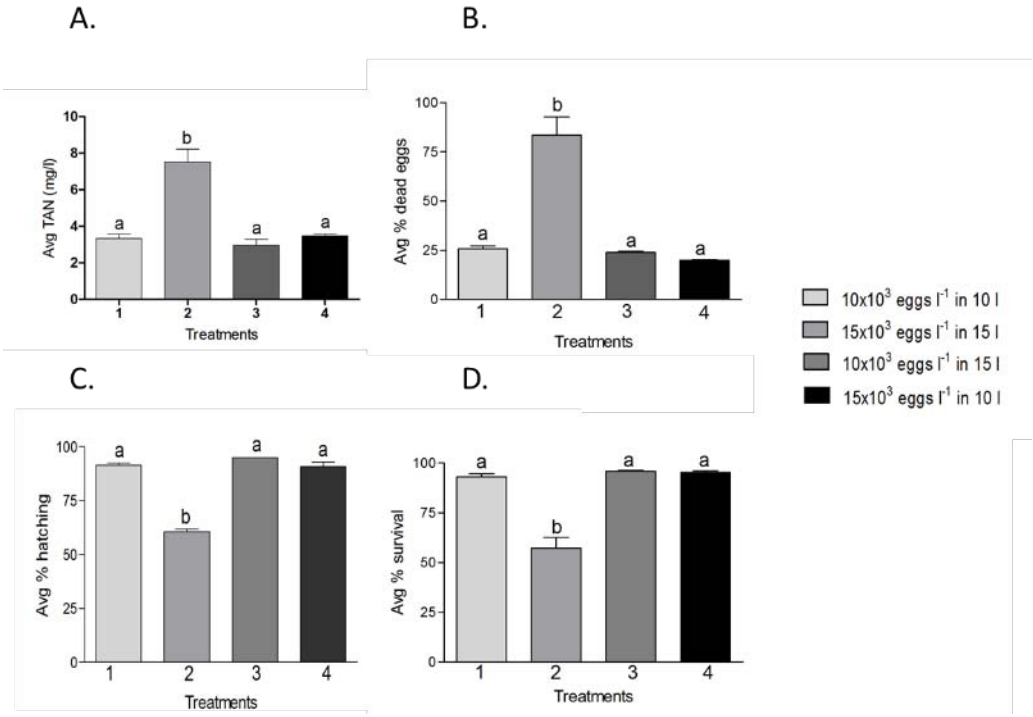

Figure 7.5.3. Average (Avg) total ammonia (TAN) levels (A), percent (%) dead eggs (B), percent (%) hatching (C) in egg sea water after 26 h of simulation in the 4 stocking and volume treatments. (D) Percent (%) survival to the end of 0 dph in the 4 stocking and volume treatments. Values with different letters were significantly ($P<0.05$) different.

CONCLUSIONS

1. Short term shipping (≤11 h) of gastrula stage, grey mullet eggs with very high egg density (55-84,000 eggs l^{-1}), can be readily carried out using cubitainers or strong plastic bags together with the addition of pure oxygen. One or no Freezer packs may be sufficient as long as the shipment does not encounter temperature extremes.
2. Long term shipping (26 h) employing the bluefin tuna protocol for gastrula stage egg transport can be successfully applied to the shipment of live grey mullet eggs (gastrula 1), provided that cubitainers stocked with a maximum of 15,000 eggs l^{-1} should not exceed a total sea water volume of 10 l (total of 150,000 eggs).

The full description of the work and results has been provided in ***Deliverable 7.4 "Protocol for shipping grey mullet eggs"***.

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Deviations from Annex I and their impact:
No deviations.

Group Work Packages

Nutrition

The overall objective of the activity done within this period with meagre nutrition was to determine the nutritional requirements and optimum levels of n-3 LC-PUFA for fingerlings, evaluating its effects on fish survival, growth performance, feed utilization and composition. Additionally, the present study aimed to improve the understanding of the modulation action of dietary n-3 LC-PUFA on hepatic lipid profile and its possible role on the development of liver steatosis and granulomatosis in meagre. Besides, the effects on elongase and desaturase gene expression, digestive enzymes and stress resistance were also evaluated. Results of this study are relevant to properly design well balance grow-out diets for this species. For this purpose, one feeding trial was conducted in order to determine the n-3 LC-PUFA requirements for meagre fingerlings optimum performance by using different lipid sources, followed by a stress challenge trial with the objective to evaluate the effect of increasing dietary n-3 LC-PUFA levels on meagre stress resistance. ***Meagre showed the ability to selectively conserve key FA, particularly DHA and ARA*** over other FA, in response to EFA-deficiency. Furthermore, ***meagre seems to have active Δ6 desaturases and Elovl5, but their activities were insufficient to produce DHA and EPA from PUFA precursors*** to sustain fast growth. Based in overall results the results obtained show that ***the requirement for n-3 LC-PUFA for meagre fingerlings is at least 2.0% DM in diets containing 16.5% DM lipids***, 0.9 EPA/DHA and 0.4% ARA of total FA contents

The work done with greater amberjack was focused in on-growing diets and effects of fatty essential fatty acids on broodstock reproduction. At juvenile stage, the ***dietary lysine requirement determined was 2.11% of diet***, based on the Broken-line model. This requirement was calculated for maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, **lysine supplementation affected the specific activity of CAT in liver and intestine** of greater amberjack fed the diet containing 2.11% lysine. At broodstock levels, the optimum level of essential fatty acids (from 2.8 to 0.96% of total fatty acids) for reproductive success was studied, focusing on the effect on reproduction reliability. The diet containing ***1.57% of total fatty acids induced a higher number of eggs per spawn and kg of female, with the highest percentage of fertilization, egg viability, hatching rate and larval survival***. Besides, the lipids and carotenoids egg profile of culture females in comparison with their wild counterpart was also studied. An experimental diet was formulated and the experiment will start by January 2018.

For pikeperch, a multifactorial experiment examining the importance of 8 dietary factors (LC-PUFAs, vitamins A, E, C, D and minerals -Ca/P-) was conducted. ***Ca/P, fatty acids and their interaction seem to be key nutritional factors influencing pikeperch larval development***. However, only two levels of Ca/P and fatty acids were tested in the multifactorial experiment. Therefore two confirmatory experiments testing gradual levels of the dietary EPA+DHA/ARA and Ca/P ratios were performed based on the multifactorial screening results. From the confirmatory EPA+DHA/ARA experiment, fish fed diet 3.7/1.2 (EPA+DHA)/ARA, showed the lowest growth performance compared to the larvae fed 1.8/0.6 (EPA+DHA)/ARA. ***Pepsin specific activity was affected by the dietary DHA content*** whereas ***the increase in dietary EPA/ARA levels enhanced the trypsin activity in fish fed low DHA level***. The effect of phospholipids was also studied confirming the importance of ***high PL levels of approximately 8 % in diets*** for pikeperch as well as the ***positive additional beneficiary effect of supplementation with DHA+ EPA in the form of concentrated TAG*** in otherwise identical formulated diets. Thus, ***combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.00 % dw DHA and 0.16% dw EPA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, improving digestive enzymes activities and liver proteomics***. Overall the results denote that ***essential FA may be directly supplemented as triglycerides to have a beneficial effect in pikeperch larvae development***. The effect of fatty acids on the

stress response of pikeperch has been also studied. Larvae fed ***low levels of DHA displayed a tendency towards delayed escape responses*** (latency time increased) and significantly ***slower peak acceleration rates during escape responses following a mechano-sensory stimulus***. This effect was consistent up to 90 days after the dietary treatment was terminated, demonstrating ***long-term effects of early nutritional history in fish***.

A comparison of nutrient retention in Atlantic halibut larvae reared in RAS *vs* FTS was conducted. ***RAS had a large positive effect on vitamin K (MK6) concentration in Atlantic halibut larvae*** probably because this vitamin was produced by micro-organisms in the system. Most ***free aminoacids, iodine, copper and zinc were also increased*** while ***glycine concentration was decreased*** in larvae reared in RAS compared to FT. There were no effects of the treatments on other metabolites identified by the FAA method used, such as neurotransmitters, metabolites of the Urea cycle, ammonia, ethanolamine and taurine. From the studies on the effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles**,** results showed that the ***inclusion of soy lecithin so that dietary phospholipid increased from 9 to 30% of total lipids had no effect on growth, but changed lipid metabolism in Atlantic halibut juveniles.***

Enrichment products for living prey (rotifers and *Artemia*) were designed for wreckfish larvae. ***Two levels of ARA content*** were used ***for enrichment product for rotifer and one level of ARA for Artemia*** and the effect of the new enrichment products on the biochemical composition of rotifers and *Artemia* was evaluated. First data of fatty acid profile were obtained from 1dph until 26 dph of larvae to complete the data obtained previously until 10dph. ***A decrease of all the groups of fatty acids*** (expressed in ng/µg DW) with the larval development was observed, while in percentage of total fatty acids, only little differences are observed. A clear ***relationship between fatty acid profile of broodstock diet*** (semi-moisture, dry food and a mixture of hake and squid) ***and fatty acid profile of oocytes and eggs from wreckfish females fed with the different diet was found***. The wreckfish diet must contain a ***big amount of proteins, low level of lipids, a high amount of n-3 PUFA and the EPA/ARA ratio must be similar to that obtained in wild females gonads*** (about 1-1.5). Nevertheless the ***diet with a mixture of hake/squid (half and a half) seems to be a diet with good quality*** because of the protein content and the big amount of n-3 PUFA (EPA and DHA) although the EPA/ARA obtained in oocytes and eggs from females fed with this diet is high comparing with the one obtained in wild females gonads. ***First data of fatty acid profile of sperm from wreckfish males*** of different broodstock were obtained.

No dietary DHA effect on larval performance, in terms of wet weight gain and rotifer consumption rate was found for grey mullet. This suggests that ***5.5% DHA in the commercial enrichment "Red Pepper"*** is sufficient for the growing of this species. ***The hepatic CSD pathway for taurine synthesis, in the absence of dietary taurine, is still active*** but that the ***expression of this key gene increases with increased levels of dietary taurine until 1% where its expression drops at the high dietary taurine level of 2%.*** Similarly, ***the gene for ADO***, which is a key enzyme in another less dominant pathway in taurine synthesis, ***was highly expressed in the 1 % taurine fish livers*** and then dropped in fish feeding on the 2% taurine diet. ***A taurine dose dependent response on the gene expression of hepatic CYP7A1 was also determined for taurine treatments up to 1%.*** No effect of dietary DHA on juvenile mullet weight gain was found. Besides, g***rey mullet juveniles have a minimum 0.5% taurine requirement***. At broodstock levels, notable differences were found in the fatty acid profiles when comparing the gonads from wild and domestic adult grey mullet. Differences were particularly marked in ***EPA in both sexes and DHA in wild males compared to captive cohorts***. ***The percent hatching of eggs from the FO broodstock was significantly (P<0.05) higher than eggs from the VO broodstock***, and no effects on larvae survival were found. Larvae from the FO broodstock showed 100% swim bladder inflation by 5 dph, and ***no swim bladder inflation was found in fish from the VO broodstock***. ***Hatchery-produced grey mullets*** showed extensive ***alpha and beta atresia of vitellogenic follicles***, a sign of cessation of the reproductive activity.

WP 8 Nutrition – meagre

WP No:	8	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Nutrition - meagre			
Other beneficiaries (from DOW):	P15. ULL	P20. SARC	P21. DTU	
Lead Scientist preparing the Report (WP leader):	Marisol Izquierdo, Lidia Robaina			
Other Scientists participating:	Covadonga Rodriguez (P15), Ramon Fontanillas (P20), Ivar Lund (P21)			

Objectives

1. Improve current larval weaning feeds for meagre,

2. Determine nutritional requirements to promote feed utilization, consistent growth rates and fish welfare to reduce size variation.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Despite the interest of meagre for aquaculture diversification, there is a lack of information on nutrition during larval development. The importance of highly unsaturated fatty acids (HUFA) and the antioxidants vitamin E and vitamin C has not been investigated in this species, despite the fact that the oxidative risk is particularly high in fast growing larvae. To improve current larval feeds and the optimum level of these nutrients, six weaning diets containing two levels of HUFA (0.4 and 3% dw), two of vitamin E (150 and 300 mg $100g^{-1}$) and two of vitamin C (180 and 360 mg $100g^{-1}$) were fed to 15 days after hatching (dah) 36,000 meagre larvae in triplicate. Low HUFA/vitamin E/vitamin C diet reduced larval growth, lipid absorption and HUFA contents. Dietary HUFA levels of 3% improved larval growth and lipid absorption and deposition. Besides, among fish fed 3% HUFA, increase in vitamin E and vitamin C significantly improved body weight, as well as lipid, 22:6n-3 and n-3 fatty acids contents in the larvae. Thus, the results demonstrated that weaning diets for meagre must be optimized increasing high HUFA levels, up to 3% and vitamins E and C over 1500 and 1800 mg kg^{-1} to spare these essential fatty acids from oxidation.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the 2nd reporting period, two major studies were conducted. First, a study was conducted to determine optimum essential fatty acids and related micronutrient levels in weaning diets for meagre. The results of this study showed that 0.4% dietary HUFA is not enough to cover the essential fatty acid requirements of larval meagre and, since their elevation up to 3% markedly improved lipid absorption, essential fatty acids levels and growth, a high HUFA requirement in weaning diets is foreseen for this species. Besides, the results also pointed out the importance of dietary vitamin E and vitamin C to protect these essential fatty acids from oxidation, increase their contents in larval tissues and promote growth, suggesting as well high vitamin E and vitamin C requirements in meagre larvae (higher than 1500 and 1800 mg kg-1 for vitamin E and vitamin C, respectively). A second study was conducted to determine the importance of dietary vitamins A, K and D in weaning diets for meagre. Results obtained demonstrated the importance of supplementation of meagre weaning diets with 2.4 mg/kg vit K, since the absence of this vitamin markedly reduced larval survival. However, meagre seemed to be very sensitive to hypervitaminosis

D and, only midly to hypervitaminosis A, since supplementation with these vitamins leaded to a growth reduction. On the contrary, taurine supplementation did not have any effect in meagre larvae performance. Both experiments were included in Deliverable 8.1. "Improvement of larval weaning diets", delivered at month 24.

Summary of progress towards objectives (31-48 Mo):

The overall objective of the activity done within this period was to determine the nutritional requirements and optimum levels of n-3 LC-PUFA for meagre fingerlings, evaluating its effects on survival, growth performance, feed utilization and fish composition. Additionally, the present study aimed to improve the understanding of the modulation action of dietary n-3 LC-PUFA on hepatic lipid profile and its possible role on the development of liver steatosis and granulomatosis in meagre. Besides, the effects on elongase and desaturase gene expression, digestive enzymes and stress resistance were also evaluated. Results of this study are relevant to properly design well balance grow-out diets for this species. For this purpose, one feeding trial was conducted in order to determine the n-3 LC-PUFA requirements for meagre fingerlings optimum performance by using different lipid sources, followed by a stress challenge trial with the objective to evaluate the effect of increasing dietary n-3 LC-PUFA levels on meagre stress resistance. Both trials were conducted within the frame of Task 8.2 (leaded by FCPCT). Meagre showed the ability to selectively conserve key FA, particularly DHA and ARA over other FA, in response to EFA-deficiency. Furthermore, meagre seems to have active Δ6 desaturases and Elovl5, but their activities were insufficient to produce DHA and EPA from PUFA precursors to sustain fast growth. Based in overall results the results obtained show that the requirement for n-3 LC-PUFA for meagre fingerlings is at least 2.0% DM in diets containing 16.5% DM lipids, 0.9 EPA/DHA and 0.4% ARA of total FA contents.

Details for each Task

Task 8.1 Improvement of larval weaning feeds (led by FCPCT(2), Marisol Izquierdo).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 8.1. Improvement of larval weaning diets.***

Task 8.2 Determination of nutritional requirements to promote feed utilization, consistent growth rates and fish welfare (Led by FCPCT(2), Lidia Robaina)

Scientist participant: FCPCT (2): Lidia Robaina, Marisol Izquierdo, Marta Carvalho, DTU(21) Ivar Lund, Manuel Gesto, ULL (15) Covadonga Rodriguez, José Pérez. SARC (20) Ramón Fontanillas

The activity presented in this report belongs to the **deliverable 8.2 Dietary requirements for essential fatty acids of meagre *Argyrosomus regius* fingerlings,** delivered in month 48. Thus a brief of the activity done within the period is presented here.

Experimental fish and rearing conditions

The feeding trial was conducted with meagre (*Argyrosomus regius*) fingerlings with an initial body weight of 2.80±0.23 g (mean±SD) and an initial total length of 6.37±0.20 cm (mean±SD). Triplicate groups of meagre fingerlings, produced at FCPCT facilities, were randomly distributed in 15 experimental tanks (200 L fibreglass cylinder tanks with conical bottom and painted with light grey colour) at a density of 45 fish per tank and fed manually one of the experimental diets until visual apparent satiety, three times a day, 6 days per week, during 30 days. Daily feed intake was calculated by recording diet uptake, and subtracting uneaten pellets. The tanks were installed in open system and supplied with filtered seawater (37 mg L^{-1} salinity). Water was continuously aerated and dissolved oxygen was maintained above 6.0± 0.2 mg L^{-1} during the trial.

Average water temperature along the trial was 23.0±0.2 ºC. The experiment was run under natural photoperiod between September and October 2016.

Experimental diets

Five isoproteic and isolipidic experimental diets were formulated containing fish oil (FO) and vegetable oils (VO; linseed, palm and rapeseed oils) as lipid sources. Five dietary increasing levels of n-3 LC-PUFAs, namely eicosatrienoic acid (20:3n-3, ETE), eicosatetraenoic acid (20:4n-3, ETA), docosapentaenoic acid (22:5n-3, DPA), EPA and DHA were defined: 0.8, 1.4, 2.0, 2.6 and 3.6% of dry matter (DM), where DHA and EPA accounted for 93% of total n-3 LC-PUFA. The desired n-3 LC-PUFA content of each experimental diet was achieved by successively replacing VO by FO. Diet composition and proximate analysis are shown in **Table 8.2.1** and dietary fatty acid composition in **Table 8.2.2**. The experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) with a pellet size of 2 mm, analysed for proximate and fatty acid composition at FCPCT laboratories and kept in a cold room at 10ºC until use.

Table 8.2.1. Composition (%) and proximate analysis of the experimental diets for meagre fingerlings. 1: Skretting, Stavanger, Norway; 2: Cargill Nordic AS, Charlottenlund, Denmark; 3: AAK AB, Karlshamn, Sweden; 4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals;Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

	Dietary n-3 LC-PUFA level (% DM)				
	0.8	1.4	2.0	2.6	3.6
Ingredients (%)					
Fish meal, N. Atlantic[1]	15.0	15.0	15.0	15.0	15.0
Corn gluten[2]	10.0	10.0	10.0	10.0	10.0
Faba beans[1]	10.0	10.0	10.0	10.0	10.0
Wheat[1]	8.0	8.0	8.0	8.0	8.0
Wheat gluten[1]	18.4	18.4	18.4	18.4	18.4
Soy protein concentrate[1]	25.0	25.0	25.0	25.0	25.0
Fish oil, S. American[1]	0.0	2.7	5.4	8.2	10.9
Linseed oil[3]	1.6	1.2	0.8	0.4	0.0
Palm oil[3]	3.3	2.5	1.7	0.8	0.0
Rapeseed oil[1]	6.0	4.5	3.0	1.5	0.0
Premix[4]	2.8	2.8	2.8	2.8	2.8
Proximate analysis (% DM)					
Protein	56.5	54.5	54.5	56.0	54.3
Lipids	16.2	17.0	16.5	16.9	16.2
Ash	4.9	5.0	5.1	5.2	5.0
Moisture	8.7	8.5	8.5	8.2	7.9

Table 8.2.2. Fatty acid composition of the experimental diets (% of total identified fatty acids)

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Σ SFA	29.3	30.9	32.2	37.9	36.8
14:0	1.3	2.0	3.0	3.8	5.1
15:0	0.1	0.2	0.2	0.3	0.4
16:0	14.8	15.1	15.4	15.2	16.2
17:0	0.1	0.2	0.4	0.5	0.7
18:0	2.8	2.8	2.8	2.9	3.1
20:0	0.5	0.4	0.3	0.3	0.3
Σ MUFA	36.1	33.4	29.7	11.6	20.8
14:1n-7	0.1	0.1	0.1	0.1	0.1
14:1n-5	0.1	0.1	0.2	0.2	0.2
16:1n-7	1.2	2.1	3.2	4.1	5.6
16:1n-5	0.1	0.1	0.2	0.2	0.3
18:1n-7	1.9	1.8	2.0	2.2	2.3
18:1n-5	0.1	0.1	0.1	0.1	0.1
20:1n-7	1.4	1.4	1.5	1.7	1.7
20:1n-5	0.1	0.1	0.1	0.5	0.2
22:1n-11	1.4	1.4	1.8	2.0	2.2
Σ n-9	31.4	27.8	22.4	15.4	10.7
18:1n-9	31.0	27.4	21.9	17.8	9.8
18:2n-9	0.0	0.0	0.0	0.0	0.1
20:1n-9	0.1	0.1	0.1	0.2	0.2
20:2n-9	0.0	0.0	0.0	0.1	0.1
20:3n-9	0.0	0.0	0.0	0.0	0.1
22:1n-9	0.2	0.2	0.3	0.3	0.3
Σ n-3	14.3	16.4	19.4	21.3	26.5
Σ n-3 LC-PUFA	5.2	8.0	12.2	15.1	21.9
16:3n-3	0.1	0.1	0.1	0.4	0.3
16:4n-3	0.2	0.4	0.6	1.4	1.1
18:3n-3	8.3	7.3	5.4	3.5	1.3
18:4n-3	0.5	0.7	1.1	1.4	1.9
20:3n-3	0.0	0.0	0.0	0.0	0.1
20:4n-3	0.1	0.2	0.3	0.3	0.5
20:5n-3	2.1	3.4	5.4	6.8	9.2
22:5n-3	0.2	0.4	0.6	0.8	1.1
22:6n-3	2.8	4.0	6.0	7.2	10.5
Σ n-6	18.3	17.1	15.9	12.1	11.8
Σ n-6 LC-PUFA	0.3	0.5	0.7	0.9	1.2
18:2n-6	18.0	16.6	15.1	12.6	10.4
18:3n-6	0.0	0.1	0.1	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1	0.2
20:3n-6	0.0	0.0	0.1	0.1	0.1
20:4n-6	0.1	0.2	0.4	0.5	0.7
22:4n-6	0.0	0.0	0.0	0.1	0.1
22:5n-6	0.0	0.1	0.1	0.2	0.2
EPA/DHA	0.7	0.8	0.9	0.9	0.9
EPA/ARA	14.0	13.7	13.6	13.7	13.9
EPA+DHA	4.9	7.9	10.9	16.5	22.0
Other FAs					
16:2n-4	0.1	0.2	0.4	0.5	0.7
16:3n-4	0.1	0.1	0.1	0.2	0.2
16:3n-1	0.0	0.0	0.1	0.1	0.1
18:2n-4	0.0	0.1	0.1	0.2	0.2
18:3n-4	0.1	0.1	0.1	0.1	0.1
18:4n-1	0.0	0.1	0.1	0.1	0.1

At the beginning and the end of the trial, fish were anaesthetized and individually weighed and sized (total length) after being unfed for 24 h. The following formulas were used for calculating survival, growth and feed utilisation parameters: Survival (%): (number of final fish- number of initial fish)/ number of initial fish x 100; Weight gain, WG (g): final weight- initial weight; Specific growth rate, SGR (% day^{-1}): (ln final mean weight – ln initial mean weight) /number of days x 100; Thermal growth coefficient, TGC: (final $weight^{1/3}$- initial $weight^{1/3}$)/(Temperature x days); Feed intake, FI (g feed $fish^{-1}$ day^{-1}): feed intake (g) / days of experiment/ number of fish; Feed conversion ratio, FCR: feed intake (g) / weight gain (g); Condition factor, K (%): body weight / total $length^3$ x 100; Protein efficiency ratio, PER: weight gain (g) / protein intake (g) (dry matter); Lipid efficiency ratio, LER: weight gain (g) / lipid intake (g) (dry matter). Besides, retention efficiency of the most relevant dietary fatty acids was calculated as following: Fatty acid retention (% FA intake): final weight x FA in final whole body x final whole body lipids – initial weight x FA in initial whole body x initial whole body lipids / FI x dietary lipids x dietary FA x 100. Resulting data are presented as means ± standard deviation (SD).

Sampling

At the beginning of the feeding trial 10 fish were euthanized with excess of clove oil, and frozen at -80ºC to determine the initial whole-body composition. At the end of the experiment, 5 fish per tank were collected for the same purpose. Livers from 15 fish were collected and weighed to calculate hepatosomatic index (HSI). Five of those livers were used for biochemical and FA composition analysis, other 5 for histological studies, and 3 for bacteriological analysis. Additionally, livers from 5 fish per tank were collected and conserved in RNA later (Sigma-Aldrich, Madrid, Spain) for gene expression studies. At the day after, RNA later was removed and liver samples stored at -80ºC until analysis.

Biochemical analysis

Moisture, ash and protein were determined according to A.O.A.C. (2000). Total lipid content was extracted with chloroform/methanol (2:1 v/v). Fatty acid methyl esters (FAMES) were obtained by transmethylation of total lipids and were separated by gas liquid chromatography. Fatty acid methyl esters (FAMES) from liver total lipid and polar fraction were obtained by transmethylation following the above-mentioned conditions.

Gene expression

Total RNA was extracted from meagre livers using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were homogenized with the TissueLyzer-II (Qiagen) with TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and centrifuged with chloroform at 12 000 g for 15 min, at 4°C. The quantity of RNA was analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity using Gel Red™ staining (Biotium Inc., Hayward, CA) on a 1.4% agarose electrophoresis gel. Synthesis of cDNA was run with iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad). Gene expression of fatty acyl desaturase (*fads2)* and fatty acyl elongase 5 (*elovl5)* genes were determined by Real-Time PCR (RT-PCR) in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad) using *β-actin* as housekeeping gene. *β-actin*, *fads2* and *elovl5* primer sequences used are shown in **Table 8.2.3**, as well their annealing temperatures.

Table 8.2.3. Sequences of primers used for running RT-PCR analysis of fads2 and elovl5 gene expression of meagre hepatic tissue. *GenBank: http://www.ncbi.nlm.nih.gov/.

Gene	Primer Sequence (5'-3')	Concentration	Temperature	Accession nos*
β-actin	F: 5'-CCATCGAGCACGGTATTGT-3'	0.4 µM	60.5ºC	GU584189
	R: 5'-CAGCTTCTCCTTGATGTCACG-3'			
Fads2	F: 5'-TGACTGGGTGACAATGCAGT-3'	0.4 µM	60.5ºC	KC261978
	R: 5'-TGGTGCTAACTTTGTGCCCT-3'			
Elovl5	F: 5'-CATCACACAGTTACAGCTGGTC-3'	0.4 µM	60.5ºC	KC261977
	R: 5'-GAATTGTGTGCACGGTTTCT-3'			

Histological studies

Samples of liver were processed and stained with haematoxylin and eosin (H&E). Additional sections of liver tissue were stained with acid-fast (Ziehl-Neesen) for searching the presence of *Mycobacterium sp.* and *Nocardia sp.* All slides were examined under light microscopy (BX51TF, Olympus, Tokyo, Japan) and blinded evaluated by three different investigators to define visual differences among treatments. A semi-quantitative score evaluation of lipid infiltration level was used, ranging from 0 to 3. Score 0-1 was defined as normal liver morphology, score 1-2 was considered as moderate steatotic alterations in hepatic tissue with moderate lipid infiltration and score 2-3 severe steatotic alterations in hepatic tissue with high lipid infiltration.

Bacteriological analysis

At the end of the trial, three liver samples from each experimental treatment were seeded using blood (sheep) agar, supplemented with 1.5% sodium chloride (AS-1.5% NaCl), brain-heart infusion agar supplemented with salt (BHIA-1.5% NaCl) and YEME as culture media to discard the presence of *Nocardia spp*. and *Streptomyces sp*. Cultures were incubated at 25ºC for 4 weeks with daily check for bacteria growth.

Digestive enzymes activity

Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were measured in samples of tissue (intestine and stomach). All enzymatic activities were expressed as specific activity defined as units per milligram of protein.

Stress challenge trial

Triplicate groups of meagre juveniles fed 0.8, 1.4 and 2.6% n-3 LC-PUFA from the previous feeding trial (initial total length body weight ~25 g), were randomly distributed in 9 experimental tanks (working volume 100 L $tank^{-1}$) at a density of 10 fish tank-1. Samples from 4 fish per tank were taken at 0 (pre-stress), 1h, and 5 hours for biochemical analysis at the end of the trial. Plasma stress markers (cortisol, glucose, lactate), as well as brain serotonergic activation (in the telencephalon), were analyzed.

Additionally, 8 fish from the three dietary treatments mentioned above (0.8, 1.4 and 2.6%) were tested in terms of fast escape response. The trial was conducted in a white semi-translucent polyethylene circular tank with a diameter of 38 cm and a water depth of 5 cm. The escape response was triggered by mechanical stimulation by releasing an iron rod (ø 10mm, l 15mm) manually from a height of 90 cm above the water surface. Fast escape was determined for single fish with two repetitions performed with 30 minutes of recovery between tests. Escape responses were analysed using Tracker (v. 4.72, www.cabrillo.edu/~dbrown/tracker). Time 0 was set as the nearest 4 ms interval at which the stimulus broke the water surface. The centre of mass was plotted every 4 ms from stimulus and 20 frames forward. These x,y coordinates were used to calculate escape latency (defined as the time elapsed between stimulus breaking the water surface and the first detectable escape motion of the fish), peak velocity during the escape response (bl s−1), distance covered during the first 80 ms of the escape response, and peak acceleration (m s−2).

Statistical analysis

All data were tested for normality and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively, and analysed by one-way ANOVA. Relative gene expression data were normalized according Livak method and required a logarithmic transformation before being analysed by one-way ANOVA. When p-values were significant ($P<0.05$), means were compared with Tukey's multiple range test (Tukey, 1949). When appropriated, response data were also subjected to regression analysis (linear or exponential), where dietary FA level (analysed) served as the independent variable. A broken-line model was applied to estimate meagre n-3 LC-PUFA requirements. All statistical analyses were done using the SPSS 21.0 software package for Windows.

Results

Growth performance

Survival was high in all treatments and was not affected by diet composition (**Table 8.2.4**). After 30 days of feeding, meagre final weight ranged from 9.5 (in fish fed 0.8 n-3 LC-PUFA diet) to 10.7 g (in those fed 2.6 n-3 LC-PUFA diet), achieving a 3-fold increase from initial body weight (**Table 8.2.4**). Fish fed the 0.8% n-3 LC-PUFA diet showed the lowest values for total length, body weight, WG, SGR and TGC. Increase in dietary n-3 LC-PUFA, significantly ($P<0.05$) increased final total length and body weight. Thus, the highest total length was found in fish fed 2.6% n-3 LC-PUFA diet, being significantly higher than fish fed 0.8%, 1.4% or 3.6% n-3 LC-PUFA (**Table 8.2.4**). Feed intake was not affected by the dietary treatment, and fish fed the 2.6% n-3 LC-PUFA diet showed the best K, but not significantly different from fish fed other n-3 LC-PUFA levels. The broken-line linear model fitted best to the dietary n-3 LC-PUFA relation to final length, final weight, WG, SGR or TGC and pointed out a requirement of 2.1% n-3 LC-PUFA for maximum growth of meagre fingerlings (i.e. WG showed in **Fig. 8.2.1**).

Table 8.2.4. Growth performance of meagre fingerlings fed the experimental diets for 30 days[1]. Values (mean ± SE) with different superscript letters in the same row are significantly different ($P<0.05$).* n-3 LC-PUFA (% total FA in DM diet)

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Survival (%)	93.3±0.7	97.8±1.3	99.3±0.7	94.8±1.5	97.8±2.2
Initial total length (cm)	6.4±0.0	6.3±0.1	6.3±0.0	6.4±0.0	6.2±0.0
Final total length (cm)	9.0±0.1^{c}	9.4±0.1^{b}	9.3±0.1^{ab}	9.6±0.1^{a}	9.3±0.1^{bc}
Initial body weight (g)	2.8±0.1	2.8±0.1	2.7±0.1	2.7±0.1	2.6±0.1
Final body weight (g)	9.5±0.3^{b}	10.4±0.3^{a}	10.2±0.4^{ab}	10.7±0.3^{a}	10.4±0.3^{a}
WG (g)	6.7±0.4	7.5±0.4	7.6±0.3	8.0±0.3	7.8±0.2
SGR (% day^{-1})	4.1±0.1	4.3±0.2	4.5±0.1	4.5±0.1	4.6±0.1
TGC	1.0±0.0	1.1±0.1	1.1±0.0	1.2±0.0	1.2±0.0
FI (g feed fish^{-1} day^{-1})	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
FCR	0.8±0.1	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0
K (%)	1.3±0.0	1.3±0.0	1.3±0.1	1.2±0.1	1.3±0.0
PER	2.4±0.2	2.6±0.1	2.5±0.1	2.6±0.1	2.6±0.1

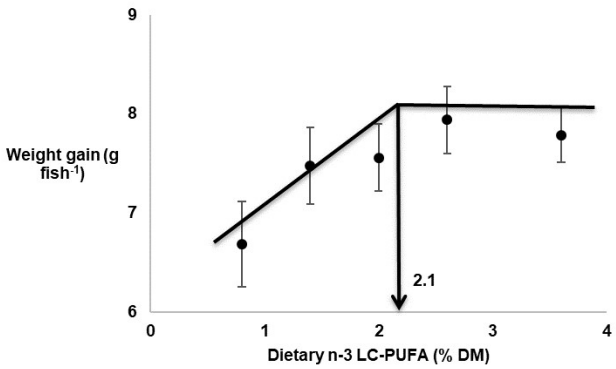

Figure 8.2.1. Broken-line linear model fitting dietary n-3 LC-PUFA levels to weight gain of meagre fingerlings fed the experimental diets for 30 days; the arrow indicates the requirement for dietary n-3 LC-PUFA (% DM).

Whole-body biochemical and fatty acid composition

Compared to the initial values, final body composition of meagre was higher in lipid and lower in ash contents (**Table 8.2.5**). At the end of the trial, no significant differences were observed in protein, lipids, ash, and water content in whole-body composition among the dietary treatments.

Table 8.2.5. Whole-body composition (% wet weight) of meagre fingerlings fed the experimental diets for 30 days. n-3 LC-PUFA (% total FA in DM diet)

	Dietary n-3 LC-PUFA level (% DM*)					
	Initial	0.8	1.4	2.0	2.6	3.6
Protein	15.6±0.5	15.8±0.2	16.1±0.1	15.6±0.3	16.3±0.5	16.0±0.6
Lipid	2.2±0.1	4.1±0.5	4.1±0.3	3.8±0.2	3.8±0.3	3.9±0.3
Ash	3.9±0.3	2.7±0.2	2.7±0.1	2.8±0.1	2.8±0.0	2.6±0.3
Moisture	78.6±0.3	78.1±0.8	77.7±0.0	78.0±0.6	78.5±0.1	78.7±0.2

Whole-body FA retention efficiency, expressed as percentage of FA intake, is presented in **Table 8.2.6**. In general, fish presented retentions below 100% for most FA, indicating a net reduction. However, retention of Δ6 desaturase activity products from OA and LA, 18:2n-9 and 18:3n-6, was over 100% in fish fed the 0.8% and 1.4% n-3 LC-PUFA diets ($P<0.05$), indicating a net accumulation. In contrast, the retention of Δ6 desaturase activity products from ALA, 18:4n-3, was lower compared to those originated from LA or OA. Furthermore, elovl5 products from ALA and LA, 20:3n-3 and 20:2n-6, respectively were also over 100% in fish fed the 0.8% and 1.4% n-3 LC-PUFA diets ($P<0.05$). Retention of EPA was unaffected by diet composition. In contrast, DHA, as well as ARA, retention was the highest in fish fed 0.8% n-3 LC-PUFA, ($P=0.18$). Furthermore, both DHA and ARA retention values were higher than EPA retentions. Additionally, retention of ALA was highest in fish fed 3.6% n-3 LC-PUFA ($P<0.05$).

Table 8.2.6. Retention efficiency (net accumulation or reduction) of specific fatty acids in whole-body of meagre fingerlings fed the experimental diets for 30 days. Values (mean ± SE) with different superscript letters, in the same row, are significantly different ($P<0.05$);* n-3 LC-PUFA (% total FA in DM diet)

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
18:2n-9	251.9±43.0^{a}	125.7±16.5^{b}	55.1±16.2^{b}	47.2±1.2^{b}	37.7±1.4^{b}
20:2n-6	227.8±57.4^{a}	165.4±11.4^{ab}	114.2±12.8^{ab}	82.2±110.7^{b}	86.5±10.3^{ab}
18:3n-6	190.8±44.8	107.8±14.7	48.2±13.7	40.9±2.4	65.5±12.6
20:4n-6	55.0±18.2	57.2±2.8	28.7±8.9	32.9±3.0	53.1±6.7
18:3n-3	25.7±5.3^{b}	38.7±4.1^{ab}	24.5±4.3^{b}	31.7±2.9^{b}	56.8±5.2^{a}
20:3n-3	315.0±88.6^{a}	256.1±24.6^{ab}	136.6±12.9^{abc}	50.8±0.6^{b}	68.2±10.7^{b}
18:4n-3	23.3±3.6	26.9±4.7	14.6±4.3	18.9±3.3	30.0±3.2
20:5n-3	25.0±6.7	31.3±4.4	13.3±4.7	17.9±4.1	30.3±4.3
22:6n-3	56.1±5.8^{a}	41.4±3.^{b}	41.5±4.7^{b}	37.5±0.7^{b}	39.9±3.7^{b}

Fads2 and elovl5 gene expression

The results of the RT-PCR showed that the relative gene expression of *fads2* was significantly higher for fish fed the lowest n-3 LC-PUFA level (0.8%) than for fish fed the highest n-3 LC-PUFA level (3.6%; P<0.05; **Fig. 8.2.2**). Although no significant differences were observed, in fish fed the lowest n-3 LC-PUFA level gene expression of *elovl5* was 2 to 14-fold higher than those fed the other n-3 LC-PUFA levels (**Fig. 8.2.3**).

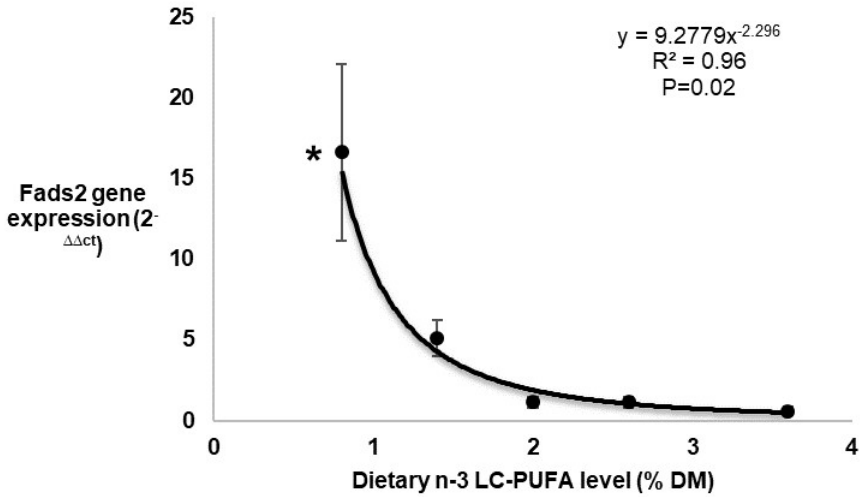

Figure 8.2.2. Fatty acyl desaturase (*fads2*) gene expression ($2^{-\Delta\Delta ct}$) of meagre hepatic tissue fed the experimental diets for 30 days; * indicates significance difference (p=0.04) compared to diet 3.6% n-3 LC-PUFA.

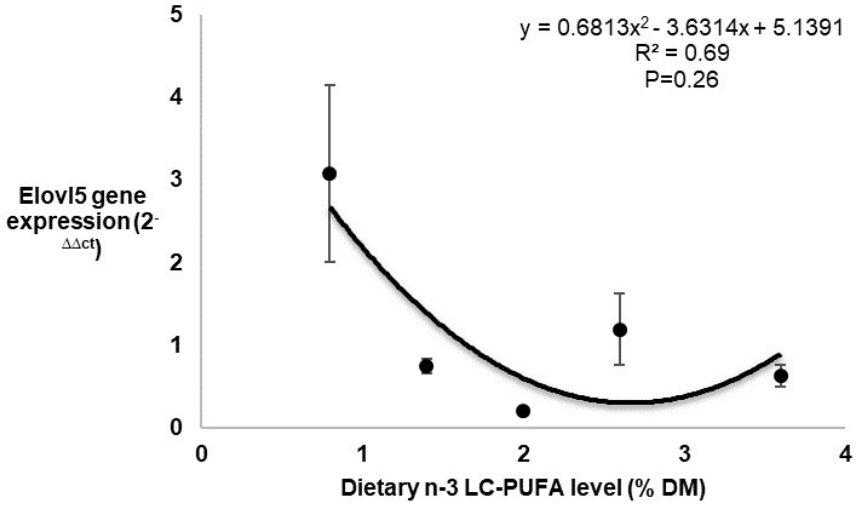

Figure 8.2.3. Fatty acyl elongase-5 (*elovl5*) gene expression ($2^{-\Delta\Delta ct}$) of meagre hepatic tissue fed the experimental diets for 30 days.

FA composition of liver total lipids reflected the dietary composition and FA composition of liver polar lipids was less affected by the n-3 LC-PUFA level of the experimental diets. A complete set of results of different fatty acids from total and polar fatty acids were detailed in deliverable 8.2.

Liver histopathology

Histological examination of cross-section of hepatic tissue showed that no necrotic tissue was found in meagre fed different dietary n-3 LC-PUFA levels. However, liver of fish fed 0.8% n-3 LC-PUFA showed a significantly higher degree of steatosis than those fed >2% n-3 LC-PUFA (**Table 8.2.7**). Liver of fish fed 0.8 and 1.4% n-3 LC-PUFA presented a severe steatosis, reflected by the hypertrophy of the hepatocytes (**Fig. 8.2.4A&B**). Furthermore, in the same fish, an extensive infiltration of lipid vacuolization, in hepatocytes was observed and consequently, nuclei were displaced from central position in the cell to the periphery. Contrarily, liver of fish fed 2.0-3.6% n-3 LC-PUFA showed smaller hepatocytes, with spherical nuclei and, mostly, located at a central position of the cell although some lipid infiltration was also observed at a lower extension (**Fig8.2.4C,D&E**). Thus, hepatic steatotic alterations decreased linearly with the increase of the dietary n-3 LC-PUFA levels (r^2=0.88, P=0.19).

Table 8.2.7. Histomorphological evaluation of hepatic tissue of meagre fed the experimental diets for 30 days. [1]Means with different superscript letters in the same row are significantly different (P<0.05). [2]Mean score value: score 0-1: normal liver histomorphology, score 1-2: moderate lipid infiltration; and score 2-3 high lipid infiltration.[3]Granulomas: measured in number of granulomas observed in each sample.* n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Steatosis[2]	$2.6^{a}\pm0.2$	$2.4^{ab}\pm0.2$	$1.7^{ab}\pm0.2$	$1.2^{b}\pm0.0$	$1.2^{b}\pm0.2$
Granulomas[3]	$5.3^{a}\pm1.6$	$2.1^{ab}\pm1.1$	$1.4^{b}\pm1.0$	$0.1^{b}\pm0.1$	$0.7^{b}\pm0.4$

Additionally, despite gross examination did not revealed the presence of granulomatous lesions in any organ of any fish, those fed the lowest dietary n-3 LC-PUFA level (0.8%) presented higher (P<0.05) number of hepatic granulomas than fish fed ≥0.2 %n-3 LC-PUFA (**Table 8.2.8**). Regardless the dietary n-3 LC-PUFA level, two main developmental stages were observed: an early stage characterized by concentric layers of macrophages and inflammatory cells (**Fig. 8.2.5A&B**); and a more developed stage characterized by a necrotic center surrounded by an external fibroblast layer and inflammatory cells (**Fig. 8.2.5C&D**).

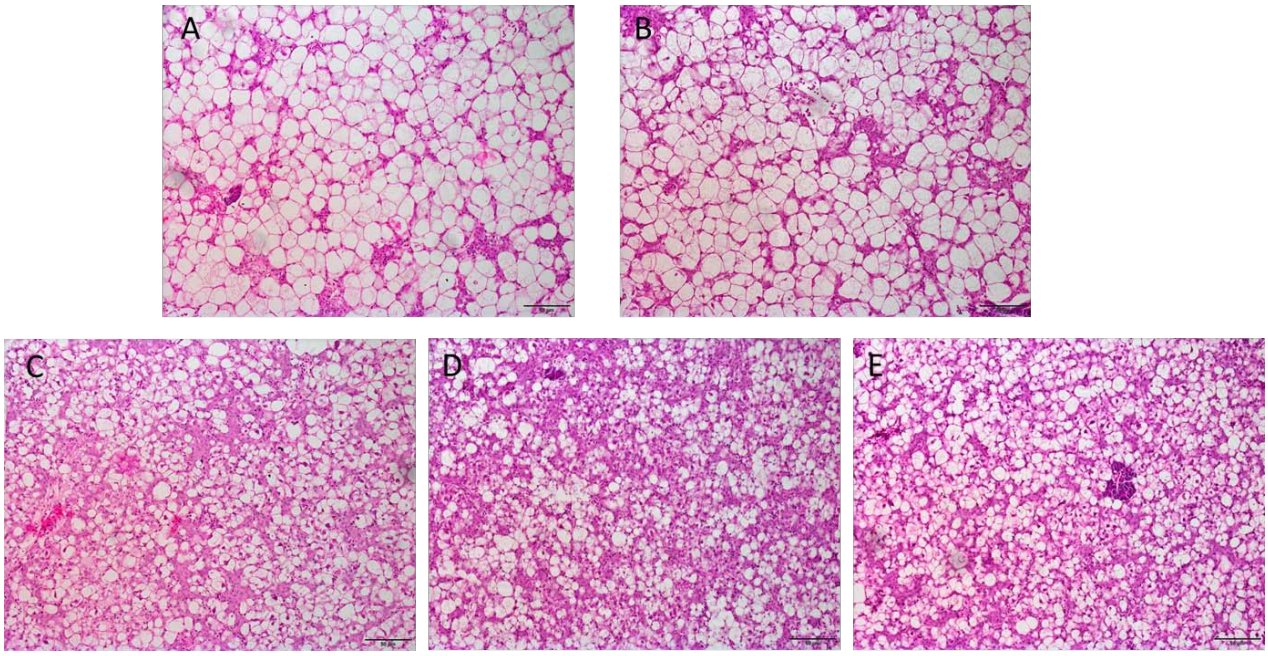

Figure 8.2.4: Liver sections from meagre fed different n-3 LC-PUFA levels stained with H&E, Bars 50µm: (A) 0.8% n-3 LC-PUFA; (B) 1.4% n-3 LC-PUFA; (C) fed 2.0% n-3 LC-PUFA; (D) 2.6 % n-3 LC-PUFA; (E) 3.6% n-3 LC-PUFA.

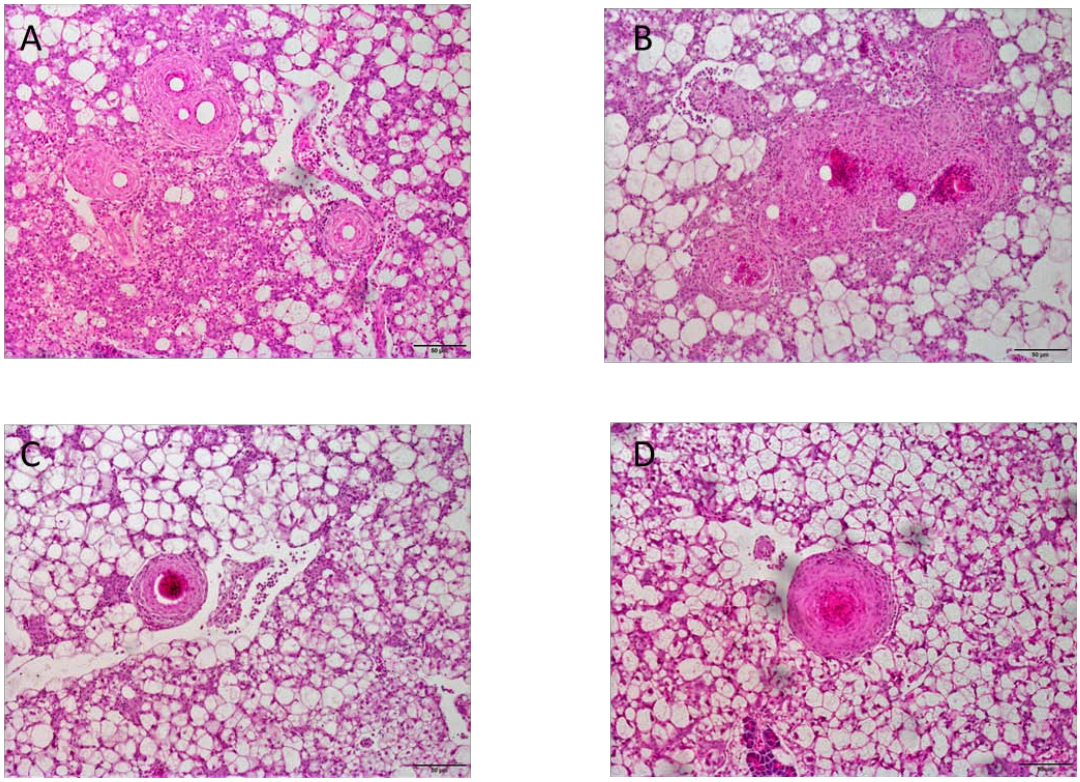

Figure 8.2.5: Liver sections of meagre with presence of granulomas at different stages of development: A and B showing concentric layers of macrophages and inflammatory cells around and C and D showing necrotic centre with external fibroblast layer and inflammatory cells (Bars 50 µm).

Bacteriological results

Liver sections stained with acid-fast (Ziehl-Neelsen) led to a negative result for the presence of *Mycobacterium sp*. and *Nocardia spp.*, and no colony forming units (CFU) of *Nocardia spp.* and *Streptomyces sp*. grown in any culture media utilised.

Digestive enzymes activity

As shown in **Table 8.2.6**, in general terms and except for the pepsin, activities measured in the gut content (inner) were clearly higher than those of the empty tissue. This is particularly evident for the pancreatic alkaline protease, which in addition, was very active at the sampled fingerlings age, and for all treatments.

Only inner amylase and inner pepsin displayed some significant differences among treatments, with amylase activity being higher in fish fed 2.6% n-3 LC-PUFA compared to 3.6% and 1.4% n-3 LC-PUFA and a higher.

Table 8.2.6. Specific enzymatic activity (U/mg protein) in meagre juveniles fed the experimental diets for 30 days. [1]Means with different superscript letters in the same row are significantly different (P<0.05). * n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

	AMYLASE		PROTEASES	
Dietary n-3 LC-PUFA level (%DM*)	Tissue	Inner	Tissue	Inner
0.8	1.1 ± 0.5	5.5 ± 1.6 ab	310.9 ± 169.5	1350.3 ± 316.9
1.4	1.6 ± 0.2	3.6 ± 1.1 b	274.8 ± 131.1	1445.6 ± 495.9
2.0	1.4 ± 0.3	4.8 ± 2.3 ab	155.6 ± 36.8	930.2 ± 162.2
2.6	1.8 ± 0.2	8.1 ± 1.5 a	262.3 ± 46.4	1205.6 ± 116.6
3.6	1.3 ± 0.3	3.6 ± 0.9 b	252.7 ± 102.6	1010.6 ± 225.1

Stress challenge trial

No mortality in fish was observed during and after the stress challenge. In general, the results of the stress challenge showed nice activation and recovery of all markers induced by the stressor. According to plasma cortisol, 1h after inducing the stress all treatments presented higher plasma cortisol levels than respective control levels (pre-stress; **Fig. 8.2.6**). However, no significant differences were detected 1h and 5h post-stress between fish fed different n-3 LC-PUFA levels (**Fig. 8.2.6**). For glucose levels, 1h after inducing the stress, fish fed 1.4% showed significant lower levels of plasma glucose than fish fed 0.8% n-3 LC-PUFA. Furthermore, fish fed 0.8% n-3 LC-PUFA showed significant higher plasma lactate concentration 1h after inducing stress than fish fed 1.4% and 2.6% n-3 LC-PUFA. Even though after 5h all treatments showed a recovery to basal levels.

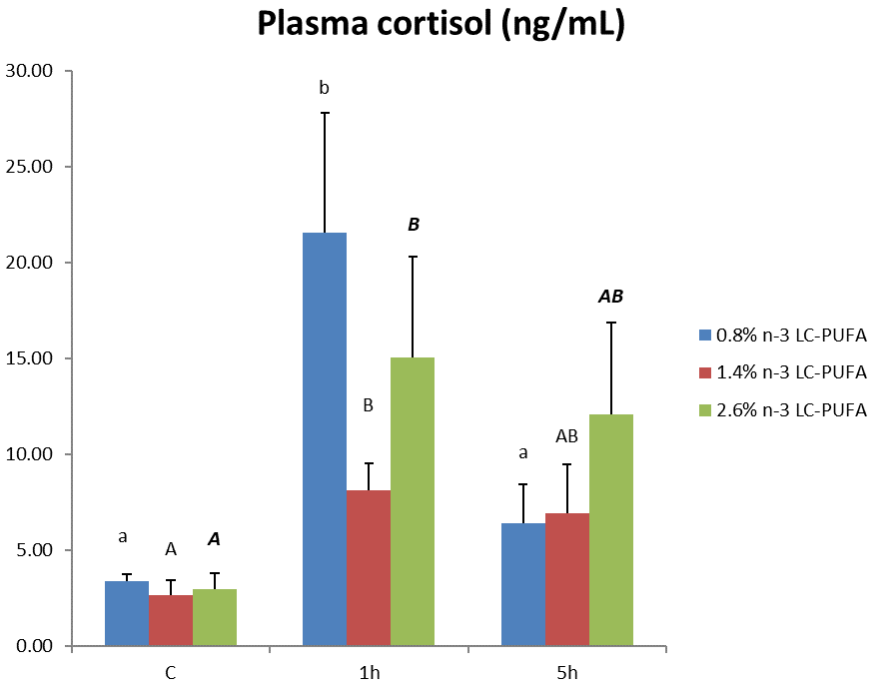

Figure 8.2.6. Plasma cortisol levels (ng/mL) in meagre fed the experimental diets before, 1h and 5h after stressor; at specific sampling times, bars with different letters are significant different (P<0.05).

The results of telencephalic serotonergic system showed that fish showed that maximum 5-hydroxyindoleacetic acid (5-HIAA) levels 1h after inducing the stress, recovering its basal levels 5h after inducing the stress, despite no differences were observed between fish fed different n-3 LC-PUFA levels (**Fig. 8.2.7**). In contrast, serotonin (5-HT) levels were unaffected by experimental diets. The 5-HIAA/5-HT ratio, an indicator of serotonergic activity, increased significantly 1h after inducing the stress in meagre fed 0.8 and 1.4% n-3 LC-PUFA, returning to values even lower than basal values 5h after inducing the stress (**Fig. 8.2.8**). However, this ratio was unaffected by stress in meagre fed 2.6% n-3 LC-PUFA.

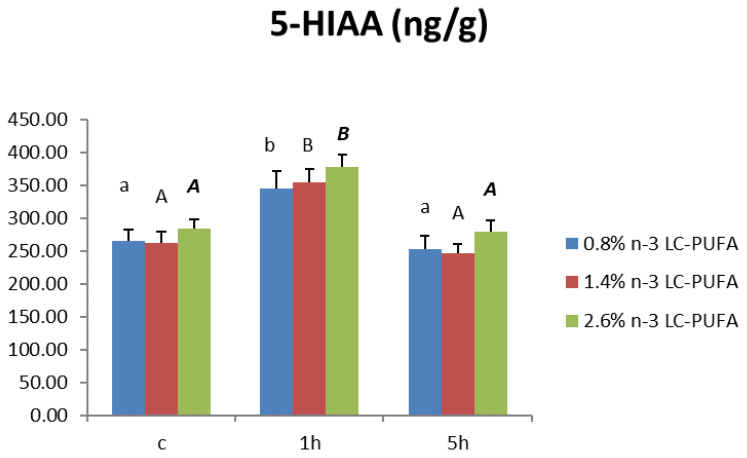

Figure 8.2.8. 5-hydroxyindoleacetic acid (5-HIAA) levels in brain of meagre fed the experimental diets before, 1h and 5h after stressor; at specific sampling times. Different letters denote significant differences (P<0.05)

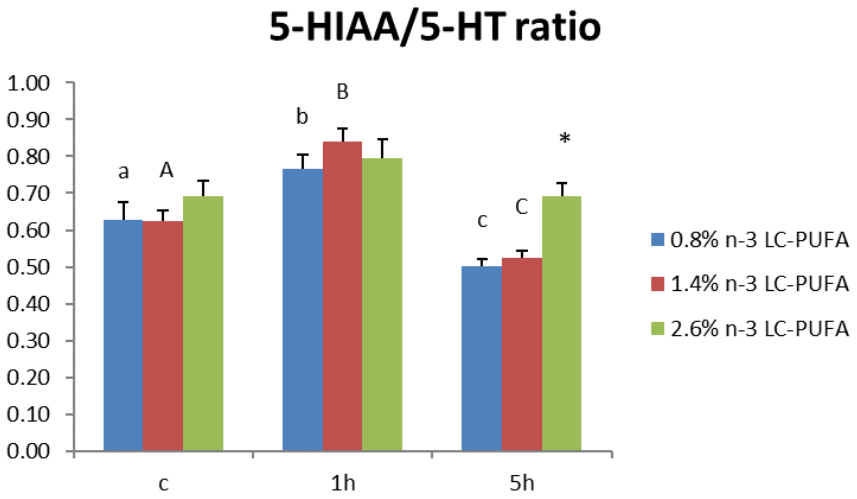

Figure 8.2.9. 5-hydroxyindoleacetic acid (5-HIAA)/ serotonin (5-HT) ratio values in brain of meagre fed the experimental diets before, 1h and 5h after stressor; at specific sampling times, bars with different letters are significant different, and asterisk denotes significant differences with respect to diet 0.8% n-3 LC-PUFA ($P<0.05$).

Deviations from Annex I and their impact:

No deviations from the plan have been recorded.

WP 9 Nutrition – greater amberjack

WP No:	9	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Nutrition – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P8. IEO	P15. ULL	P.20 SARC
P.28 CANEXMAR				
Lead Scientist preparing the Report (WP leader):	Marisol Izquierdo			
Other Scientists participating:	Yannis Kotzamanis (P1), Jerez Salvador (P8), Covadonga Rodriquez (P15), Ramon Fontanillas (P20), Rafael Guirao (P28), Hipólito Fernández-Palacios (P2), Daniel Montero (P2)			

Objectives

1. Improve of larval enrichment products for live-preys to enhance production of larvae and juvenile,
2. Develop diets for grow-out in order to maximize growth potential,
3. Development of an appropriate broodstock diet to improve unreliable reproduction in amberjack.

Summary of work reported in the previous Reporting Period (1-12 Mo):

To improve larval enrichment products for greater amberjack, an experiment was conducted with larvae fed *Artemia* enriched with five levels of the essential docosahexaenoic acid (DHA) (Task 9.1.1).

- The lowest DHA content in the emulsion lead to poor survival, total length and body weight.
- DHA levels in the *Artemia* up to 1-2% produced the highest survival total length, body weight and fish welfare.
- Excess levels of DHA were toxic for amberjack larvae and reduced growth.
- Increase in DHA content in Artemia lead to improved utilization of dietary lipids, as well as increase in DHA contents in Artemia, but it did not affect other fatty acids.
- Increased DHA content over 2% in *Artemia* increased cranial anomalies.

The results demonstrated the importance of adequate levels of DHA in enrichment products for *Artemia* (1-2% DHA) to prevent bone malformations and promote maximum growth and survival in greater amberjack.

To examine the combined effect of PUFA-rich lipids and carotenoids (Task 9.1.2), rotifers were enriched according to the lipid composition of wild greater amberjack eggs, testing four lipid enrichment treatments and one commercial product combining different times of enrichment with different sources and levels of LC-PUFA rich lipids. A range of lipid sources mainly rich in polar lipids (PL) (E1), triacylglycerols (TAG) (E3), or a mixture of them (E2) was used.

- Treatments E1 and E3 produced similar survival than the commercial product (C), whereas treatment E2 produced lower survival.
- Overall, the experimental treatment E1 showed the best results in terms of survival and ovigerous females in the rotifer population.
- Longer enrichment protocols and higher total lipid levels in rotifers increased the proportions of TAG.

The results indicate that rotifer enrichment treatment E1 (100% marine lecithin) is the best protocol for LC-PUFA enrichment according to the lipid composition of wild greater amberjack viable eggs. To achieve objective 3, information on the nutritional requirements and spawning quality determination in greater

amberjack and related species were collected in order to define a basal diet formulation for amberjack broodstock (Task 9.3).

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the 2nd Reporting Period, this WP has addressed three of the main bottlenecks identified in greater amberjack: Limited production of larvae and juvenile, scarce information on nutritional requirements during grow-out and the lack of reliable reproduction and egg availability. Specifically, enrichment products were improved by determining the optimum EPA levels, a trial on the effect of nutritionally enhanced grow out diets on juvenile performance has been conducted and another one tried to improve broodstock feeding regimes to boost reproduction.

Summary of progress towards objectives (31-48 Mo):

Regarding greater amberjack juveniles, the effects of different dietary levels of lysine on growth, voluntary feed intake, nutrient utilization, body proximate composition and antioxidant capacity of fish fed diets with low fishmeal inclusion were studied. Result obtained indicate that the dietary lysine requirements, based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, was 2.11% of diet. Lysine supplementation affected the specific activity of CAT in liver and intestine of greater amberjack fed the diet containing 2.11% lysine

At broodstock levels, an experiment to determine the optimum level of essential fatty acids for reproductive success was conducted. Different groups of broodstocks were fed diets containing different essential fatty acids levels (from 2.8 to 0.96% of total fatty acids) in order to determine the effect on reproduction reliability. The effects on gonad maturation, frequency of spawns, fecundity, fertilization rates, hatching rates and larval survival rates were determined. Proximate composition of diets and eggs were also analysed. The diet containing 1.57% of total fatty acids induced a higher number of eggs per spawn and kg of female, with the highest percent of fertilization, egg viability, hatching rate and larval survival.

The lipids and carotenoids egg profile of culture females in comparison with their wild counterpart was also studied. An experimental diet was formulated and the experiment will start by January 2018.

Details for each Task

Task 9.1. Improve larval enrichment products to enhance production of larvae and juveniles (led by FCPCT).

Sub-task 9.1.1 (FCPCT, Marisol Izquierdo)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D9.1 Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids in greater amberjack enrichment products:***

Sub-task 9.1.2 (IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez)

This Sub-task was completed during this 2nd Reporting Period and results were submitted in ***Deliverable 9.1. "Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids in greater amberjack enrichment products".***

Task 9.2. Development of diets for grow-out of amberjack to maximize growth (led by HCMR).

Sub-task 9.2.1 (HCMR, Yannis Kotzamanis, E. Kouroupakis, V. Ilia, SARC, Ramón Fontanillas.

Description

In this task, we investigated the effects of different dietary levels of lysine on growth, voluntary feed intake, nutrient utilization, body proximate composition, antioxidant capacity and protein expression of heat shock proteins of juvenile greater amberjack fed diets with low fish meal inclusion.

Materials and Methods

A basal diet (L1) with low lysine concentration (1.93 g/100g diet) based mainly on plant ingredients such as wheat meal (28.6%), corn gluten meal (10%), wheat gluten meal (22%) and soya concentrate meal (1%), and with low fish meal inclusion (25%), was formulated to contain ca. 45% crude protein (CP), 18% crude lipid (supplemented mostly by fish oil) and 22 MJ/kg gross energy. Graded levels of crystalline L-lysine-HCl were added to the basal diet at the expense of wheat meal to produce five isonitrogenous and isoenergetic diets containing each of them a final lysine concentration of, 2.01 (L2), 2.11 (L3), 2.15 (L4), 2.20 (L5), and 2.29 (L6) g/100g diet, respectively (**Table 9.2.1**). The extruded feeds (2.5 mm) were manufactured by Skretting ARC (Norway) and shipped to the experimental facilities of the Hellenic Centre for Marine Research (HCMR) in Ag. Kosmas, Athens, Greece.

Table 9.2.1. Diet formulation and analysed chemical composition of the experimental diets based mainly on plant ingredients and supplied with different levels of lysine. *Calculated by difference: 100 - (%protein + %fat + %ash + %moisture) (i.e. N-free extractives + crude fiber).

Ingredients **(% diet)**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**
Fish meal (71%)[a]	25.00	25.00	25.00	25.00	25.00	25.00
Wheat meal	28.65	28.55	28.40	28.30	28.20	28.10
Corn gluten	10.00	10.00	10.00	10.00	10.00	10.00
Wheat gluten	21.95	21.95	21.95	21.95	21.95	21.95
Soya concentrate	1.01	1.01	1.01	1.01	1.01	1.01
Fish oil	12.33	12.33	12.33	12.33	12.33	12.33
Lysine HCl	***0.00***	***0.10***	***0.21***	***0.31***	***0.41***	***0.52***
Dicalcium phosphate	0.61	0.61	0.61	0.61	0.61	0.61
Mineral & Vitamin premix	0.50	0.50	0.50	0.50	0.50	0.50
Analyzed chemical composition of diets (% or specified)						
Protein	44.58	44.83	44.63	44.52	44.53	44.68
Fat	17.65	17.47	17.24	17.19	17.01	17.38
Ash	5.14	5.34	5.31	5.23	5.16	5.15
Moisture	7.87	8.66	8.41	8.65	8.52	8.13
Carbohydrate*	24.76	23.70	24.21	24.41	24.78	24.66
Gross energy (MJ kg^{-1})	21.90	21.63	21.55	21.58	21.52	21.78

Juvenile greater amberjack fish were obtained from a brood stock that reproduced in captivity at the Institute of Marine Biology, Biotechnology and Aquaculture, HCMR. Once acclimated, all fish with an initial average body weight (BW) of 32.8 ± 3.0 g (mean ± standard deviation; n = 450) were assigned to 18 experimental small cages (1.0 x 5 x 1.0 m; 5 m), at a density of 25 fish per cage (3 replicates/cages per diet). All cages were placed in two large rectangular concrete tanks of 36 m^3 water capacity that were continuously supplied

with filtered sea water (salinity 35 ppt). Seawater was distributed in each 36 m^3 tank from 10 different pipes at 400 L/h and aerated to over 80% oxygen saturation. Water temperature followed the ambient seasonal temperature throughout the experiment with an average value of 19.8 ± 1.7 ^oC.

Fish were hand-fed twice a day (09:00 and 15:00 h) to apparent satiation, six days a week with the experimental diets for a period of 55 days (started on October 21, 2015). Uneaten feed was collected by siphoning and weighed after each meal to monitor daily feed consumption.

At the end of the feeding trial, all fish were anaesthetized and weighed individually after being deprived of feed for one day. Ten fish were randomly sampled and pooled from each tank (30 fish per diet) for carcass composition. In addition, five fish from each tank were sampled for assessing the activity of catalase (CAT) and protein expression of heat shock proteins (HSP70 and HSP90) in the liver and mid intestine.

Results and Conclusion

The survival of fish in all treatments was ranged from 88% to 98%, while fish fed the L4 diet showed the highest mortality. However, a significant number of fish during the trial, ranging from 10-30%, in all feeding treatments had failed to grow, lost weight (< initial average body weight) and finally died without any obvious clinical signs. Those emaciated fish were not found to be associated with a specific diet and were not taken into account for the growth calculations and survival.

Approximately a 3-fold increase in average final body weight (FBW) was found over the course of the 8-week growth trial. No significant differences in ABW were found among the diets (P>0.05). Fish fed the L1, L2 and L5 diets showed lower final mean weights (88 g, 92 g and 91 g, respectively) among the experimental diets. The highest growth was exhibited by L3 and L6 diets (99 g and 96 g, respectively).

In this trial, feeds were offered to visual satiety twice daily and voluntary feed intake (TFI) was found to increase from L1 - L3 diet and then decreased in diet L5, although was not significantly affected by the dietary lysine level. Diets L4 and L5 exhibited higher or equal TFI values, respectively, compared to L1 diet. Those differences in TFI among the diets were mirrored in FBW, weight gain (WG) and daily growth index (DGI) respectively, and can partly justify the observed variations. No significant differences in FCR were found among the diets (Fig. 1). Diet L3 showed the lower FCR (1.18), whereas the L5 diet the higher (1.27). FCR of the rest of diets was ranged within those values. Protein utilization (PER) was found similar among the treatments showing a slight higher value in diet L3. Similarly, L3 diet was not found to be statistically different compared to the rest of the diets, although showed the highest SGR (**Fig. 9.2.1**)

Figure 9.2.1. Specific growth rate and feed conversion ratio of juvenile greater amberjack fed the six experimental diets with different lysine levels. Data are based on tank means, n=3.

Final body weight and weight gain of fish increased with the increase of dietary lysine levels from 1.93% to 2.11%. Both parameters were lower in fish fed the diets supplemented with 2.15% or higher lysine levels than in those fed 2.11% lysine.

The results from the present study indicated that the dietary lysine requirements, based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, was 2.11% of diet. No significant effect of lysine levels on the expression of HSP in liver or intestine was found. Lysine supplementation found to affect the specific activity of CAT in liver and intestine of greater amberjack fed the diet containing 2.11% lysine.

Sub-task 9.2.2 (CANEXMAR, Lara Soares)

No activities have been reported. Experiment depends on the diets defined in task 9.2.1.

Task 9.3. Design adequate feeding regimes for broodstock to optimize reproduction (led by IEO).

Sub-task 9.3.1 Optimum ARA, DHA and EPA levels as essential fatty acids for reproductive success of greater amberjack (*Seriola dumerili*) (FCPCT, Hipólito Fernandez Palacios and Daniel Montero)

Groups of greater amberjack broodstock were fed diets containing different essential fatty acids levels (SARC) in order to determine the effect on reproduction reliability. The effects on gonad maturation, frequency of spawns, fecundity, fertilization rates, hatching rates and larval survival rates were determined. Proximate composition of diets and eggs were also analysed.

Groups of fish with a mean ± SD weight of 3.41 ± 1.12 kg for females and 2.37 ± 1.07 kg for males, (captured from the wild in 2011) were conditioned in tanks of 10 m^3 (3 m x 3 m x 1.5 m depth) (**Fig. 9.3.1.**), at the facilities of the Grupo de Investigación en Acuicultura (GIA), located in the Parque Cientifico Tecnologico Marino (PCTM), of the Universidad de Las Palmas de Gran Canaria (ULPGC). Fish were kept under natural photoperiod using seawater at a temperature range 20.83 ± 0.32 °C in winter and 23.84 ± 0.18 °C in summer. In January 2013, greater amberjack (8.27 ± 1.11 kg females body weight and 8.12 ± 1.82 kg males body weight) were transferred to three circular tanks of 40 m^3 (5 m x 2.35 m) (**Fig.9.3.2**). There were no mortalities during this acclimation period. Before starting the experiment (3 June 2016), in late March 2016, all fish were anesthetized with clove oil (Guinama SL, Valencia, Spain; 50 ppm), weighted and sized. Weight evolution of broodstock lot from 2011 to 2016 is shown in **Figure 9.3.3**.

Figure 9.3.1. Set of tanks of 10 m^3 for greater Amberjack broodstock at FCPCT facilities

Figure 9.3.2. Set of tanks of 40 m^3 for greater amberjack broodstock at FCPCT facilities.

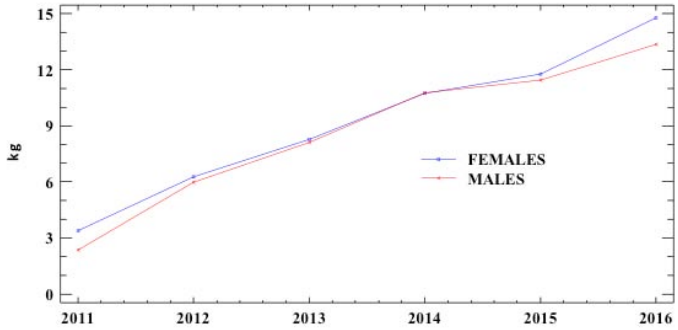

Figure 9.3.3. Weight evolution of broodstock lot from 2011 to 2016 held at FCPCT broodstock station.

The selected fish were distributed (sex ratio 1:1) in four circular tanks of 40 m^3, 2♀ and 2♂ in each one. The tanks were filled with natural seawater of 37 ‰ salinity and natural photoperiod following the day extension in concordance with the geographical position (27º 59' 28" N; 15º 22'05" W). The flow rate set at 6 cycles per day and the temperature was determined continuously through a system of sensors monitored by computer (Miranda, Innovaqua, Sevilla, Spain), and was ranged from 21.58 ± 0.36ºC and 23.30 ± 0.17°C (June–October).

Given that all females used in the present experiment spawned the previous years and to avoid an excessive handling, they were not cannulated, neither the abdominal massage was performed on males. The selected broodfish were intramuscularly injected with gonadotropin releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 $\mu g.kg^{-1}$ (Fernández-Palacios et al., 2015) (**Fig.9.3.4**). These hormonal treatments were weekly applied to avoid excessive stress for the broodstock, from June 3th to October 11th, 2016.

At the beginning of the spawning season, from June 3th to July 12th, broodfish were fed twice a week with commercial diet (13 mm, Vitalis CAL, Skretting, Burgos, Spain) at 1% of their estimated total biomass, and once a week with Atlantic mackerel (*Scomber scombrus*) at 2% (diet GIA), to ensure that there were no significant differences in the spawning quality of the different experimental groups. During this period, a total of 12 induction per tank were performed (6 per couple), in tank 1 (diet 1) 9 spawns were obtained, in tanks 2 and 3 (diet 2 and 3, respectively) 7 spawns, and in tank 4 (diet 4) 8 spawns.

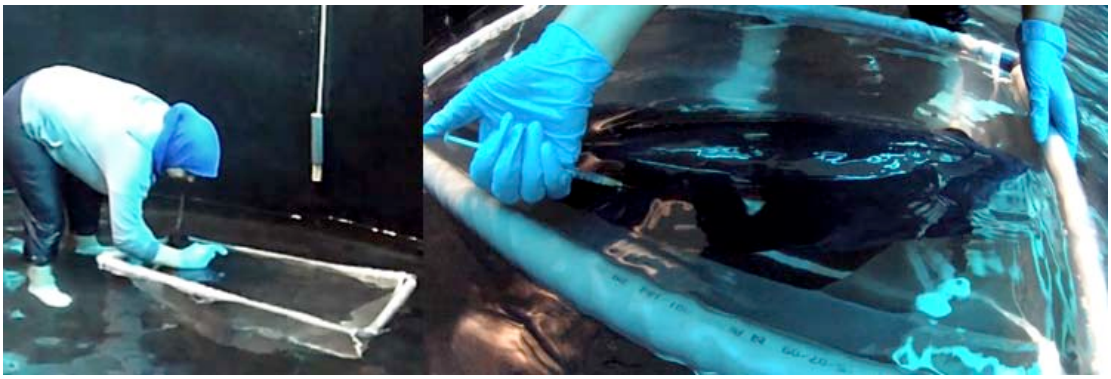

Fig. 9.3.4. Hormonal induction of spawning: Handling procedures.

Spawning quality was determined as: the percentage of fertilization, viable eggs at 24 hours (%), hatching and larval survival at 1, 3 and 5 days post-hatching (dph), using two replicates of 96-well microtiter plates according to the protocol described by Panini et al. (2001) and Fernández-Palacios (2005). With these percentages, the total numbers of fertilized, 24h viable and hatched eggs and larvae produced at 1 and 5 dph were calculated (Fernández-Palacios et al., 2011). Also, for each spawn the total number of eggs, the number of eggs per spawn and the number of eggs per spawn and weight of female (per kg) were determined.

Egg samples of all spawns per tank were collected during the experimental period and immediately frozen at −80 °C for biochemical analysis. Proximate composition analysis of eggs from each treatment was conducted following standard procedures (AOAC, 2012). Dry matter content was determined after drying the sample in an oven at 105 ºC until reaching constant weight, ash by combustion in a muffle furnace at 600 ºC for 12 h, protein content (N x 6.25) was determined by Kjeldahl method, and crude lipid was extracted following the Folch method (Folch et al., 1957). All analyses were conducted in triplicate.

Total number of eggs, total number of eggs per spawn and the total number of eggs per spawn and kg female are shown in **Figures 9.3.5.**, **9.3.6.,** and **9.3.7**. In **Table 9.3.1** and **9.3.2**, the results corresponding to the egg quality and the composition of fatty acids in eggs are indicated, during the feeding period with the GIA diet.

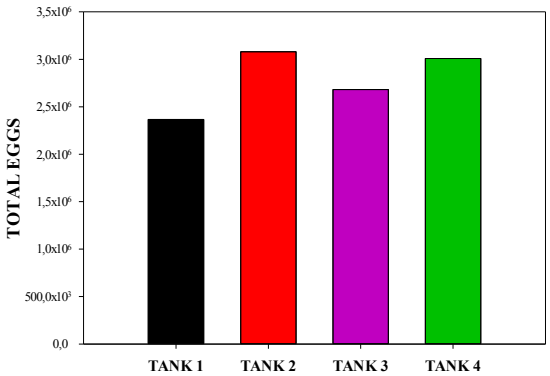

Figure 9.3.5. Number of eggs obtained during the feeding period with the GIA diet.

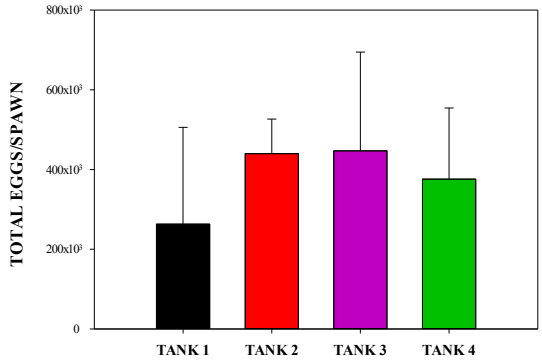

Figure 9.3.6. Number of eggs per spawn obtained during the feeding period with the GIA diet.

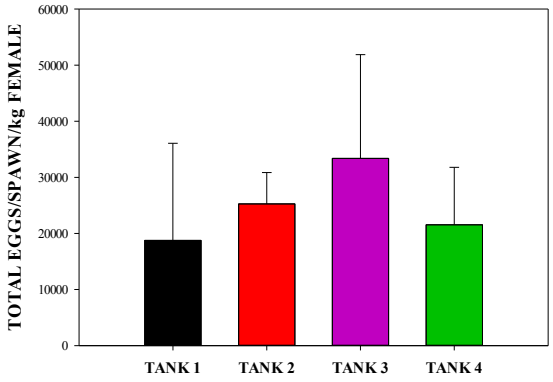

Figure 9.3.7. Number of eggs per spawn and kg female obtained during the feeding period with the GIA diet.

Table 9.3.1. Quality of egg and larvae obtained during the feeding period with the GIA diet.

Tank	% Fertilization	% Viable 24h	% Hatching
1	55.66±26.22	83.43±7.50	81.04±8.03
2	61.65±15.94	88.71±0.76	86.90±0.96
3	41.93±29.61	89.58±4.55	86.67±6.01
4	64.92±14.86	86.67±7.21	82.29±8.05
Tank	% 1 dph survival	% 3 dph survival	% 5 dph survival
1	63.68±14.93	39.81±14.13	2.84±1.63
2	74.34±9.70	28.32±7.35	4.27±1.66
3	70.45±9.33	29.78±8.25	3.17±2.27
4	72.04±11.08	37.39±12.95	3.21±2.27

Table 9.3.2. Fatty acid composition (% total fatty acids) of eggs obtained during the feeding period with the GIA diet.

Fatty acid	1- Diet G	2- Diet H	3- Diet I	4- Diet K
14:0	1.48 ± 0.21	1.59 ± 0.08	1.81 ± 0.34	1.74 ± 0.22
16:0	15.09 ± 0.65	15.87 ± 0.53	16.44 ± 0.56	16.33 ± 1.55
16:1n-7	4.31 ± 0.21	4.33 ± 0.22	4.76 ± 0.54	4.43 ± 0.45
16:1n-5	0.14 ± 0.01	0.13 ± 0.00	0.13 ± 0.01	0.14 ± 0.01
16:2n-4	0.22 ± 0.05	0.23 ± 0.03	0.24 ± 0.06	0.22 ± 0.06
17:0	0.17 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.25 ± 0.15
16:3n-3	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.13 ± 0.06
16:3n-1	0.13 ± 0.01	0.13 ± 0.03	0.10 ± 0.01	0.14 ± 0.05
16:4n-3	0.09 ± 0.03	0.09 ± 0.02	0.09 ± 0.03	0.06 ± 0.04
18:0	5.26 ± 0.57	5.31 ± 0.67	4.51 ± 0.26	5.98 ± 1.38
18:1n-9	23.00 ± 0.53	23.31 ± 1.57	24.27 ± 1.03	23.99 ± 0.68
18:1n-7	4.04 ± 0.06	3.94 ± 0.16	4.05 ± 0.08	4.10 ± 0.04
18:1n-5	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
18:2n-9	0.17 ± 0.01	0.33 ± 0.10	0.30 ± 0.10	0.20 ± 0.06
18:2n-6	10.15 ± 0.19	10.21 ± 0.45	9.77 ± 0.51	9.51 ± 0.32
18:3n-6	0.24 ± 0.01	0.36 ± 0.04	0.31 ± 0.07	0.25 ± 0.02
18:3n-3	1.31 ± 0.07	1.29 ± 0.11	1.21 ± 0.08	1.29 ± 0.13
18:4n-3	0.55 ± 0.09	0.59 ± 0.07	0.57 ± 0.07	0.53 ± 0.10
20:0	0.18 ± 0.04	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.05
20:1n-9	0.15 ± 0.02	0.14 ± 0.01	0.15 ± 0.02	0.15 ± 0.02
20:1n-7	0.89 ± 0.13	0.81 ± 0.03	0.86 ± 0.05	0.93 ± 0.11
20:1n-5	0.15 ± 0.02	0.13 ± 0.00	0.14 ± 0.01	0.16 ± 0.02
20:2n-9	0.09 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.02
20:2n-6	0.35 ± 0.02	0.30 ± 0.00	0.29 ± 0.02	0.33 ± 0.03
20:4n-6 (ARA)	1.45 ± 0.07	1.34 ± 0.04	1.21 ± 0.10	1.56 ± 0.07
20:3n-3	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.02
20:4n-3	0.63 ± 0.02	0.57 ± 0.02	0.59 ± 0.02	0.59 ± 0.08
20:5n-3 (EPA)	5.99 ± 0.37	5.99 ± 0.28	5.65 ± 0.50	5.16 ± 0.45
22:1n-11	0.13 ± 0.03	0.10 ± 0.02	0.11 ± 0.03	0.12 ± 0.03
22:1n-9	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.09 ± 0.07
22:4n-6	0.11 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.11 ± 0.02
22:5n-6	0.43 ± 0.01	0.39 ± 0.01	0.37 ± 0.03	0.41 ± 0.02
22:5n-3	2.42 ± 0.06	2.11 ± 0.01	2.32 ± 0.19	2.13 ± 0.22
22:6n-3 (DHA)	18.63 ± 0.53	17.84 ± 0.80	17.09 ± 0.44	16.62 ± 1.66
Total saturates	27.56 ± 0.46	27.58 ± 0.68	27.79 ± 0.44	26.87 ± 1.03
Total monoenoic	22.43 ± 0.27	23.36 ± 1.16	23.37 ± 0.68	24.83 ± 2.97
Total n-3	33.10 ± 0.58	33.21 ± 1.53	34.81 ± 0.47	34.47 ± 0.50
Total n-6	29.91 ± 0.53	28.73 ± 1.25	27.78 ± 0.53	26.66 ± 2.11
Total n-9	12.93 ± 0.22	12.88 ± 0.41	12.24 ± 0.72	12.44 ± 0.28
Total n-3 HUFA	23.52 ± 0.50	24.00 ± 1.67	24.93 ± 1.08	24.67 ± 0.65
EPA/ARA	3.12 ± 0.24	2.98 ± 0.04	3.04 ± 0.33	3.23 ± 0.35
DHA/EPA	12.82 ± 0.35	13.36 ± 0.34	14.20 ± 1.20	10.67 ± 1.10
DHA/ARA	4.12 ± 0.32	4.48 ± 0.08	4.70 ± 0.69	3.32 ± 0.43
EPA+DHA	24.62 ± 0.49	23.83 ± 1.07	22.74 ± 0.35	21.77 ± 1.85
n-3/n-6	2.31 ± 0.05	2.23 ± 0.07	2.27 ± 0.18	2.14 ± 0.18
18:1n-9/n-3 HUFA	0.83 ± 0.03	0.88 ± 0.09	0.94 ± 0.06	0.97 ± 0.06

After checking that there were no significant differences ($P < 0.05$) with the GIA diet, in any of production parameters and egg quality, we started to feed broodstock with the four experimental diets. The formulation and proximate composition of the experimental diets are presented in **Table 9.3.3**. The fatty acid

composition of the four diets is shown in **Table 9.3.4**. Fish were hand feed twice a day and 5 days a week (2% of biomass day^{-1}).

Table 9.3.3. Proportion of ingredients and proximate of the experimental diets (Skretting Aquaculture Research Center, Stavanger, Norway).

Diet	1	2	3	4
Lineseed oil	0.00	1.52	3.01	4.50
Wheat	19.09	19.13	19.13	19.13
Wheat gluten	13.62	14.99	14.99	14.99
Fish meal	44.97	43.46	43.46	43.46
Squid meal	10.00	10.00	10.00	10.00
Fish oil	10.93	7.48	4.04	0.61
Palm oil	0.00	2.03	3.98	5.93
Premix vit. Min.	0.64	0.64	0.64	0.64
EPA+DHA (% total fatty acids)	2.80	2.17	1.57	0.96
Proximate composition (%)				
Crude protein	58.50	58.91	58.91	59.06
Crude fat	24.25	24.89	24.35	25.61
Moisture	7.27	5.41	7.22	8.30
Ash	7.46	7.19	7.25	7.30

Table 4. Fatty acid composition (% total fatty acids) of the experimental diets.

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
14:0	6.51	5.35	4.26	2.19
16:0	18.55	20.39	21.22	23.06
16:1n-7	6.73	5.29	4.08	1.78
16:1n-5	0.28	0.20	0.16	0.06
16:2n-4	0.82	0.64	0.50	0.20
17:0	0.80	0.62	0.47	0.16
16:3n-3	0.23	0.18	0.13	0.05
16:3n-1	0.11	0.08	0.06	0.03
16:4n-3	1.19	0.93	0.72	0.28
18:0	3.64	3.59	3.49	3.47
18:1n-9	11.03	15.32	17.80	23.69
18:1n-7	2.68	2.22	1.86	1.14
18:1n-5	0.17	0.14	0.12	0.08
18:2n-9	0.06	0.06	0.04	0.01
18:2n-6	4.58	6.72	8.41	11.26
18:3n-6	0.22	0.17	0.13	0.05
18:3n-3	1.73	6.50	9.89	17.11
18:4n-3	2.37	1.86	1.50	0.77
20:0	0.30	0.28	0.28	0.20
20:1n-9	0.34	0.29	0.26	0.21
20:1n-7	3.44	2.98	2.83	2.33
20:1n-5	0.31	0.24	0.18	0.07
20:2n-9	0.06	0.04	0.03	0.01
20:2n-6	0.18	0.13	0.18	0.14
20:3n-9	0.08	0.06	0.05	0.02
20:4n-6 (ARA)	0.86	0.65	0.52	0.24
20:3n-3	0.10	0.06	0.08	0.06
20:4n-3	0.59	0.43	0.34	0.15
20:5n-3 (EPA)	11.21	8.51	6.70	2.95
22:1n-11	4.84	4.07	3.96	3.28
22:1n-9	0.46	0.37	0.34	0.26
22:4n-6	0.10	0.05	0.05	0.02
22:5n-6	0.08	0.06	0.05	0.03
22:5n-3	1.21	0.88	0.68	0.31
22:6n-3 (DHA)	12.29	9.17	7.49	3.79
Total saturates	30.31	30.43	30.06	29.25
Total monoenoic	30.60	31.59	31.79	32.99
Total n-3	30.93	28.51	27.52	25.47
Total n-6	6.14	7.98	9.47	11.78
Total n-9	11.95	16.08	18.48	24.17
Total n-3 HUFA	25.41	19.05	15.28	7.25
EPA/ARA	13.03	13.00	13.00	12.39
DHA/EPA	1.10	1.08	1.12	1.29
DHA/ARA	14.29	14.01	14.54	15.93
EPA+DHA	23.51	17.67	14.18	6.74
n-3/n-6	5.04	3.57	2.90	2.16
18:1n-9/n-3 HUFA	0.43	0.80	1.16	3.27

Total number of eggs, total number of eggs per spawn and the total number of eggs per spawn and kg female are shown in **Figures 9.3.8, 9.3.9** and **9.3.10**.

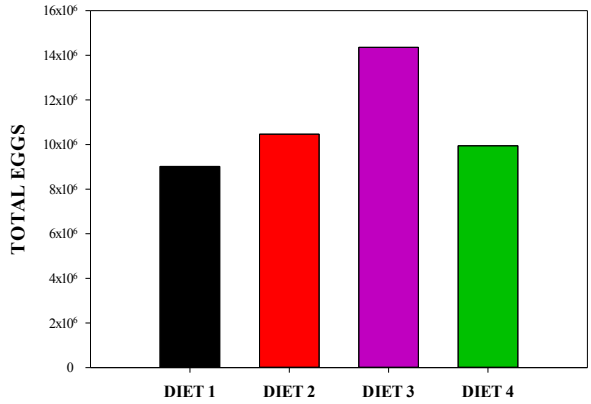

Figure 9.3.8. Number of eggs obtained after feeding with the experimental diets.

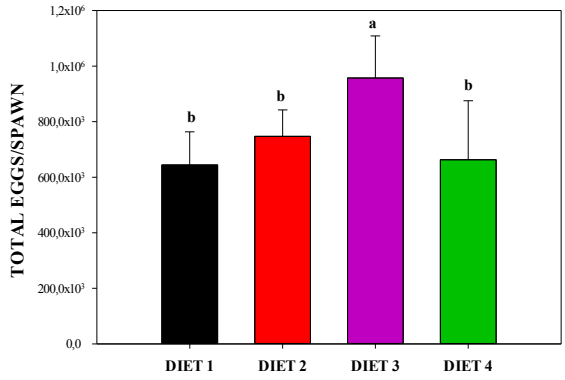

Figure 9.3.9. Number of eggs per spawn obtained after feeding with the experimental diets. *Different letters denote significant differences ($P < 0.05$).

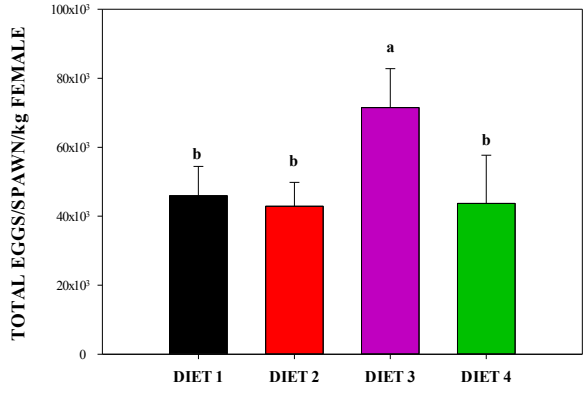

Figure 9.3.10. Number of eggs per spawn and kg female obtained after feeding with the experimental diets. *Different letters denote significant differences ($P < 0.05$).

In **Table 9.3.5**, the results corresponding to the egg quality, obtained starting from August 4th, after three weeks of feeding with experimental diets (Watanabe *et al*., 1985; Fernández-Palacios *et al*., 1995; Tandler *et al*., 1995). A total of 20 inductions were performed (10 per couple). In Tanks 1 and 2, 14 spawns were obtained, and in Tanks 3 and 4, 15 spawns. The composition in fatty acids of the fertilized eggs from broodstocks fed the different experimental diets is shown in **Table 9.3.6**.

Table 9.3.5. Quality of egg and larvae obtained after feeding with the experimental diets. Means ± SD. Different superscripts in the same column indicate significant differences (P <0.05)

Diet	% Fertilization	% Viable 24h	% Hatching
1	52.42±10.64^{c}	90.28±3.28^{c}	76.99±8.94^{b}
2	69.02±7.38^{b}	85.07±1.73^{b}	79.68±3.74^{b}
3	91.76±3.12^{a}	95.99±2.81^{a}	94.22±3.62^{a}
4	86.32±1.67^{a}	93.88±2.48^{a}	92.51±2.27^{a}
Diet	% 1 dph survival	% 3 dph survival	% 5 dph survival
1	57.44±3.08^{b}	16.15±4.96^{b}	1.56±1.04^{b}
2	59.85±2.94^{b}	11.59±2.22^{c}	2.95±1.98^{a}
3	85.25±9.97^{a}	28.33±8.01^{a}	3.98±1.52^{a}
4	87.04±2.92^{a}	28.12±2.05^{a}	3.73±1.08^{a}

Table 9.3.6. Fatty acid composition (% total fatty acids) of eggs obtained after feeding with the experimental diets.* Means ± SD. Different superscripts in the same column indicate significant differences (P <0.05).

Fatty acid	1- Diet G	2- Diet H	3- Diet I	4- Diet K
14:0	2.61 ± 0.33	2.30 ± 0.12	2.05 ± 0.04	1.81 ± 0.37
16:0	18.73 ± 0.86	18.18 ± 0.62	18.52 ± 0.23	18.50 ± 0.45
16:1n-7	5.45 ± 0.33 [a]	4.63 ± 0.09 [b]	4.11 ± 0.02 [b]	4.17 ± 0.11 [b]
16:1n-5	0.15 ± 0.04	0.11 ± 0.01	0.08 ± 0.01	0.12 ± 0.02
16:2n-4	0.36 ± 0.09	0.35 ± 0.03	0.25 ± 0.02	0.23 ± 0.09
17:0	0.25 ± 0.06	0.24 ± 0.01	0.16 ± 0.01	0.17 ± 0.05
16:3n-3	0.14 ± 0.00	0.12 ± 0.00	0.10 ± 0.00	0.11 ± 0.00
16:3n-1	0.10 ± 0.00	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.01
16:4n-3	0.11 ± 0.04	0.12 ± 0.00	0.07 ± 0.01	0.07 ± 0.04
18:0	5.78 ± 0.48	6.16 ± 0.22	4.90 ± 0.15	5.48 ± 0.66
18:1n-9	20.88 ± 2.43	20.85 ± 0.12	24.05 ± 0.57	24.43 ± 2.21
18:1n-7	4.09 ± 0.29	3.26 ± 0.17	3.04 ± 0.07	3.41 ± 0.48
18:1n-5	0.22 ± 0.00 [a]	0.20 ± 0.01 [ab]	0.17 ± 0.00 [b]	0.19 ± 0.01 [ab]
18:2n-9	0.15 ± 0.03	0.08 ± 0.01	0.09 ± 0.01	0.16 ± 0.02
18:2n-6	6.82 ± 0.45 [c]	9.02 ± 0.56 [ab]	10.39 ± 0.01	8.66 ± 0.21 [b]
18:3n-6	0.21 ± 0.01 [a]	0.17 ± 0.01 [b]	0.16 ± 0.01 [b]	0.19 ± 0.01 [ab]
18:3n-3	1.02 ± 0.06 [c]	4.67 ± 0.21 [b]	6.54 ± 0.40 [a]	4.07 ± 0.44 [b]
18:4n-3	0.89 ± 0.25	0.92 ± 0.03	0.65 ± 0.04	0.64 ± 0.25
20:0	0.15 ± 0.00	0.20 ± 0.02	0.18 ± 0.01	0.15 ± 0.01
20:1n-9	0.28 ± 0.04	0.28 ± 0.01	0.28 ± 0.01	0.26 ± 0.04
20:1n-7	1.30 ± 0.23	1.50 ± 0.05	1.39 ± 0.05	1.19 ± 0.27
20:1n-5	0.17 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.13 ± 0.02
20:2n-9	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.06 ± 0.01
20:2n-6	0.26 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.24 ± 0.02
20:3n-9	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
20:4n-6	1.33 ± 0.01 [a]	1.16 ± 0.08 [ab]	0.93 ± 0.01 [c]	1.07 ± 0.07 [bc]
20:3n-3	0.11 ± 0.01 [b]	0.13 ± 0.01 [b]	0.15 ± 0.00 [a]	0.14 ± 0.01 [ab]
20:4n-3	0.69 ± 0.07 [a]	0.56 ± 0.01 [ab]	0.50 ± 0.01 [b]	0.56 ± 0.04 [ab]
20:5n-3 (EPA)	6.35 ± 1.10	6.22 ± 0.07	4.86 ± 0.11	4.98 ± 0.97
22:1n-11	0.38 ± 0.11	0.46 ± 0.04	0.39 ± 0.01	0.31 ± 0.14
22:1n-9	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
22:4n-6	0.10 ± 0.02	0.10 ± 0.04	0.07 ± 0.01	0.11 ± 0.07
22:5n-6	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
22:5n-3	2.23 ± 0.01 [a]	1.60 ± 0.07 [b]	1.70 ± 0.03 [b]	1.85 ± 0.23 [ab]
22:6n-3 (DHA)	16.88 ± 0.84	14.47 ± 0.35	12.43 ± 0.04	15.05 ± 0.28
Total saturates	27.82 ± 0.00	27.32 ± 0.72	26.05 ± 0.02	26.33 ± 0.61
Total monoenoic	33.13 ± 2.00	31.66 ± 0.25	33.82 ± 0.52	34.37 ± 2.06
Total n-3	28.40 ± 2.36	28.80 ± 0.14	26.98 ± 0.54	27.46 ± 1.80
Total n-6	8.94 ± 0.40 [b]	10.84 ± 0.61	11.97 ± 0.01	10.48 ± 0.36
Total n-9	21.50 ± 2.40	21.38 ± 0.13	24.56 ± 0.58	24.99 ± 2.16
Total n-3 HUFA	26.24 ± 2.01	22.97 ± 0.37	19.63 ± 0.07	22.57 ± 1.06
EPA/ARA	2.69 ± 0.34	2.33 ± 0.08	2.56 ± 0.06	3.08 ± 0.54
DHA/EPA	12.79 ± 0.71	12.57 ± 1.12	13.38 ± 0.13	14.08 ± 1.20
DHA/ARA	4.81 ± 0.86	5.39 ± 0.28	5.23 ± 0.08	4.68 ± 1.22
EPA+DHA	23.22 ± 1.94	20.69 ± 0.29	17.29 ± 0.09	20.02 ± 1.24
n-3/n-6	3.19 ± 0.41	2.66 ± 0.16	2.26 ± 0.05	2.63 ± 0.26
18:1n-9/n-3 HUFA	0.80 ± 0.16	0.91 ± 0.01	1.23 ± 0.04	1.09 ± 0.15

Sub-task 9.3.2 (IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez)

With the aim to mimic the lipid and carotenoid profiles of eggs released by cultured females to those of their wild counterparts, experimental diets with optimized EFA and carotenoid contents have been designed. These diets will be tested in groups of amberjack broodstock for three months prior to the spawning. Fecundity, egg quality and haematological and biochemical indicators of fish health will be studied (IEO). Sperm, eggs and larvae will be also analysed for lipid contents and lipid classes, EFA and carotenoids profiles (ULL).

Background

In previous studies conducted by our research group (Rodríguez-Barreto et al., 2012, 2014), it has been shown that cultured females displayed lower proportions of arachidonic acid (20:4n-6, ARA) and higher proportions of linoleic acid (18:2n-6) and eicosapentaenoic acid (20:5n-3, EPA) than wild specimens for all tissues. Based on these results, an experimental diet was formulated and tested (Rodríguez-Barreto et al., 2014). When greater amberjack broodstock born in captivity were fed on this experimental diet, the lipid and fatty acid composition of the muscle, liver and ovary of these specimens approached that of the wild fish, although some imbalances in certain fatty acids were still observed.

Results from Deliverable 3.3 ("Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating and apoptotic germ cells, vitellogenin accumulation, yolk content in the oocytes and nutritional status") have shown that the proportions of total polar lipids, and specific lipid classes and EFA proportions particularly differed among wild and captive-reared fish gonads, with the latter displaying clearly lower contents of specific phospholipids, essential fatty acids (ARA and DHA) and DHA/EPA and ARA/EPA ratios, all crucial factors for reproductive success and sperm and egg and larval quality (Zupa et al., 2017). These differences are particularly evident at early and advanced gametogenesis periods, with testes and ovaries of captive fish displaying around 30-40% less DHA and ARA, and clearly higher contents of 18:2n-6. As a consequence, DHA/EPA and ARA/EPA ratios, also suffer marked decrements in the gonads of the captive fish. These nutritional differences are presumably the result exclusively of differences in the diet between wild and captive fish.

Formulation of diets

Taking into account these previous results, the aim was to approach the lipid and carotenoid profiles of eggs released by cultured females to their wild counterparts. To this end, three treatments with optimized EFA and carotenoid contents have been designed and will be tested in 2018:

- A control diet based exclusively on local frozen mackerel (*Scomber scombrus*) will be used as reference due to the optimal spawning results obtained in wild broodstock so far, compared to formulated diets. In order to supplement mackerel with a vitamin mix, 500ppm (500mg kg^{-1}) of vitamin C, 200 ppm of vitamin E and 50 ppm of astaxanthin for each kg of raw fish in dry matter will be included in this dietary regime.
- A commercial diet provided by Skretting.
- A new experimental diet to resemble the above mentioned requirements, accordingly to the lipid composition of wild amberjack gonads and eggs.

  According to a recent meeting with Ramón Fontanilla at Dubrovnik AE2017 Congress, Skretting España S.A. is already working in the manufacture of feeds with these characteristics:

  - Total lipid content 16%
  - Mainly marine ingredients (protein and lipids) in order to avoid or decrease as much as possible the linoleic acid. Fish meal contributes with 40% of the fat to the diet and the rest is fish oil.
  - Some lecithin is included (probably soybean lecithin since the required marine lecithin LC60 is not viable for these quantities according to our recent meeting).

- EPA and DHA contents: 6-7% EPA, 25% DHA. DHA level has been increased by adding algae meal.
- ARA content around 2% of total fatty acids or 3 g kg^{-1} feed. (EPA/ARA ratio 2-2.5/1)
- Carotenoids: Astaxanthin and other HUFA antioxidants

The expected fatty acid composition of the three diets is shown in **Table 9.3.7**.

Table 9.3.7. Expected main fatty acid composition (% of total fatty acids) of the experimental diets

	Control diet	Commercial Diet	Experimental diet
18:1n-9	12.2	21.9	
18:2n-6	2.1	7.7	
20:4n-6	2.8	1.0	2
20:5n-3	5.9	10.2	6-7
22:6n-3	25.3	11.0	25
DHA/EPA	4.3	1.1	3.6-4.2
EPA/AA	2.1	0.1	2-2.5

.

This diet design process shall take into account physical characteristics of diets: size particle, palatability (adequate feed intake), and buoyancy (integrity). The estimated need for feed per experimental treatment assay will be:

- Broodstock biomass about 140 kg per treatment
- Fish fed three times a week
- Estimated daily ration: 1.0 -1.5 % body weight day-1
- Duration of trial: 6 – 8 months
- Weekly feed: 4.2 - 6.3 kg
- Monthly feed: 16.8 – 25.2 kg
- Annual feed: 201.6 – 302.4 kg

Formation of experimental broodstock groups of Seriola dumerili

There is currently a new stock of *S. dumerili* broodstock available for the implementation of this task at the Culture Unit of Canary Island Oceanographic Centre – Spanish Institute of Oceanography (IEO). The fish, with an average weight of 10-12 Kg, are between 4 and 5 years old, more than the age considered to be of first maturity for this species.

These specimens are actually stocked in raceway 500 m3 outdoor tanks, with light intensity muted with tank covers, under natural conditions of water temperature and photoperiod. Water flow rate into the tanks is set to 1000 l min^{-1} from 5 inlet pipes, allowing a complete water renewal every 8 hours. Feeding consist in low commercial value fish (mackerel, *Scomber scombrus*) supplied once a day and three days a week in a quantity adjusted to 1% of their biomass per day.

The stock of 42 *S. dumerili* broodstock will be divided into 3 groups of 14 fish, which will be fed on three different experimental diets.

- Group A: fed on control diet (frozen mackerel + vits. premix)
- Group B: fed on commercial diet
- Group C: fed on experimental diet

Each group will be placed in a raceway 500 m^3 outdoor tank with tank covers, supplied with a water flow rate of 1000 l min^{-1}, under natural conditions of water temperature and photoperiod. Tanks are provided with an overflow egg collector.

The formation of the broodstock groups will be held 3 months before the beginning of each spawning season (January). From the time of the formation of groups until the end of the spawning season, broodstock groups will be fed with the experimental diets. Food will be supplied once a day and three days a week, in a quantity adjusted to 1% of the biomass per day.

The trial will start in January 2018. Fecundity, egg and sperm quality and haematological and biochemical indicators of fish health will be studied (IEO). Diets, sperm, eggs and larvae samples will be analyzed for lipid contents and lipid classes, EFA and carotenoids profiles (ULL).

Deviations from Annex I and their impact:

Project Coordinator comment: Although no deviation has been reported by the WP leader, I see in the report submitted that subtask 9.2.2 (lead beneficiary P28. CANEXMAR) has not be initiated yet, based on the claim that this subtask depends on the data obtained from the previous subtask (9.1.1, lead beneficiary P1. HCMR). However, the latter task has been completed already and the corresponding deliverable "*D9.2 Lysine requirements of greater amberjack juveniles*" has been submitted to the EU on month 39 (20 February 2017). So, nothing is missing based on the DOW to implent Subtask 9.2.2. As this task requires a full grow out period (12-24 months) to be implemented and collect the samples, and then some more time to analyze fillet quality, it seems questionable if deliverable "*D9.3. Performance of grow-out diets for greater amberjack developed in order to maximize growth potential*" will be submitted at the end of the project.

WP 10 Nutrition – pikeperch

WP No:	10	**WP Lead beneficiary:**		P21. DTU
WP Title (from DOW):	Nutrition – pikeperch			
Other beneficiaries (from DOW):	P2. FCPCT	P15. ULL	P16. FUNDP	P.39 F2B
Lead Scientist preparing the Report (WP leader):	Ivar Lund			
Other Scientists participating:	Marisol Izquierdo (P2); David D. Montesdeoca (P2); Covadonga Rodriguez (P15); Jose A. Perez (P15); Patrick Kestemont (P16); Najlae El Kertaoui (P16); Manuel Gesto (P21); Peter Skov (P21) Jiri Bossuyt (P39)			

Objectives

1. Increase knowledge on the effect of nutrients essential for first feeding of pikeperch.
2. Develop specific enrichment products and formulated diets to improve pikeperch larval performance.

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the first 12 months the partners in WP10 exchanged ideas and designed studies to be carried out in the project period. During the first year the experimental feed types were formulated and prepared and 1 study was planned on pikeperch larvae starting by month 12, involving the effect of phospholipid levels and levels of single HUFAs in formulated diets. However due to high cannibalism and subsequent mortality, the study was repeated from month 14 and is included in the present report.

Summary of work reported in the previous Reporting Period (13-30 Mo):

Several studies have been performed to increase our knowledge on how essential nutrients are important for first feeding pikeperch larvae. Within the period there has been some delay due to some technical failures (1 experiment) or high cannibalism of larvae (2 experiments) meaning that three trials had to be repeated, this means that some analytical work was delayed, but is expected included in the deliverables in month 36. In **Task 10.1**, trials have shown that pikeperch larvae require both high dietary inclusion levels of phospholipids and HUFAs to perform optimally. A multifactorial screening trial of importance of 8 dietary factors (high or low levels) has been initiated at the end of the 2nd Project Reporting period and is still ongoing. In **Task 10.2**, adding saline water to rearing conditions does not improve growth, but can change the ability of pikeperch larvae to elongate and desaturate different fatty acids (FA) and phospholipids. An experiment investigating the consequence of various phospholipid levels and LC HUFAs on welfare indicators and stress physiology, behaviour and respiratory metabolism was started at the end of the 2nd Reporting Period and is ongoing.

Summary of progress towards objectives (31-48 Mo):

Several studies have been conducted or initiated. **Task 10.1.** A multifactorial exp. examining the importance of 8 dietary factors started in period 2 was finished. Likewise a nutritional confirmatory HUFA experiment based on results from the multifactorial study was performed in month 42. A second confirmatory exp. was

initiated in month 43, but will need repetition due to mortality and is to be started at the beginning of period 4 (month 50). **Task 10.2**. An experiment (started end of period 2) investigating the consequence of various dietary levels of LC HUFAs on welfare indicators and stress physiology, behavior and respiratory metabolism was completed.

Remaining analytical work that was delayed in period 2 has been performed, while some analytical work of the confirmatory exp. is still under analysis.

The completion of deliverable 10.1 (due month 36) is awaiting the final experiment to be performed in month 50 - and subsequent analytical work.

The completion of deliverable 10.2. (due month 36) is expected this month (month 48)

Deliverable 10.3 (due month 48) is awaiting results from ongoing exp.to be started month 50.

Details for each Task

Task 10.1 Effect of selected dietary nutrients on pikeperch larval development and performance (led by DTU, Ivar Lund).

- Exp. 1

A multifactorial screening experiment for investigating the effects of dietary fatty acids, vitamins and minerals on early development of pike-perch (*Sander lucioperca*).

DTU (21): Ivar Lund, FUNDP(16): Najlae El Kertaoui, Patrick Kestemont, S.N.M. Mandiki, FCPCT(2): David Montesdeoca, Mariol Izquierdo

Objectives:

The study intended to determine optimal levels for major essential nutrients; LC-PUFAs, vitamins (Vit A, E, C, D) and minerals (Ca/P) during the early weaning to dry feeds by studying the impact of these nutrients at different larval organismic levels by anatomo-histological, biochemical and molecular biomarkers. The specific objectives of the studies were:

a) Investigate the dietary effects of LC-PUFAs and their interactions with vitamins and minerals on larval development and performance, digestive capacity, skeleton malformation, bone mineralization, and molecular markers of oxidative status and bone development.
b) Determine the combined effect of n-3 and n-6 LC-PUFAs (DHA and ARA) and n-3/n-6 ratio and its influence on larval development, digestive enzymes activity, deformity occurrence and lipid metabolism.
c) To assess the effect of the dietary Ca/P taken into account P and Ca effect especially its implication in bone mineralization.

Experimental design

Sixteen isonitrogenous and isolipidic diets containing different levels of Ca/P, EPA+DHA, ARA, Se, vitamin A, C, D and E were formulated and fabricated by SPAROS as cold extruded feed pellets of 200-400 µm and 400-700 µm. The experimental diets were tested in factor-modality design (**Table 10.1.1**, which represented a unique variant nutrient combination. No replicates were allotted in this experiment (1 tank per treatment). The experiment was repeated at the facilities of UNamur, because of a high cannibalism related mortality observed in the first trial carried when out at DTU Aqua in Denmark.

Diets and feeding

Experimental diets were formulated (**Table 10.1.2**) using a mix of oil as source EPA, DHA and ARA to reach the required fatty acids content and to equalize the lipid content in each diet. Lutavit E, Lutavit C, Rovimix A and Rovimix D3 were used as vitamin source of vitamin C, E, A and D Vitamin E, C, A and D respectively. Selplex-Se yeast was used as a source of Se, while Ca/P levels were obtained by changing the P

levels in diets using NaH_2PO_4 as source of P (**Table 10.1.2**). The analysed proximate content of the main important nutrients and vitamins/minerals is shown in **Table 10.1.2.** The Proximate composition and the analytical content of FA are shown, respectively, in **Tables 10.1.3** and **10.1.4.**

Table 10.1.1: Experimental factors-modalities (Diet = experimental conditions)

Exp. diets (n°)	Ca/ P	EPA+DHA %	ARA %	Vitamin E mg/kg	Vitamin D IU/kg	Vitamin C mg/kg	Vitamin A IU/kg	Se mg/kg
1	0.6	1.25	0.8	1000	2800	2000	8000	3
2	1.2	1.25	0.8	1000	28000	3600	8000	12
3	0.6	3.5	0.8	1000	2800	3600	30000	12
4	1.2	3.5	0.8	1000	28000	2000	30000	3
5	0.6	1.25	1.6	1000	28000	2000	30000	12
6	1.2	1.25	1.6	1000	2800	3600	30000	3
7	0.6	3.5	1.6	1000	28000	3600	8000	3
8	1.2	3.5	1.6	1000	2800	2000	8000	12
9	0.6	1.25	0.8	3000	28000	3600	30000	3
10	1.2	1.25	0.8	3000	2800	2000	30000	12
11	0.6	3.5	0.8	3000	28000	2000	8000	12
12	1.2	3.5	0.8	3000	2800	3600	8000	3
13	0.6	1.25	1.6	3000	2800	3600	8000	12
14	1.2	1.25	1.6	3000	28000	2000	8000	3
15	0.6	3.5	1.6	3000	2800	2000	30000	3
16	1.2	3.5	1.6	3000	28000	3600	30000	12

Rearing conditions

A stock of pikeperch larvae (3 dph) were obtained from a spawning broodstock from Viskweekcentrum Valkenswaard located in Leende, Nederland. First, an initial larval rearing was carried out in 3 tanks (500l) from 3 dph until weaning period, Larvae were fed *Artemia nauplii* enriched with DHA Protein Selco® (INVE, Dendermonde, Belgium) each hour (from 8:00 am to 6:00 pm) until they reached 17 days old, followed by a co-feeding period from 18 to 24 dph using *Artemia* nauplii and mixture of the 16 diets (200-400 µm pellets). The multifactorial experiment started with completely weaned larvae in order to avoid any additional stress. Then 25 dph larvae (9.44±4.42 mg) were randomly distributed in 16 experimental tanks respecting a density of 770 larvae tank-1 and fed one of the experimental diets for 14 days. All tanks (90 L aquarium) were supplied with filtered water at an increasing rate of 8% h^{-1} to ensure water renewal and maintain high water quality during the trial. Water was continuously aerated. Temperature and oxygen were daily measured; average water temperature along the trial was 20 °C and water dissolved O2 reach 7.8±0.32 mg. Photoperiod was kept at 12h light: 12h dark. Tanks were daily manually cleaned between 03:00 pm and 06:00 pm with a hose by a siphon system. During the experiment a daily mortality counting and a regular check out by measuring nitrite and nitrate concentrations were respected.

Table 10.1.2: Formulation of the experimental diets. *dietary content per g/100g, **vitamin A&D presented per IU/kg, †vitamin C&E presented per mg/kg

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
Ingredients (%)																
MicroNorse	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
CPSP 90	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Squid meal	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0	17.0
Krill meal (Aker Biomarine)	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Fish gelatin	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Wheat Gluten	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Potato starch gelatinised (Pregeflo)	10.45	12.95	9.15	13.37	8.95	13.27	9.49	13.85	8.68	13.04	9.14	13.46	9.04	13.26	9.58	12.08
Fish oil - SAVINOR	1.20	1.20	0.80	0.80	1.15	1.15	0.00	0.00	1.20	1.20	0.80	0.80	1.15	1.15	0.00	0.00
Incromega DHA 500TG	0.00	0.00	3.40	3.40	0.00	0.00	3.58	3.58	0.00	0.00	3.40	3.40	0.00	0.00	3.58	3.58
VEVODAR	2.10	2.10	2.10	2.10	4.25	4.25	4.25	4.25	2.10	2.10	2.10	2.10	4.25	4.25	4.25	4.25
Soybean oil	3.00	3.00	0.00	0.00	1.05	1.05	0.00	0.00	3.00	3.00	0.00	0.00	1.05	1.05	0.00	0.00
Vit & Min Premix PV02	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Lutavit C35	0.58	1.02	1.02	0.58	0.58	1.02	1.02	0.58	1.02	0.58	0.58	1.02	1.02	0.58	0.58	1.02
Lutavit E50	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Rovimix A (5000000 IU/kg)	0.00	0.00	0.44	0.44	0.44	0.44	0.00	0.00	0.44	0.44	0.00	0.00	0.00	0.00	0.44	0.44
Rovimix D3 (5000000 IU/kg)	0.015	0.51	0.015	0.51	0.51	0.015	0.51	0.015	0.51	0.015	0.51	0.015	0.015	0.51	0.015	0.51
Brewer's yeast	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Soy lecithin - Powder	6.00	6.00	6.00	6.00	6.00	6.00	4.50	4.50	6.00	6.00	6.00	6.00	6.00	6.00	4.50	4.50
Binder (sodium alginate)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
NaH2PO4	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35	4.20	0.35
SelPlex - Se yeast	0.05	0.47	0.47	0.05	0.47	0.05	0.05	0.47	0.05	0.47	0.47	0.05	0.47	0.05	0.05	0.47
L-Taurine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Table 10.1.3: Proximate composition of the experimental diets. *dietary content per g/100g, **vitamin A&D presented per IU/kg, †vitamin C&E presented per mg/kg

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
Proximate composition																
Moisture*	6.40	6.20	6.20	6.30	6.50	6.30	6.30	6.50	6.40	6.40	6.30	6.50	6.60	6.40	6.30	6.50
Crude protein*	51.1	51.3	51.5	51.1	51.3	51.1	51.1	51.2	51.0	51.3	51.3	51.0	51.2	51.1	51.1	51.2
Crude fat*	17.4	17.5	17.5	17.6	17.6	17.7	17.7	17.5	17.5	17.5	17.6	17.5	17.6	17.6	17.6	17.5
Crude ash*	7.60	6.60	7.60	6.60	7.50	6.70	7.60	6.60	7.50	6.50	7.60	6.60	7.60	6.60	7.60	6.60
Phosphorus*	1.97	1.06	1.99	1.07	1.96	1.07	2.01	1.05	1.98	1.06	2.01	1.07	1.95	1.06	2.03	1.05
Calcium*	1.24	1.26	1.25	1.24	1.25	1.24	1.25	1.24	1.24	1.24	1.26	1.23	1.23	1.24	1.24	1.24
Selenium*	4.00	14	14	4	15	4	4	14	5.0	14	14	4	14	4	4	14
Vitamin A**	7794	7775	28933	28896	28866	28917	7825	7794	28877	28891	7816	7777	7800	7814	28891	28843
Vitamin C†	1944	3428	3415	1952	1941	3409	3418	1948	3417	1933	1947	3416	3401	1946	1956	3417
Vitamin D3†	2893	28544	2835	28707	28006	2794	28430	2829	28397	2881	28208	2884	2831	28777	2848	28805
Vitamin E**	997	1006	992	1004	997	1001	1006	994	2952	2947	2956	2946	2938	2871	2967	2897

Table 10.1.4: Mean analysed TFA content (±SEM) (mg g-1 d.w.) and FA composition (% of TFA) of the 16 experimental feed types

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
FA																
16:0	13.4	13.2	12.2	12.80	13.30	13.10	10.40	10.70	13.50	13.50	12.00	12.20	13.30	13.50	10.60	10.30
18:0	4.10	4.10	3.50	3.60	4.20	4.20	3.90	3.90	4.10	4.10	3.50	3.40	4.20	4.30	3.90	4.00
Total SFA	27.0	27.10	25.80	26.6	30.10	29.9	26.0	26.50	27.30	27.10	26.10	25.90	30.00	30.60	26.20	26.10
16:1 (n-7)	2.60	2.70	2.70	2.70	2.70	2.60	2.10	2.20	2.60	2.60	2.60	2.60	2.60	2.70	2.10	2.10
18:1 (n-9)	15.50	15.50	11.80	11.90	12.90	12.70	10.10	10.30	15.40	15.40	11.50	11.50	12.90	12.90	10.10	9.90
Total MUFAs	24.35	23.65	21.85	21.40	21.40	21.10	18.30	18.60	23.70	23.90	21.00	21.50	21.00	21.20	18.10	18.00
18:2 (n-6)	16.50	15.70	12.80	13.5	14.30	14.10	10.60	10.60	16.50	16.40	12.50	12.10	14.60	14.30	11.00	10.30
20:4 (n-6) ARA	9.00	9.40	10.30	10.0	17.90	18.20	17.80	17.70	9.20	9.10	11.30	10.30	17.70	18.00	17.40	18.10
Total (n-6) PUFA	26.40	27.00	25.20	26.00	35.40	35.50	31.90	31.50	27.50	27.50	26.20	24.60	35.40	35.50	31.90	31.70
18:3 (n-3)	14.00	14.00	1.40	1.40	5.80	5.60	1.10	1.10	13.70	14.0	1.40	1.30	5.70	5.80	1.10	1.00
20:3 (n-3)	0.80	0.10	0.20	0.30	0.10	0.20	0.30	0.30	0.20	0.20	0.30	0.30	0.20	0.20	0.20	0.20
20:5 (n-3) EPA	3.90	3.90	6.50	6.30	3.90	3.90	5.40	5.40	3.90	3.90	6.20	6.60	3.90	3.50	5.30	5.30
22:6 (n-3) DHA	4.80	4.90	19.80	18.80	4.80	5.00	17.70	17.30	4.90	4.70	19.20	19.90	4.80	4.60	17.40	17.90
Total (n-3) HUFA	9.85	9.25	27.55	26.45	9.15	9.45	24.25	23.85	9.35	9.15	26.75	28.05	9.25	8.65	23.75	24.35
DHA/EPA	1.23	1.26	3.05	2.98	1.23	1.28	3.28	3.20	1.26	1.21	3.10	3.02	1.23	1.31	3.28	3.38
DHA/ARA	0.53	0.52	1.92	1.88	0.27	0.27	0.99	0.98	0.53	0.52	1.70	1.93	0.27	0.26	1.00	0.99
EPA/ARA	0.43	0.41	0.63	0.63	0.22	0.21	0.30	0.31	0.42	0.43	0.55	0.64	0.22	0.19	0.30	0.29
Oleic/DHA	3.23	3.16	0.60	0.63	2.69	2.54	0.57	0.60	3.14	3.28	0.60	0.58	2.69	2.80	0.58	0.55
(n-3)/(n-6)	0.93	0.91	1.19	1.11	0.44	0.44	0.82	0.81	0.87	0.87	1.11	1.23	0.44	0.43	0.80	0.82

Samplings and analyses

Several parameters were evaluated including husbandry variables, biochemical assays, digestive enzymatic activities, skeleton anomalies and related gene expression.

Final survival was calculated by individually counting all the living larvae at the end of the experiment. Survival was calculated as the percentage between final and initial number of fish. Mortality did not consider missing larvae due to type II cannibalism (i.e. fish completely ingested, usually head first, by a cannibal), while the mortality due to cannibalism was thus estimated by adding missing larvae to dead larvae due to cannibalism, partly consumed by their conspecifics (N_c+N_m).

Growth was monitored by sampling 40 larvae per tank at the beginning and the end of the experiment. At the end of the experiment the weighed larvae were fixed in 10% buffered formalin and kept until analysis to determine the rate of malformations. The number of sampled larvae was taken into account for survival calculation. Staining procedures with alizarin red were conducted to evaluate the skeletal anomalies following a modified method from previous studies (Izquierdo et al., 2013).

Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Data obtained were normalised and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA).

Statistics

Statistical analyses were done following appropriate methods to a fractional factorial design as developed by Kobilinski (2000) and Gardeur *et al.* (2007). Calculations were done using the Planor-Analys software developed by Kobilinski (2000). All the significant effects were tested with the Statistica™ software for windows (Stat Soft, USA) with one- or two-ways ANOVA. When significant ($p < 0.05$), means were compared according to the Tukey post hoc test.

RESULTS AND DISCUSSION

Husbandry and skeletal deformities

At the end of the experiment, the highest survival was recorded in larvae fed high Ca/P (**Fig.10.1.1, A**), but final weight and specific growth rate (SGR) were significantly lower in larvae fed high Ca/P, associated with a higher incidence of kyphosis and pectoral deformities in these larvae (**Fig.10.1.1, B**).

The higher kyphosis and pectoral element deformities were recorded in larvae fed high Ca/P levels (**Fig.10.1.2, A and B**). In these sense, the growth was probably reduced, in these larvae, due to the higher incidence of bone deformities, since skeletal deformation may affect various ecophysiological performances of fish larvae such as swimming behavior and feeding efficiency. Indeed, high incidence of kyphosis is often reported as typical consequences of P deficiency in fish larvae (Fjelldal et al., 2016).

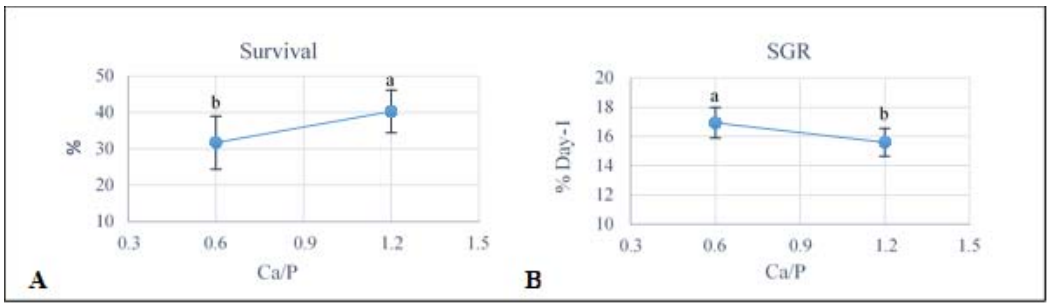

Figure 10.1.1: Effect of dietary Ca/P on Husbandry variables: Survival (A); Survival (B). Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (n=8).

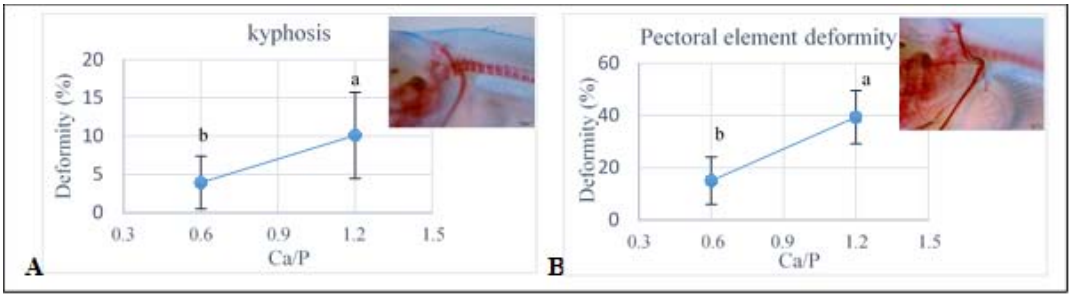

Figure 2: Effect of dietary Ca/P on larval deformities: Kyphosis (A) and Pectoral element (B). Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (n=8).

Results showed that the increase in EPA+DHA reduced jaws deformities in larvae fed 3600 mg Vit. C, (**Fig.10.1.3, B**), a high Vit. C dietary content seemed to be efficient in reducing the incidence of deformities when high levels of n-3 HUFA (especially DHA) were included in the diets pointing the antioxidant function of this vitamin (Betancor et al., 2012, Izquierdo et al., 2012). In addition, a possible explanation may be related to dietary effect on the ossification of cartilaginous- origin bone process, since vitamin C affects collagen synthesis in structural organs such as cartilage and bone (Padayatty et al., 2003).

In this study, high vitamin C associated with high EPA+DHA level resulted in higher cannibalism rate, mainly because of a decrease in incidence of jaws deformities in these larvae (**Fig.10.1.3, A**).

The increase of EPA+DHA seemed to reduce the prevalence of scoliosis in larvae fed 0.8% ARA (Fig. 3,C), while the high levels of ARA had the opposite effect. Beside scoliosis, high levels of EPA+DHA and ARA increased lordosis, while the decrease in EPA+DHA with the high ARA level reduced skeletal deformities (**Fig. 10.1.3, C** and **D**). In this regard, a possible explanation can be related to EPA+DHA/ARA, suggesting the importance of a balanced n-3 HUFA/n-6 HUFA ratio in this species.

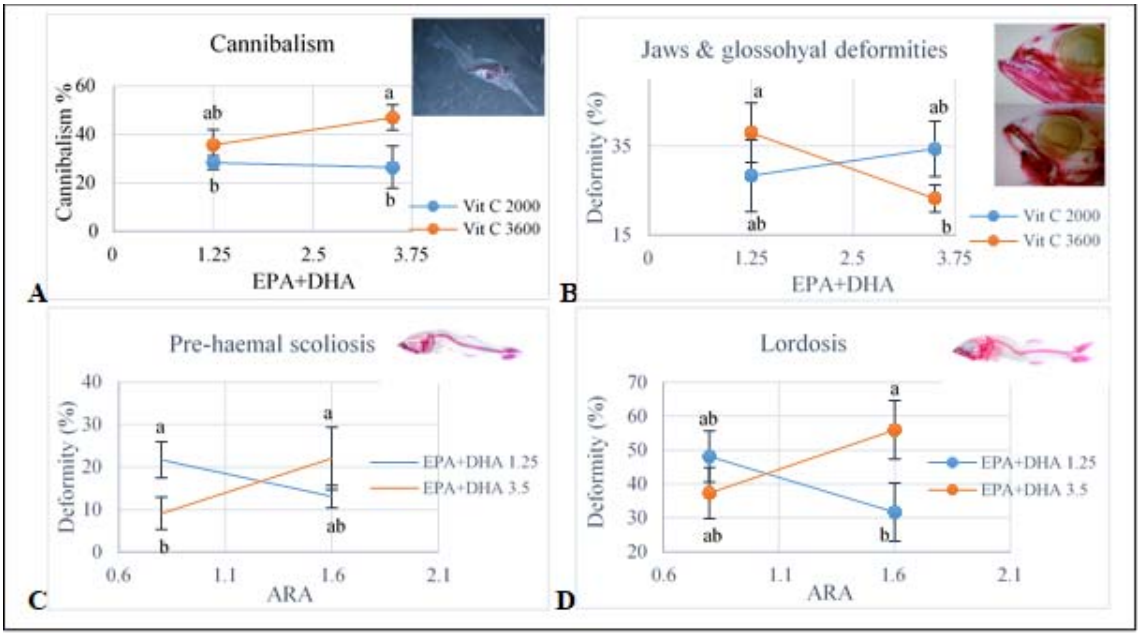

Figure 10.1.3: Effect of fatty acids and vitamin C on Larval deformities and cannibalism: A EPA+DHA and vitamin C interaction effect on cannibalism rate; B EPA+DHA and vitamin C interaction effect on jaws deformities, C EPA+DHA and ARA interaction effect on pre-hemal scoliosis; D EPA+DHA and ARA interaction effect on lordosis. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (n=4).

Activity of digestive enzymes

A differential expression pattern in the ontogenetic development of digestive enzyme system was observed depending on the dietary content. Our results showed that a high dietary n-3 HUFA content enhanced trypsin activity in larvae fed a low Ca/P level (**Fig. 10.1.4, B**). Previous studies showed that n-3 HUFA are potent stimulators of cholecystokinine (CCK) secretion (Little et al., 2007); thus EPA+DHA effect may reflect the endocrine modulation of the pancreatic digestive function which is regulated by CCK (Saleh et al., 2013, Zhao et al., 2013, Kamaszewsk et al., 2014). In fact, trypsin is secreted as a trypsinogen activated by an enterokinase requiring Ca ions. Consistently, the better utilization of Ca under specific condition of HUFA and Ca/P should be further investigated. Moreover, the opposite interaction found between EPA+DHA and Ca/P could be linked to the effect of Ca/P on growth. A higher aminopeptidase activity was also observed in larvae fed low Ca/P (**Fig. 10.1.4, A**), this result may be related to the growth improvement obtained in these larvae, denoting a higher maturation status of the gut (Zambonino Infante and Cahu, 2001; Zambonino-Infante et al., 2008).

Pepsin and the intestinal enzymes (leucine alanine, aminopeptidase and alkaline phosphatase) activities were negatively correlated with ARA levels (**Fig. 10.1.4, C, D, E** and **F**). High ARA level in the diet may delay enterocyte maturation (Yuan et al., 2015), thus pointing to its potential involvement in regulating the development of the digestive tract.

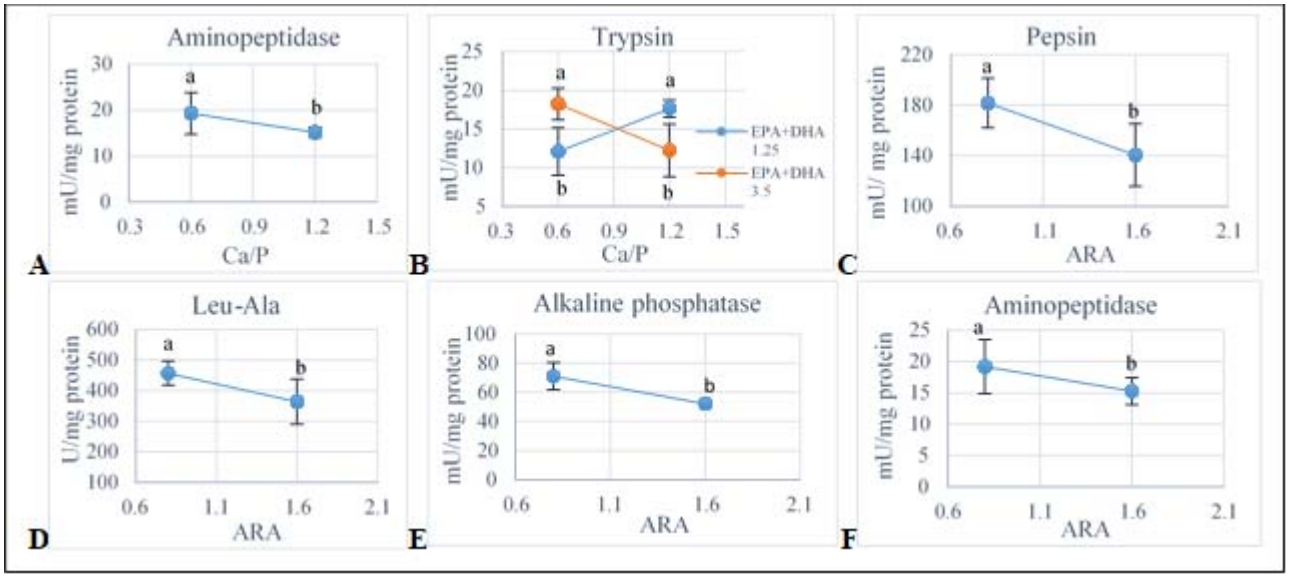

Figure 10.1.4: Larval enzymatic activity (aminopeptidase, trypsin, pepsin, leucine alanine and alkaline phosphatase) of 39 dph pikeperch larvae fed the different experimental diets. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect : n=8; interaction effect : n=4).

Fatty acids larval content

In terms of fatty acid body content, DHA, EPA and the total n-3 HUFA were significantly higher in the group of larvae fed the high n-3 HUFA treatment (3.5%) as consequence of the abundance of these fatty acids in diets indicating that the fatty acid levels in the diet affect its content in larval tissues. Previous studies also showed that the fatty acid composition reflect the composition of fatty acids in the diet (Montero et al., 1996; 2003; 2005, 2008; Izquierdo et al., 2000; 2003, 2005)

On the other hand, the increase in dietary ARA seemed to reduce EPA content in larval tissues (**Fig. 10.1.5, A**); this result might indicate a selective deposition and retention of HUFAs in pikeperch larvae, likely due to the inhibition of EPA incorporation by dietary ARA (Bell et al., 1995). In fact, the relation among dietary EPA and ARA has been proposed to be a critical factor for larval performance due to competition interaction among them (Bell and Sargent, 2003, Izquierdo, 2005).

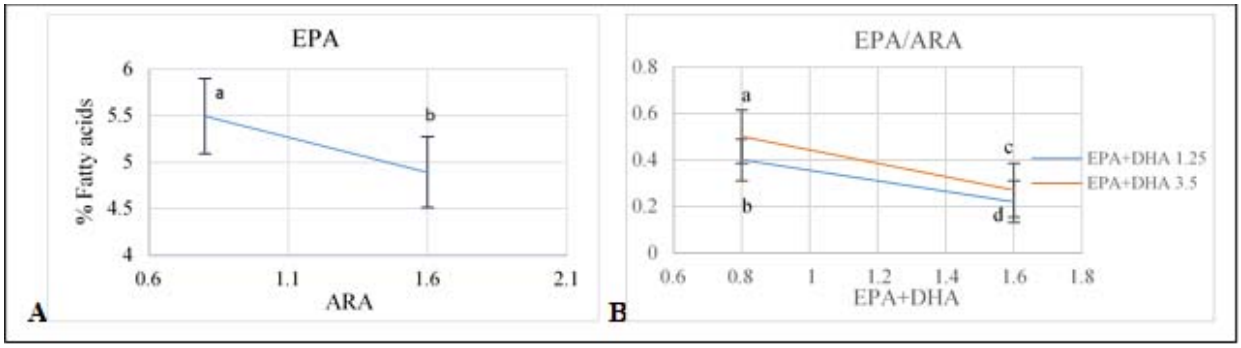

Figure 10.1.5: Larval fatty acid content of 39 dph pikeperch larvae fed the different experimental diets. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect : n=8; interaction effect : n=4).

Molecular study

Gene expression results reflected the decrease of twist expression in larvae fed high EPA+DHA with Se supplement (**Fig. 10.1.6, A**). This result reflects the antioxidant role of Se, since the twist gene antagonizes osteoblast formation, and usually induced at post-proliferative stage of osteoblast differentiation.

Mef2c22 was over expressed in larvae fed high vitamin C levels (**Fig. 10.1.6, B**); this points to the effect of vitamin C on the ossification process, and the collagen synthesis as mentioned above.

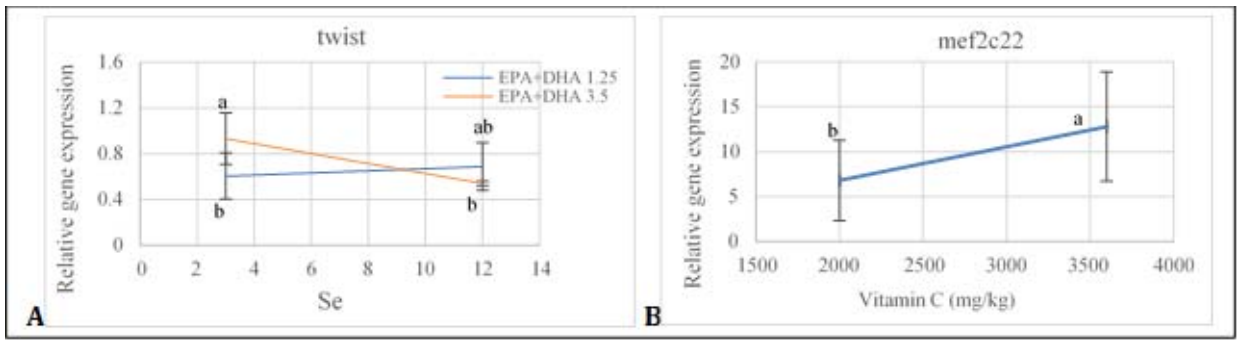

Figure 10.1.6: twist (A) and msf2c2 (B) gene expression mesured in larvae fed the 16 experimental diets. Only graphs with significant effects are shown. Results are expressed as the Mean ± SD (single effect : n=8; interaction effect : n=4).

Conclusion

In light of the results obtained in the multifactorial experiment; Ca/P, fatty acids and their interaction seem to be key nutritional factors influencing pikeperch larval development. However, only two levels of Ca/P and fatty acids were tested in the multifactorial experiment. Therefore two confirmatory experiments testing

gradual levels of the dietary EPA+DHA/ARA and Ca/P ratios were performed based on the multifactorial screening results.

- Exp. 2.

Confirmatory DHA/EPA/ARA experiment. DTU(21): Ivar Lund, FUNDP (16): Najlae El Kertaoui, Patrick Kestemont

This confirmatory experiment investigated the combined effects of graded levels ARA and two DHA dietary levels (low and high) in early weaning diets on larval performance, digestive capacity, biochemical composition, oxidative status, skeletal deformities and bone mineralization of pikeperch larvae. The study was conducted to examine the effect of dietary EPA/DHA/ARA ratios by studying the possible alteration in phospholipids synthesis due to competition among these fatty acids, as well as the possible effect of further elevation of dietary ARA on gut maturation. The study was conducted at DTU Aqua in Denmark.

Methodology

Larvae and rearing conditions

Larvae were obtained from AQUPRI, Denmark as newly hatched. Larvae were until 10 dph reared at 16-17 C and hereafter at 19-20 C. Larvae were fed on non-enriched *Artemia* nauplii (AF and EG strain) (INVE, Dendermond, Belgium) until they reached 14 day post-hatching (dph), followed by a co-feeding period from 15 to 17 dph using *Artemia* nauplii and mixture of the experimental diets. At 18 dph larvae were randomly distributed into 18 experimental tanks (50 L) at a density of 1350 larvae tank^{1} in a flow through system with adjustable light and temperature control. Larvae in each tank were fed with one of six experimental diets tested in triplicates 8 (3 tanks per diet). To assure feed availability, daily feed supplied was maintained at app. 15-20 % of larval wet weight per tank during the first week (200-400 µm/ 400-700 µm) and 10-15 % per tank during the rest of experiment period. Daily feed was administered by automatic feeders from 8 A.M to 6 P.M. and fed approximately every 20-30 min. Feed was fed in surplus and daily bottom of tanks were vacuum cleaned to remove feed waste.

Diet composition

Six diets with two levels of DHA (low and high levels) and 3 levels of EPA/ARA (1, 2 and 4) were tested. Diets were formulated by SPAROS, Portugal (**Table 10.1.5**).

Feeds were analysed for proximate composition of nutrients and fatty acid profiles

Table 10.1.5: Dietary composition of the experimental diets.

As feed basis	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Crude protein, % feed	54.22	54.22	54.22	54.22	54.22	54.22
Crude fat, % feed	20.16	20.16	20.16	20.15	20.15	20.15
Fiber, % feed	0.00	0.00	0.00	0.00	0.00	0.00
Starch, % feed	9.67	9.67	9.67	9.72	9.72	9.72
Ash, % feed	8.98	8.98	8.98	8.98	8.98	8.98
Total P, % feed	1.67	1.67	1.67	1.62	1.62	1.62
Ca, % feed	1.52	1.52	1.52	1.52	1.52	1.52
Ca/P	0.91	0.91	0.91	0.93	0.93	0.93
LNA (C18:2n6), % feed	0.53	0.40	0.33	0.50	0.37	0.30
ALA (C18:3n3), % feed	0.13	0.13	0.13	0.10	0.10	0.10
ARA, % feed	**1.20**	**0.59**	**0.30**	**1.19**	**0.59**	**0.30**
EPA, % feed	**1.19**	**1.19**	**1.19**	**1.22**	**1.22**	**1.22**
DHA, % feed	**0.61**	**0.61**	**0.61**	**2.49**	**2.49**	**2.49**
EPA/ARA	**0.99**	**2.00**	**3.95**	**1.02**	**2.07**	**4.12**
DHA/EPA	**0.52**	**0.52**	**0.52**	**2.04**	**2.05**	**2.05**
Total phospholipids, % feed	7.76	7.76	7.76	6.22	6.22	6.22

Samplings and analyses

Final survival was calculated at 40 dph by individually counting all the living larvae at the end of the experiment. The growth was determined by measuring body weight of 30 fish tank^{-1} at the beginning, the middle and at the end of the trial. To evaluate the level of maturation of the digestive system, larvae (20-50 larvae) were sampled for measuring gastric (pepsin), pancreatic (trypsin) and brush border intestinal enzymes (alkaline phosphatase AP, and aminopeptidase) according to the literature (Zambonino and Cahu, 1999).

Larvae were analysed for body proximate composition and fatty acid profiles (DTU). This study includes bone ossification and skeleton morphogenesis analyses using quantitative PCR for gene expression (still under analysis), with the possibility to add quantitative PCR for gene involved in oxidative stress.

Results and discussion

No significant differences were recorded in growth between the different groups of larvae during the trial (**Fig.10.1.7A, B** and **C**), while at the end of the experiment fish fed diet 4, exhibited the lowest growth performance compared to the larvae fed diet 2.

Survival was slightly higher, but not significant, in larvae fed diet 4 (**Fig. 10.1.7, D**) which may have an interaction with the growth recorded in these larvae. Mortality registered was affected by the high cannibalistic behavior of pikeperch larvae.

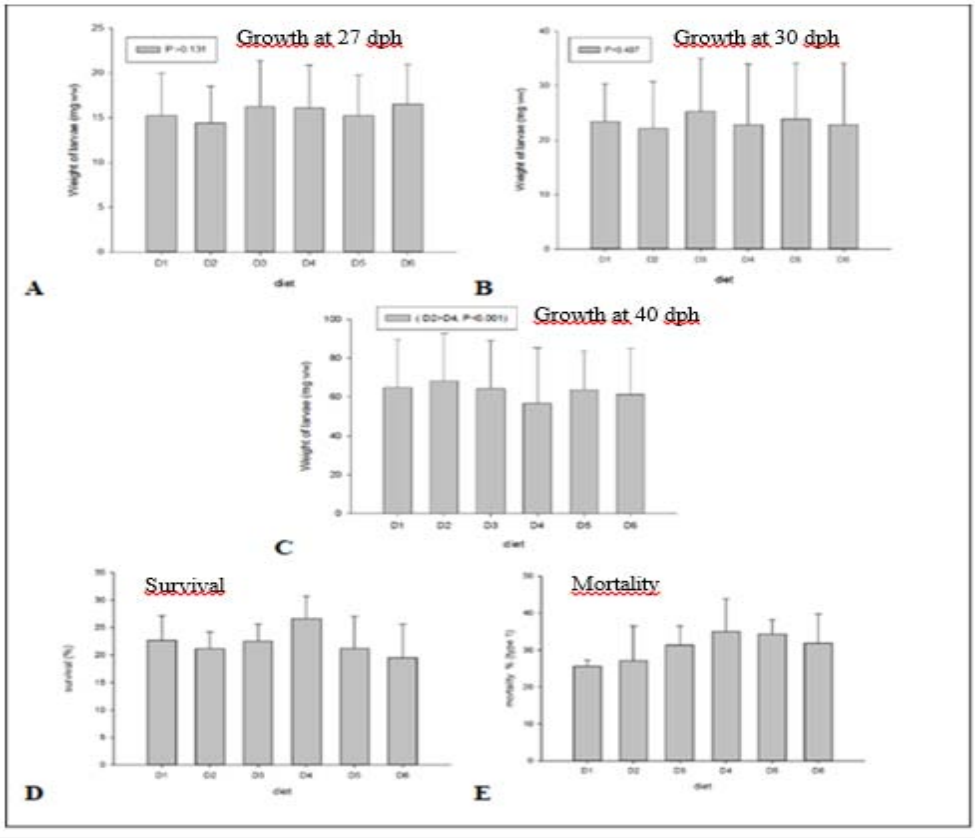

Figure 10.1.7 : Husbandry response after 24 days of feeding with the different experimental diets

Pepsin specific activity was affected by the dietary DHA content being higher at 22 dph in larvae fed diet 6 than in diets 1, 2 and 3 (**Fig. 10.1.8, A**). At 40 dph, the increase in dietary EPA/ARA levels enhanced the trypsin activity in fish fed low DHA level (**Fig. 10.1.8, C**). No differences evidenced within alkaline phosphatase and aminopeptidase activities between treatments, however a decrease in AP activity was observed at 32 dph in all groups. In this sense, the higher activity of AP recorded at 22 dph, could be explained by an alteration in the secretion process due to the weaning effect (Cahu and Zambonino, 1994; Hamza et al., 2007).

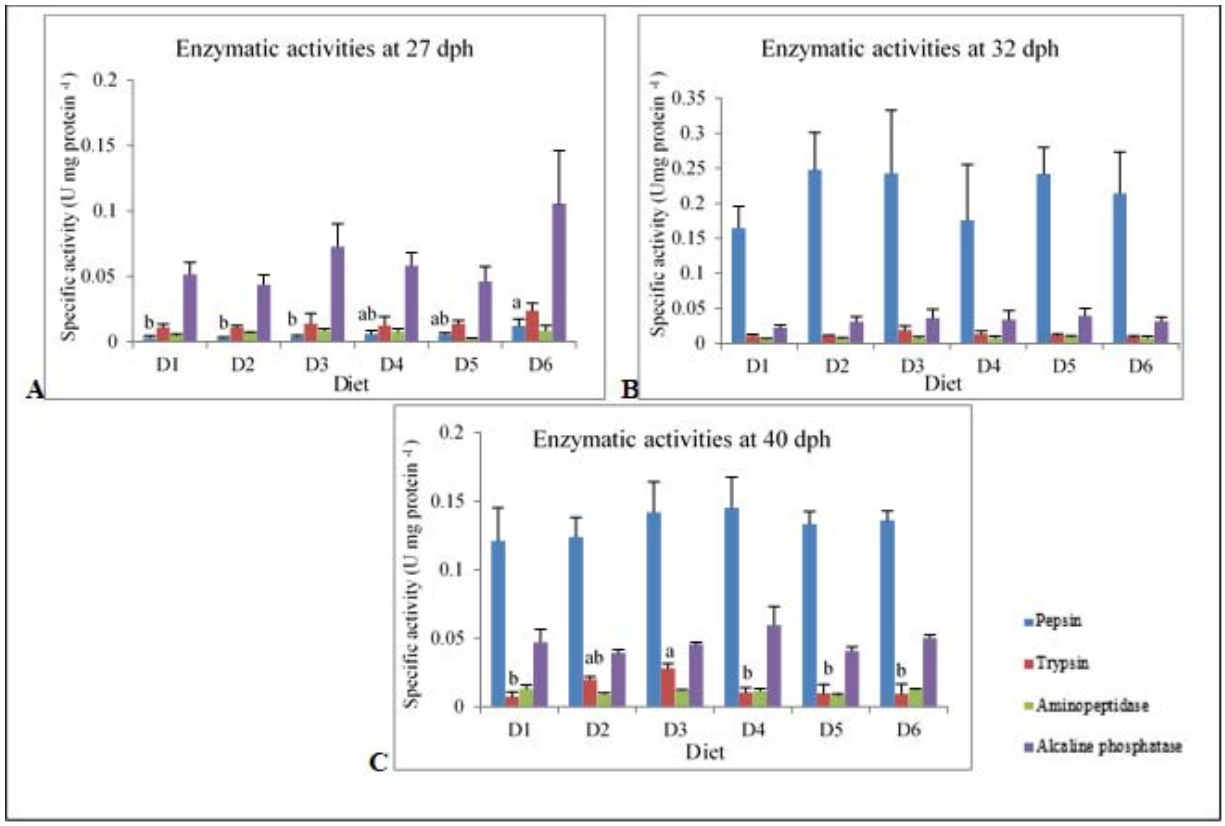

Figure 10.1.8 : Larval enzymatic activity (aminopeptidase, trypsin, pepsin, and alkaline phosphatase) of pikeperch larvae fed the different experimental diets.

- Exp. 3

Confirmatory Ca/P experiment. DTU(21): Ivar Lund, FUNDP(16): Najlae El Kertaoui, Patrick Kestemont

Based on the multifactorial results, the increase of Ca/P levels resulted in a higher incidence of kyphosis which were reported as consequences of P deficiency. Thus, a confirmatory experiment will be carried out in January 2018 at UNamur facilities, the aim of this study is to test the effect of graded levels of Ca/P regardless the Ca and P levels. The experiment will investigate the dietary Ca/P effect not only by varying one of the two minerals, but also varying both.

Diet composition

Six diets with three Ca/P levels (0.3, 0.6 and 1.2) will be tested (**Table 10.1.6**).

Table 10.1.6. Proximate composition of experimental diets

As fed basis	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Crude protein, % feed	51.16	51.15	51.14	51.14	51.16	51.17
Crude fat, % feed	18.46	18.46	18.46	18.46	18.46	18.46
Fiber, % feed	0.16	0.16	0.16	0.16	0.16	0.16
Starch, % feed	9.97	8.02	4.20	4.21	11.48	15.17
Ash, % feed	9.04	10.96	14.72	12.95	8.46	6.18
Total P, % feed	**2.68**	**2.68**	**2.68**	**3.97**	**2.01**	**1.01**
Ca, % feed	**0.80**	**1.61**	**3.21**	**1.20**	**1.20**	**1.20**
Ca/P	**0.30**	**0.60**	**1.20**	**0.30**	**0.60**	**1.19**

- Exp. 4

PL experiment. DTU(21): Ivar Lund, FUNDP(16): Najlae El Kertaoui, Patrick Kestemont, FCPCT (2): David Montesdeoca., Mariol Izquierdo

A PL experiment was performed to investigate effects of increasing inclusion of phospholipids and the additional effect of single HUFAs (EPA and DHA) on pikeperch larval performance. Several parameters such as Husbandry and digestive enzymatic activities were evaluated (reported in the WP10 2nd report). In addition, liver proteomics, skeletal deformities and gene expression involved in skeleton morphogenesis were examined.

Methodology

Samplings and analyses

For the proteomics study, the whole liver (10 larvae per tank) was extracted and immediately frozen in liquid nitrogen and kept at -80°C until analysis. The material and methods of the 2D-DIGE technique as well as for the mass spectrometry analyses are detailed in Roland et al. (2013). Staining procedures with alizarin red were conducted to evaluate the skeletal anomalies following a modified method from previous studies (Izquierdo et al., 2013).

Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Data obtained were normalised and the Livak method (2–ΔΔCt) used to determine relative mRNA expression levels. Pikeperch specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA).

Results and discussion

Proteomics

The main results of liver proteomics showed a differential pattern of expression of protein involved in lipid metabolism, protein synthesis, endoplasmic reticulum (ER) stress, and the cytoskeletal and structural protein (**Table 10.1.7**). FAS was down-expressed in larvae fed high PL and DHA levels. In addition, for the same PL content, the increase of DHA led to a decrease of the FAS expression. The combined elevation of PL and n-3 LC-PUFA suggesting a high energy demand of the small larvae. Previous studies of FAS regulation have focused on the control of gene expression, showed similar result and suggested that LC-PUFA may decrease FAS expression through the inhibition of SREBP-1c (Yahagi et al., 1999; Hannah et al., 2001). A down-regulation of SCP2 (and its product HSDL2) was observed in this study in PL3H3 larvae, which might be explained by a deficient PPARα activation due to the decrease in FAS expression in these larvae as a response to the high dietary n-3 LC-PUFA, since FAS is required for generating the phospholipid 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), an endogenous ligand for PPARα

(Chakravarthy et al., 2009*)*. Equally, result showed a low expression of ATP-citrate synthase, another protein related to lipid metabolism, in larvae fed the highest n-3 PUFA/PL diet.

An increase in abundance of PDI was observed in the liver of larvae fed on PL2 and PL2H2 compared to PL1. On the other hand, a significant difference in PDI expression was recorded between larvae fed the same n-3 LC-PUFA – PL2 compared to PL1H1. Considering the growth results (WP10 2nd report), the over-expression in PDI in the biggest larvae may reflect an enhanced protein synthesis through the high energy mobilization for growth. In this sense, Hamza et al. (2010) suggested the enhanced ability to allocate nutrient and energy into tissue formation in pikeperch larvae fed high PL.

Both Grp and Grp94 were over-expressed in larval group PL2 compared to PL3H3 in the present study, suggesting a reduced sensitivity to stress thanks to the dietary PL supplementation (Kontara et al., 1997; Coutteau et al., 2000, Hamza et al., 2010).

Regarding the reduction in the different isoforms of the keratin type II detected in liver of pikeperch fed increase PL and n-3 LC-PUFA, could indicate an advanced developmental stage in these larvae, since it has been demonstrated that keratin type II displays a differential expression pattern during the early ontogeny of fish, with a higher abundance in younger larvae (Sveinsdóttir et al., 2008).

Table 10.1.7: Protein differentially expressed in liver of 30 dph pikeperch larvae fed different PL / n-3 HUFA

Spot	accession	Protein identification	p	Fold change
794	A0A0F8AHC2	Glucose-regulated	0.007	-1.70 in PL3H3/PL2
518	A0A0F8AWU1	Glucose-regulated protein (GRP94)	0.031	-1.48 in PL1H1/PL2
	UPI000557CE3B	Glucose-regulated protein (GRP94)		-1.52 in PL3H3/PL2
369	A0A0F8AWU1	Glucose-regulated protein (GRP94)	0.033	-1.63 in PL3H3/PL1
	UPI000557CE3B	Glucose-regulated protein (GRP94)		
	UPI00055340E4	Ubiquitin carboxyl-terminal hydrolase 5		
795	UPI000556131D	fatty acid synthase-like	0.002	4.36 in PL1/PL3H3
				3.65 in PL2/PL3H3
				3.54 in PL3/PL3H3
				3.50 in PL1H1/PL3H3
1102	G3P216	ATP-citrate synthase	0.036	-2.60 in PL3H3/PL2
1633	H2U634	non-specific lipid-transfer protein	0.042	2.03 in PL1H1/PL3H3
	UPI000551760C	non-specific lipid-transfer protein		
	H2SWA2	hydroxysteroid dehydrogenase-like protein 2		
1232	G8G8Y1	Keratin 8 (Fragment) n=2	0.035	-2.27 in PL2H2/PL1
	G3NI19	keratin, type II cytoskeletal 8-like		-2.33 in PL3H3/PL1
	Q4QY72	type II keratin E3-like protein		
1376	UPI00054B498F	protein disulfide-isomerase	0.047	NS
1947	U3LRB6	Protein disulfide-isomerase	0.005	1.85 in PL2/PL1
				1.99 in PL2H2/PL1
				1.67 in PL2/PL1H1

Skeleton anomalies and related genes expression

The lowest incidence of severe anomalies was found in PL3H3 pikeperch, followed by PL3 (**Fig. 10.1.9, A**). An increase in dietary PL from PL1 to PL3 tended to reduce the incidence of severe anomalies. Moreover, a dietary increase in both PL and DHA from PL1H1 to PL3H3 significantly reduced the occurrence of severe anomalies (**Fig. 10.1.9, A**). This was more evident on anomalies affecting endochondral bones, such as the cranium, where an increase in dietary PL reduced the prevalence of these anomalies (**Fig. 10.1.9, B**). These results are in agreement with those obtained in European sea bass (*Dicentrarchus labrax*) (Cahu et al., 2003). Expression of bone morphogenesis related genes did not show a clear effect of the different treatments (**Table 10.1.8**). Hence, bone anomalies reduction by PL was not related to the relative expression of the bone-development related genes studied such as *alp* (early mineralization indicator), *twist2, mef2c* or *sox9* (endochondral bone development-related genes). This lack of effect could be due to an earlier origin of the anomalies during the first part of the feeding trial, when cartilage was being developed and bones ossified.

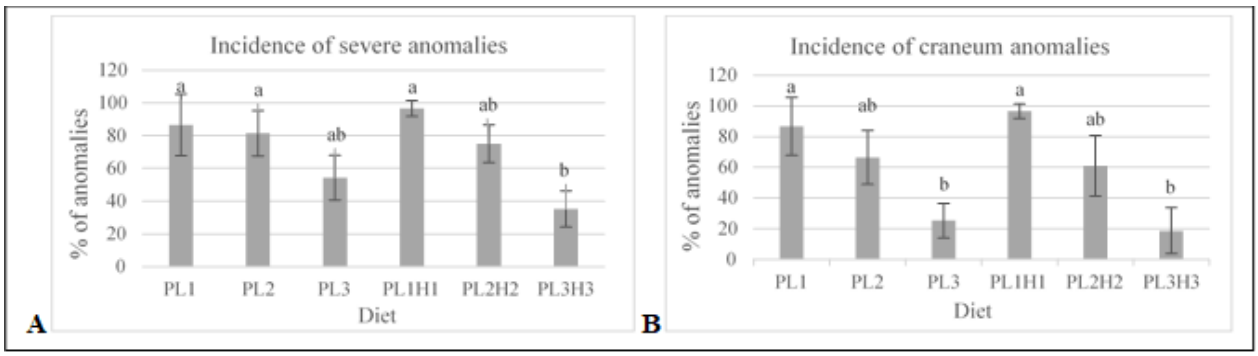

Figure 10.1.9: Incidence of anomalies (A : severe anomalies ; B : craneum anomalies) in pikeperch larvae fed experimental dry diets containing different PL and DHA levels for 20 days

Table 10.1.8. Gene expression found in 30 DAH pikeperch larvae after 20 days feeding experimental dry diets containing different PL and DHA levels

Diet	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
Alp	2.03 ± 2.70	0.81 ± 0.28	1.05 ± 0.85	3.02 ± 2.08	0.68 ± 0.79	1.28 ± 1.47
Twist2	1.33 ± 1.18	0.24 ± 0.07	0.79 ± 0.79	0.51 ± 0.48	0.15 ± 0.09	0.08 ± 0.02
Mef2c	1.15 ± 0.75	0.41 ± 0.14	1.41 ± 1.55	0.61 ± 0.52	0.12 ± 0.07	0.07 ± 0.02
Sox9	1.11 ± 0.64	0.53 ± 0.13	1.08 ± 0.78	1.52 ± 1.93	0.27 ± 0.19	0.14 ± 0.01

Conclusion

In conclusion, the present study confirms the importance of high PL levels of approximately 8 % in diets for pikeperch as well as the positive additional beneficiary effect of supplementation with DHA+ EPA in the form of concentrated TAG in otherwise identical formulated diets. Thus, combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.00 % dw DHA and 0.16% dw EPA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, improving digestive enzymes activities and liver proteomics. Overall the results denote that essential FA may be directly supplemented as triglycerides to have a beneficial effect in pikeperch larvae development. Confirmatory larval studies in pikeperch larvae

should involve effects of TAG and PL supplemented LC-PUFA and resultant FA composition in PL and NL tissue fractions.

Task 10.2. Effects of pikeperch early fatty acid nutrition on long-term stress sensitivity (led by DTU, Ivar Lund).

Exp.1. Influence of dietary HUFA levels on behavioral responses to challenges in the larval and fry stages. DTU (21): Ivar Lund, Peter Skov, Manuel Gesto

The aim of the experiment was to examine if dietary fatty acid composition in larval feed of pike perch affected behavioral responses to challenges in the larval and fry stages, and if they affect learning and the endocrine stress response in the fry stage. This was carried out by studying behavioral responses to visually simulated predator attacks and fast escape responses to mechano-sensory stimuli during the larval stage. During the fry stage the fast escape response test was repeated, spatial learning ability was studied by a maze test and effects on the endocrine stress response were quantified by post stress plasma cortisol levels.

Materials and methods

Formulation of emulsions

Four dietary emulsions were made by the substitution of extra refined virgin olive oil (Seatons 790.1 mg oleic acid/g) with either DHA oil (Incromega DHA500TG, DHA content N500 mg DHA/g; ≤100 mg EPA/g) or a fish oil rich in phospholipids from TripleNine, Esbjerg Denmark (PL: 44.3% weight (i.e. phosphatidyl choline, PC: 16.1%; lysophosphatidylcholine, LPC: 5.4%; phosphatidylethanolamines, PE: 4.5%; APE: 6.3%; spingomyelin, SPH 3.5%, others 8.5%). The main FA in the oil constituted 16:0: 188 mg g−1 oil; 18:1: 109 mg g−1 oil; DHA: 193 mg g−1 oil; EPA: 135 mg g−1 oil. The sum of polyunsaturated FA was 400 mg/g oil. Three emulsions contained either A: 0 g, B: 50 g or C: 500 g kg−1 DHA oil and one emulsion D: 500 g kg−1 phospholipid rich fish oil (i.e. 440 g phospholipids kg−1) (**Table 10.2.1**). In all emulsions soy lecithin was included (70 g kg−1) as emulgator and E-vitamin mix was added (40 g kg−1) as antioxidant (**Table 10.2.1**). Olive oil and DHA oil were obtained from Croda Chemicals Europe, Snaith, UK. Fish oil, soy lecithin and E vitamin mix were obtained from BioMar, Brande, Denmark.

Table 10.2.1. Analysed TFA Artemia content (mg g^{-1} d.w.) and FA composition (% of TFA) enriched by 4 emulsions. Formulation of emulsions (% of inclusion) is shown below

	A: OO[a]	B: OO[b] +5 DHA	C: OO[c] +50 DHA	D: OO[d] +50 PL
TFA	97.1 ± 37.6	122.1 ± 6.3	128.3 ± 75.5	79.7 ± 20.4
FA				
16:0	11.1 ± 0.0	10.1 ± 0.6	10.6 ± 0.9	11.1 ± 0.0
18:0	6.6 ± 1.2	6.0 ± 0.2	6.2 ± 0.0	6.1 ± 0.0
Total SFA	21.5 ± 2.6	19.4 ± 1.2	21.4 ± 2.1	22.5 ± 1.2
16:1 (n-7)	1.0 ± 0.3	1.1 ± 0.0	1.2 ± 0.0	1.4 ± 0.2
18:1 (n-9)	36.5 ± 0.7[c]	36.6 ± 0.4[c]	25.6 ± 0.9[a]	29.9 ± 0.8[b]
Total MUFAs	40.6 ± 1.8	43.1 ± 1.8	34.2 ± 3.5	39.1 ± 2.2
18:2 (n-6)	5.1 ± 0.2[b]	4.8 ± 0.2[b]	4.2 ± 0.2[a]	4.4 ± 0.4[ab]
18:3 (n-6)	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
20:3 (n-6)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
20:4 (n-6) ARA	0.3 ± 0.1[a]	0.4 ± 0.1[ab]	0.7 ± 0.0[b]	0.6 ± 0.2[ab]
Total (n-6) PUFA	5.9 ± 0.5	5.8 ± 0.6	5.5 ± 0.5	5.6 ± 0.7
18:3 (n-3)	28.9 ± 2.4	27.8 ± 1.1	27.5 ± 1.5	22.9 ± 0.5
20:3 (n-3)	1.3 ± 0.4	1.1 ± 0.0	1.4 ± 0.2	1.1 ± 0.0
20:5 (n-3) EPA	0.5 ± 0.4[a]	1.4 ± 0.1[b]	3.3 ± 0.2[c]	4.4 ± 0.2[d]
22:6 (n-3) DHA	0.1 ± 0.1[a]	0.6 ± 0.1[a]	5.5 ± 0.2[c]	3.1 ± 0.2[b]
Total (n-3) PUFA	30.9 ± 3.3	30.9 ± 1.3	37.8 ± 2.0	31.6 ± 0.9
DHA/EPA	0.2 ± 0.4[a]	0.4 ± 0.1[a]	1.7 ± 0.0[b]	0.7 ± 0.1[a]
ARA/DHA	3.2 ± 7.0	0.7 ± 0.2	0.1 ± 0.0	0.2 ± 0.0
ARA/EPA	0.5 ± 0.5	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
(n-3)/(n-6)	5.2 ± 0.5[a]	5.4 ± 0.2[ab]	6.9 ± 0.2[b]	5.6 ± 0.3[ab]

Values in a row followed by a different superscript are significantly different $P < 0.05$.
All emulsions included 7% soya lecithin and 4% E vitamin mix.

[a] OO: 89% OO; (OO (olive oil, Seatons refined, ≥79.1% oleic acid).

[b] OO + 5 DHA: 84% OO. 5% Incromega DHA500TG, DHA content ≥51% of total fatty acids.

[c] OO + 50 DHA: 39% OO. 50% Incromega DHA500TG, DHA content ≥51% of total fatty acids.

[d] OO + 50PL: 39% OO. 50% fish oil with phospholipid (PL) content ≥44% total lipids.

Larval and juvenile rearing and feeding

Larvae were obtained from a commercial farm AquaPri Innovation,Egtved, Denmark at 2 day post hatching (dph). Approximately 1600 larvae were distributed into each of 12 tanks at a density of approximately 36 larvae per litre. The larval rearing tanks had a volume of 46 L, and received a water flow of 8–10 L h−1 froma 10m3 temperature controlled reservoir. Each tank had separate inlet taps with adjustable flowmeters, 500 μm drainage filters and aeration. Larvae were kept under constant dim light provided by light bulbs above the tanks. Temperature and oxygen saturation were monitored daily using a portable DO meter (OxyGuard Handy, OxyGuard, Birkerød, Denmark). The temperature was maintained at 16.6±0.7 ° C during the first 28 days of experimentation. Oxygen content was kept around 7.1–7.5 mg/L in all tanks. Larvae for each treatment were reared in triplicate tanks. Newly hatched un-enriched Artemia (MC 450 type, N260.000 nauplii/g, INVE Artemia Systems, Belgium) were used as starter feed from dph 3 until 6 dph for all larval groups. From 7–27 dph, randomly chosen triplicate larval groups were fed EG type *Artemia* (INVE-Artemia Systems) enriched by one of 4 emulsions (0.6 g emulsion L−l for 24 h). *Artemia* were enriched according to normal enrichment procedures at a temperature of 21–22 °C, providing vigorous aeration by airstones (by amix of air and pure oxygen to ensure oxygen levels N 4 mg/L) at a density of 500–1000 *Artemia* /ml.

Artemia were harvested in the morning and administered continuously for 2 periods of 6 hours (each morning and afternoon) by automatic dispensers each holding a suspension of *Artemia* in seawater. Buckets containing the remaining *Artemia* of each enrichment type were kept aerated by airstones in a refrigerator between feedings at 5 °C. The tank bottom of each larval tank was gently vacuumed on a daily basis to remove uneaten *Artemia*, debris and to examine for mortality of larvae, which were counted. From dph 29–40 all larval groups were fed *Artemi*a enriched by emulsion D (phospholipid rich fish oil) and gradually weaned to an extruded experimental feed composed of fish meal (50%); soy protein concentrate (12.5%); wheat (17.2%); fish oil (10%); rape seed oil (10%); vitamin/mineral (0.3%). Protein and lipid content was 43.6% and 28.1% respectively. The feed was initially crushed to match the size of the growing fish fry and was fed to the fry during the remaining of the study until dph 140 by 12 h band feeders. Fry were kept in their initial tanks during the entire study and tanks regularly cleaned. Temperature was kept at 19.3–20.4 ° C and oxygen above 5.1 mg/L.

Behavioral studies on larvae and fry;

Avoidance tests

The protocol used for examining avoidance behavior in pike perch larvae was similar to the bouncing ball assay described by Colwill and Creton (2011) and Pelkowski et al. (2011), with slight modifications. The imaging system consisted of a PC running a Microsoft Power Point presentation on a 19 inch LCD monitor placed in horizontal position. The presentation displayed the outline of 4 petri dishes and a 30 mm black bouncing ball animation traversing the upper third of each dish at a velocity of 50 mm sec−1. A camera (HD-4110, Hewlett Packard) with a resolution of 1920 × 1080 pixels was positioned approximately 60cm above the petri dishes, to record the observations from the experiment using Debut Video Capture Software Professional (v. 1.64, NCH Software) at a rate of 5 fps onto a local PC. The entire setup was fitted within a cabinet, which was closed during experimentation. Larvae for avoidance experiments were sampled at random from different dietary treatments at 33 and 34 dph. Fish larvae were isolated individually in 50 mL beakers overnight at room temperature (20 °C). The following day, 4 fish larvae at a time were transferred to individual test arenas (petri dishes with an internal diameter of 115mm) placed on the monitor. Care was taken to avoid air exposure of fish during transfer. The final water volume in each petri dish (test arena) was 70 ml, proving a water level of ~7 mm. Following transfer, fish were allowed to acclimatize for 30 min. Each experimental round was initiated by recording a 5min period without an animated predator stimulus to determine baseline behavior, followed by 25min recordings of behavioral responses to visual predator simulation. All avoidance experiments were completed on 2 consecutive days. Baseline behavior was analyzed during a 30 s period following the first minute in the experimental round and the response to the predator stimulus was analyzed during a 30 s period following 20 min predator simulation. Adobe Photoshop (Adobe Systems Software) was used to export one frame from every second (every fifth frame), yielding 2 × 30 frames for further analysis for each fish. Video frames were analysed using Image J (v. 1.46r,Wayne Rasband, NIH, USA). The centre x,y coordinates for each petri dish and the length of each fish was established from the first suitable image. For all other images, the x,y coordinates for the snout and centre of mass were recorded (centre of mass was defined as the posterior border of the abdominal cavity which was clearly visible). All coordinates were transferred to a Microsoft Excel, and were used to determine orientation, time spent at the edge of the petri dish (defined as the outermost 10% of the radius), the upper or lower half of the petri dish, swimming speed (body lengths per second, bl s^{-1}) and time spent immobile (defined as moving less than 0.1 bl s^{-1}).

Fast escape response

The fast escape performance studies on larvae (dph 35–38) and juveniles (dph 121–124) were conducted in a white semi-translucent polyethylene circular tank with a diameter of 38 cm and a water depth of 5 cm, using a slight modification of previously described methods (Marras et al., 2011). Fish were transferred to the tank without air exposure and allowed to acclimatize to the tank for a period of 1 hour. The experimental setup was covered in black opaque plastic to prevent visual disturbance of the fish. Video recordings were made at

a rate of 250 fps using a Casio high-speed camera (EX-FH100) mounted 80 cm above the water surface. The setup was illuminated from below using a 28Wfluorescent light. The escape response was triggered by mechanical stimulation by releasing an iron rod (ø 10mm, l 15mm) manually from a height of 90 cm above

the water surface. To avoid visually stimulating the test subject, the iron rod fell inside a vertical PVC pipe suspended approximately 1 cm above the water surface. Fast escape was determined for single fish and only once per fish larvae, while two repetitions were performed for juveniles with 30 minutes of recovery between tests. There was no water replacement or water current during experiments. Fish were subsequently anaesthetized and measured for standard and total length to the nearest half mm, blotted dry and weighed to the nearest mg or g. Escape responses were analysed using Tracker (v. 4.72, www.cabrillo. edu/~dbrown/tracker). Time 0 was set as the nearest 4 ms interval at which the stimulus broke the water surface. The centre of mass was plotted every 4 ms from stimulus and 20 frames forward. These x,y coordinates were used to calculate escape latency (defined as the time elapsed between stimulus breaking the water surface and the first detectable escape motion of the fish), peak velocity during the escape response (bl s−1), distance covered during the first 80 ms of the escape response, and peak acceleration (m s−2).

Maze spatial learning test. Long-term effects on learning ability and stress responsiveness were investigated at dph 121–140 by a maze test and cortisol response to confinement. The test was performed in a maze consisting of a 40 × 40 cm square with access to a 10 × 15 cm compartment at each corner. The maze was white and light was provided by two fluorescent tubes (20W,warm white) placed 1.3m above the water surface in the maze. One of the corner compartments was fitted with an exit, leading fish out of the maze to a darker area with cover. The day before the maze test and in between training sessions during the maze test, fish were kept individually isolated in 20 litre aquaria provided aeration and water exchange. During the training sessions, fish was transported from the isolation aquaria in a 2 litre beaker and gently inserted in the mid-section of the maze. The behavior of the fish was video recorded (HD-4110, Hewlett Packard). Fish showed two stereotypical behavioral patterns in the maze. First, after being inserted in the maze fish did not move, displaying “freezing behavior”. After this, fish showed “seeking behavior”, exploring the maze and corner compartments until locating the exit. Time spent in freezing and seeking behavior was recorded. Time spent freezing and time to leave maze was recorded was 30 min if the fish did not move for a period of 30 min after being inserted in the maze. Fish were exposed to six training sessions during a period of three days (two to three daily training sessions, with a minimum of 3 h in between). Since the behavior and fatty acid profiles were similar within the groups given feed containing low levels of DHA (diet A and B) and high levels of DHA (Diet C and D) at the larval stage, the behavioral data for these two groups was pooled. The group fed low levels of DHA consisted of four fish given diet A and five fish given diet B. The group fed with high levels of DHA consisted of two fish given diet C and eight fish given diet D. Following the maze test, fish were exposed to standardized confinement stress test. Fish were kept in submerged transparent chambers (10 × 5 × 3 cm) for 30 min, whereupon they were anesthetized with an overdose of tricaine methanesulphonate (MS-222, 50mg/L) and frozen (−80 °C) for later whole body cortisol analyses.

Cortisol levels

Whole fish cortisol analyses were carried out on all experimentally used fry. Data presented as pooled values of dietary groups (A and B, n = 11) and (C and D, n = 12). To analyze for whole body cortisol, fish fry were thawed and about 1 g of tissue collected behind the anal fin was dissected out and weighed. The tissue was then homogenized in PBS (1 ml PBS g tissue−1), thereafter cortisol from the homogenate was extracted with ethyl acetate (the relation between homogenate and ethyl acetate was 1 to 5). After vortexing the homogenate / ethyl acetate was centrifuged at 1500 g for ten minutes.1 ml of the supernatant was evaporated using a vacuum centrifuge and the remaining residue was re-suspended in an extraction buffer (ELISA kit extraction buffer). Cortisol content in re-suspended samples was quantified using the ELISA kit standard method (Neogen, Product #402710).

Statistics

Larval dry weight (d.w.); mortality; tissue FA composition; and escape-response tests were compared by one way ANOVA and all pairwise Holm Sidak comparison. Percent data were arcsine transformed prior to analysis. Avoidance behaviour andmaze testswere carried out by two way repeated measurements ANOVA and all pairwise Holm Sidak comparison. Normality of data was tested by Shapiro Wilks test. All statistics were performed using Sigma Plot (v. 12.5) and P b 0.05 was considered statistically significant.

Growth and mortality

Pikeperch larvae had a dry weight of 0.14 ± 0.0 mg at 1 dph. At 28 dph larval dw was A: 1.81 ± 0.36; B: 1.94 ± 0.36; C: 2.34 ± 0.54; D: 2.41 ± 0.46 mg individual−1 with no significant differences (P = 0.07) between dietary treatments.. Specific Growth Rate, SGR (LnWf−LnWi × 100)/t from 1–27 dph was A: 21.4 ± 0.8; B: 21.6 ± 0.7; C: 22.6 ± 1.1; D: 22.8± 0.9% d−1, and was not significantly different between treatments (P= 0.06). Mortality was relatively low (1.6–4.8% for the 4 treatments, P = 0.73) until the onset of cannibalism.

Avoidance and fast escape response

Baseline (before predator simulation) positioning of larvae in the test arena differed between dietary treatments. (**Fig. 10.2.1a–d**). Larvae fed a diet deficient or low in DHA oil (treatment A and B) spent a high proportion of time (~90%) at the edge of the test arena compared to larvae fed a diet with a high DHA or phospholipid inclusion (C and D), which spent b10% of the time at the edge (**Fig. 10.2.1a**). When a visual predator simulation was presented in the upper half of the test arena, larvae fed all dietary treatments increased the fraction of time spent at the edge to 50–70%. Simulation of a predator in the upper half of the test arena caused fish larvae to spend a significantly greater amount of time in the lower half (**Fig. 10.2.1b**). There was no overall effect of diet, although a preference for diet C to occupy the upper half of the arena was significantly higher than for the other diets. Larvae on dietary treatment A and B with low levels of DHA had significantly higher routine swimming speeds under control conditions, averaging 2 and 1.3 BL s−1 respectively, compared to dietary treatments C and D in which routine swimming speeds were significantly lower (0.1–0.2 BL s−1) (**Fig. 10.2.1c**). During predator simulation, diet A and B showed no change in swimming speeds, while diets C and D responded with a 5–8 fold increase in swimming speed. Under control conditions, the fraction of time that fish spent immobile decreased significantly with decreasing DHA oil enrichment, to a minimum of 10% in the groups fed diet A (**Fig. 10.2.1d**). In the face of a simulated predator, all dietary treatments decreased the amount of time they were immobile, with no significant differences between dietary treatments. In the series of experiments to a mechano-sensory stimulus and assessment of fast-escape response (**Fig. 10.2.2a–d**), larvae reared on diet C and D had higher peak accelerations with a magnitude of 29–33% than larvae fed diets poor in DHA (A). No significant differences were observed in peak velocity or distance covered in 80 ms. There was a tendency towards decreasing escape latencies with increasing DHA content, but this finding was not significant. Escape response experiments performed on juvenile groups 86 days after the larval experiment (**Fig. 10.2.3a–d**) showed similar results, with no significant differences observed, except for peak acceleration response in which treatment C was significantly different from treatment A (**Fig. 10.2.3d**).

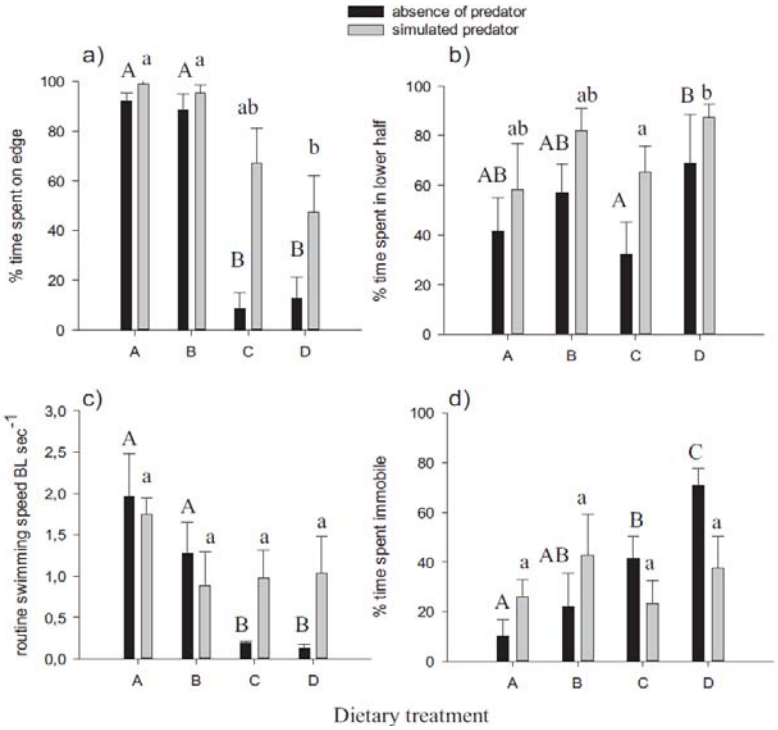

Figure 10.2.1. a-d. Avoidance behavior of larvae in the absence and presence of a predator. 1a: Time spent at edge of petri dish; there was a statistically significant interaction between dietary treatment and predator (P = 0.029). 1b: Time spent in lower half of petri dish; There was not a statistically significant interaction between dietary treatment and predator (P = 0.768). 1c: Maximum swimming speed; there was a statistically significant interaction between dietary treatment and predator (P = 0.032); 1d: Time spent holding station: there was a statistically significant interaction between dietary treatment and predator (P = 0.030). Values are presented as the mean ± SEM. Significant differences (P < 0.05) between treatments in presence - or absence of a stressor are shown by different capital or lower case letters respectively. n = 5–8 per treatment. Dietary treatment A: OO;B: OO5DHA; C: OO50DHA; D: OO50PL.

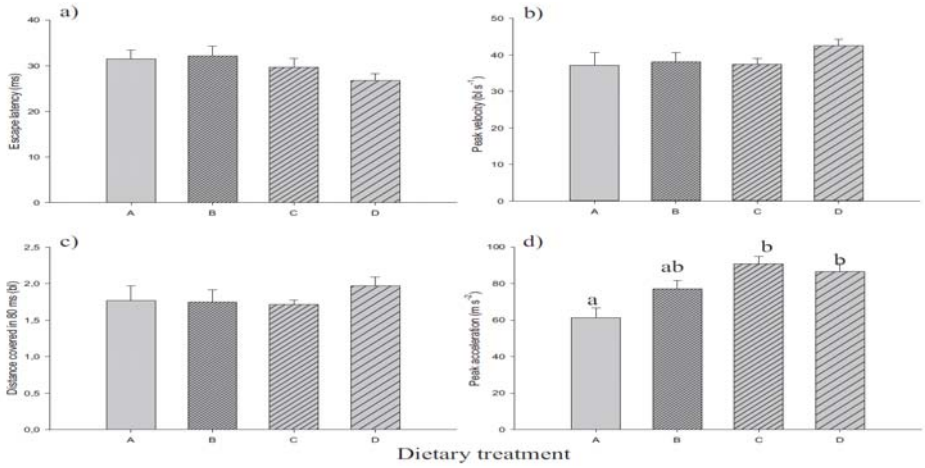

Figure 10.2.2. a–d. Larval escape-response to a visual mechano-sensory stimulus. 2a: Escape latency, (P= 0.192); 2b: Peak velocity, (P= 0.399); 2c: Distance covered in 80 ms (P= 0.439); 2d:Peak acceleration (P= 0.001). Values are presented as the mean ± SEM. Significant differences (P =0.192) between treatments are shown by different letters. n= 6–9 per treatment. Dietary treatment A: OO; B: OO5DHA; C: OO50DHA; D: OO50PL.

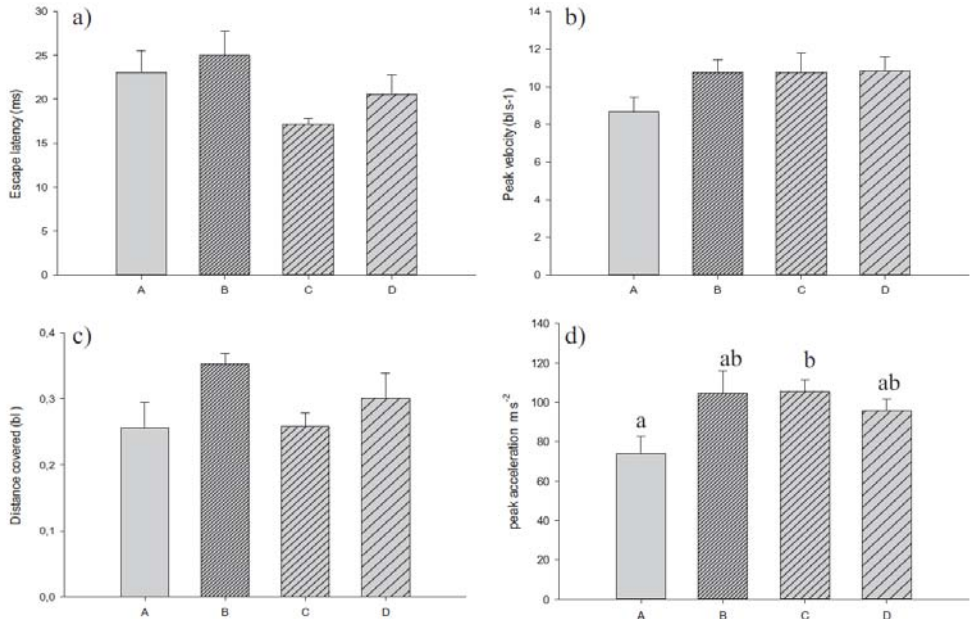

Figure 10.2.3. a–d. Fry escape-response to a visual mechano-sensory stimulus. 3a: Escape latency, (P = 0.107); 3b: Peak velocity, (P= 0.206); 3c: Distance covered in 80 ms, (P = 0.093); 3d: Peak acceleration, (P= 0.049). Values are presented as the mean±SEM. Significant differences (P < 0.05) between treatments are shown by different letters. n=6 per treatment. Dietary treatment A: OO; B: OO5DHA; C: OO50DHA; D: OO50PL.

Maze spatial learning test and fry cortisol content

The time fry spent to solve a maze decreased with training (**Fig. 10.2.4a–b**), an effect that was related to a decrease in initial freezing time (**Fig. 10.2.4b**). Moreover, fry fed diets low or deficient in DHA (A+B) as larvae had longer initial freezing time compared to fry fed diet C or D given diets high in content of DHA and phospholipids, this was independent of training. There was no significant difference (P= 0.25) in tissue cortisol level (mean ± SEM between dietary treatments groups (A and B): 35±3.7 ng g−1 tissue and (C and D): 30±2.5 ng g−1 tissue, respectively. Thus, dietary DHA content or phospholipids did not affect the magnitude of a stress-induced cortisol release in fry.

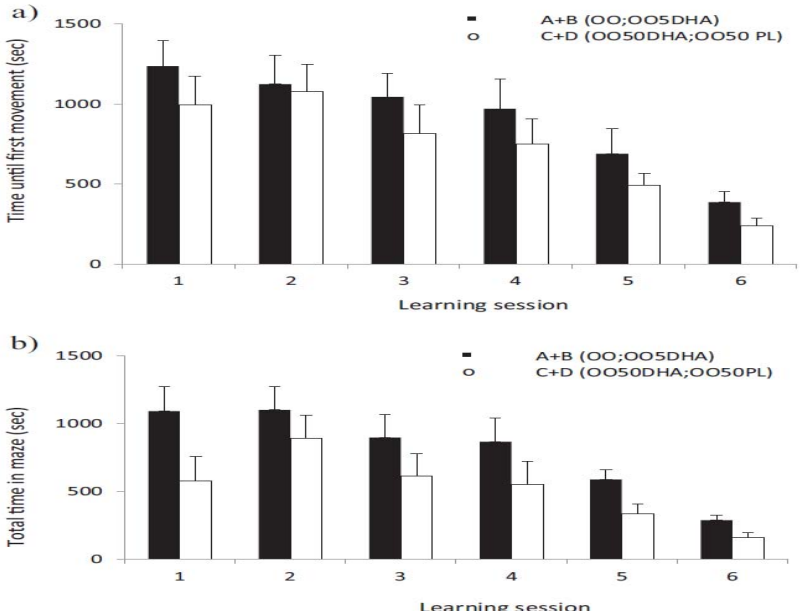

Figure 10.2.4. a–b. Learning ability of fry fish to find way out of a maze during 6 repetitious training sessions. 4a: Total time in maze. Two way repeated ANOVA: Learning session: $P < 0.001$; Fatty acid: $P < 0.20$; Fatty acid x learning session: $P= 0.55$. 4b: Time until first movement. Two way repeated ANOVA: Learning session: $P < 0.001$; Fatty acid: $P < 0.05$; Fatty acid x learning session: $P= 0.56$; Values are presented as the mean ± SEM. Dietary treatment A + B: Pooled fish, OO;OO5DHA (n = 7); C + D: Pooled fish, OO50DHA; OO50PL (n = 10).

The present study showed a trend towards lower response times with higher dietary DHA and EPA content in both larvae and in fry. As no effects were observed on the escape latency, it could be hypothesised that effects on peak acceleration rates in larvae from dietary levels of DHA and EPA are related to efferent sensory signalling from Mauthner cells.

The present study demonstrated a temporal consistency in effects and tendencies for effects of dietary fatty acid composition indicating that a diet with an adequate HUFA profile could not compensate for deficiencies experienced during early ontogeny. The behavior responses to mechano-sensory stimuli at the larval stage were maintained in fry until 95 days after the dietary treatment period (7–27 dph) had ceased

The observation that all dietary treatments responded by avoidance to the simulated predator suggests that visual acuity in fish on diets with low DHA or EPA content was not impaired to any significant degree.

In the present study, irrespectively of habituation, the time lag after being transferred to the maze was consistently longer for DHA deficient fish. The longer time lag before first movement for groups low or deficient in DHA and EPA (A + B) was independent of training, and may thus reflect a more anxious behavioural profile of these fish.

Conclusions

In this study we present a number of behavioral effects correlated to n-3 LC-PUFA levels in diets for pike perch larvae. Larvae fed low levels of DHA displayed a tendency towards delayed escape responses (latency time increased) and significantly slower peak acceleration rates during escape responses following a mechano-sensory stimulus. This effect was consistent up to 90 days after the dietary treatment was terminated, demonstrating long-term effects of early nutritional history in fish. A more anxious behavioral profile of the fry low in DHA lends supports to long-term central effects, such as brain developmental pattern, being the cause of these behavioral effects.

Deviations from Annex I and their impact:

The completion of deliverable 10.1 (due month 36) is awaiting a final confirmatory experiment to be performed in month 50 - and subsequent analytical work.

The completion of deliverable 10.2. (due month 36) is expected month 48.

Deliverable 10.3 (due month 48) is awaiting results from ongoing exp. to be started month 50.

The main reason for the postponement of the deliverables has been redoing experiments due to mortality/cannibalism and analytical work that has taken longer time than anticipated. Besides more studies have been carried out that described in the DOW (Annex 1).

Deliverable 10.2 will be submitted in month 48, while the two other deliverables will be handed in before end of project period 4 (month 60).

WP 11 Nutrition – Atlantic halibut

WP No:	11	**WP Lead beneficiary:**		P17. NIFES
WP Title (from DOW):	Nutrition – Atlantic halibut			
Other beneficiaries (from DOW):	P7. IMR	P15. ULL	P20. SARC	
Lead Scientist preparing the Report (WP leader):	Kristin Hamre			
Other Scientists participating:	Øystein Sæle (P17), Torstein Harboe (P7), Covadonga Rodriquez (P15). Ramon Fontanillas (P20)			

Objectives

1. Develop a protocol for early weaning,
2. Develop a production strategy for on-grown *Artemia,*
3. Improve growth in late larval stages, and juvenile quality, through feeding with on-grown *Artemia,*
4. Better understand the effects of RAS vs FTS on Atlantic halibut larval nutrient utilization,
5. Investigate how dietary phospholipids after weaning affects growth and lipid metabolism.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Although in the DOW it was indicated that the work should start from the beginning of the project, we only had planning activities in the period 1-12 Mo. The actual experiments started in Mo 13. This has to do with the relatively few activities in this WP and the need to organize the work in a practical way.

Summary of work reported in the previous Reporting Period (13-30 Mo):

1. A protocol for weaning of Atlantic halibut at 28 days post first-feeding (dpff) has been developed and almost 100% of the larvae fed Ottohime diet (Japan) were filling up their guts with feed after a 5 d adaptation period.
2. A production strategy for ongrown *Artemia* has been established, which improves the nutritional value of *Artemia* with respect to protein, lipid and micronutrient contents.
3. Growth and juvenile quality was excellent in larvae fed *Artemia* nauplii in this experiment and was not improved by feeding ongrown *Artemia.*
4. Objectives 4 and 5 have not been addressed yet.

Summary of progress towards objectives (31-48 Mo):

1. *Research under objective 4 found that RAS had a large positive effect on vitamin K (MK6) concentration in Atlantic halibut larvae. Most free amino acids, iodine, copper and zinc were also increased, while glycine concentration was decreased in larvae reared in RAS compared to FT.*
2. *Inclusion of soy lecithin so that dietary phospholipid increased from 9 to 30% of total lipids had no effect on growth, but changed lipid metabolism in Atlantic halibut juveniles*

Details for each Task

Task 11.1 Early Weaning of Atlantic halibut (led by IMR, Torstein Harboe) This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.2*** **Report on optimal characteristics of feed particles and feeding environment for early weaning of Atlantic halibut larvae.**

Task 11.2. Development of a production strategy for on-grown *Artemia* (led by IMR, Torstein Harboe). This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.1*** **Report on the nutrient profile of *Artemia* nauplii and on-grown *Artemia***.

Task 11.3. Nutrient retention and digestive physiology of Atlantic halibut juveniles fed *Artemia* nauplii or on-grown *Artemia* (led by NIFES, Kristin Hamre). This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.3*** **Report on the nutrient retention and digestive physiology in Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia.***

Task 11.4. Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS (led by NIFES, Kristin Hamre). This task has been completed and the results have been provided in ***Deliverable 11.4*** **Report on the nutrient retention and digestive physiology in Atlantic halibut larvae reared in RAS vs FTS.** A brief summary follows below:

Introduction

Atlantic halibut larvae kept in a RAS system will encounter matured water, which can affect their gut flora (Nayak, 2010) in a way that may have a positive effect on intestinal health. Gnotobiotic and conventional studies indicate the involvement of gut microbiota in nutrition and epithelial development (Nayak, 2010). Gastrointestinal bacteria may also produce essential nutrients such as vitamins and polyunsaturated fatty acids, and enzymes that can aid digestion (Ray et al., 2012). These considerations favour the hypothesis that the general nutrient absorption and retention in the fish is affected by RAS. Iodine retention must have an extra focus, since NO3- at levels found commonly in recirculation systems block iodide uptake by the sodium iodide symporter and may cause goiter in the fish (Morris et al., 2011; Ribeiro et al., 2011).

This study aims to determine the potential effects of RAS *vs* FTS on water chemistry, water microbiology, microbial colonisation of larvae and fish performance. One group of Atlantic halibut was held in a flow-through system while another group was held in a RAS system. Both groups were fed enriched *Artemia* nauplii. In the present task, analyses of the nutritional profile of the larvae at 38 dpff (days post first-feeding) as well as main digestive enzymes activities at 30 dpff were measured, in order to compare nutrient retention and digestive system physiology between the two fish groups.

Results and discussion

The larval final weight was 2.3 fold higher in larvae held in FTS, compared to larvae held in RAS, e.g. 0.122 and 0.054 g, respectively. Both lipid, measured as total fatty acids, and protein content was also higher in the FTS larvae. This is probably a result of the size difference, since dry matter, protein and lipid levels increase with increasing size in halibut larvae and juveniles, at least up to a body weight of 4 g (Hamre unpublished). There were differences between the groups in a few micronutrients, the most interesting being MK6, which was higher in RAS larvae than in FTS larvae, possibly because it was produced by microorganisms in the system. Iodine was higher in fish from the RAS system, although recirculation is known to block iodine uptake from the water (Morris et al., 2011; Ribeiro et al., 2011). In this experiment, sufficient iodine appears to have been supplied by the diet. Copper and Zinc were also higher in larvae from RAS. Differences in fatty acid composition were small and probably biologically insignificant. The concentrations of most of the free amino acids were higher in RAS larvae than in FTS larvae, however, the concentration of glycine was lower in the FTS larvae. Glycine is a nonessential amino acid, so there may have been an inhibitory effect on glycine synthesis or increased breakdown of glycine caused by some component in the RAS system. Glycine

is also an inhibitory neurotransmitter. There were no effects of the treatments on other metabolites identified by the FAA method used, such as neurotransmitters, metabolites of the Urea cycle, ammonia, ethanolamine and taurine.

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Task 11.5 Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles (led by NIFES, Øystein Sæle)

Introduction

The benefit, or even essential need for high inclusion of phospholipids (PLs) in feeds for marine fish larvae is well documented (Coutteau et al., 1997). But adding PLs to the feed of juvenile fish has also shown to be beneficial for a wide spectre of species (Atar et al., 2009; Niu et al., 2008; Sotoudeh et al., 2010). PLs are vital for lipid transfer from the intestinal tissue to the blood, probably due to limited capacity of de novo PL synthesis in enterocytes. Limited PL synthesis will also inhibit membrane metabolism in the larval body, and may thereby affect growth.

Lipids are transported from enterocytes to other tissues in chylomicrons. Besides proteins, chylomicrons consist of a core of TAG and cholesterol esters and a monolayer of PL on the surface. Chylomicron production starts with the formation of PL rich particles, thus PL synthesis is a potential bottleneck for lipid transport out of the intestine.

We have shown that juvenile Ballan wrasse increase the growth rate by up to 40% when lipids are added as phospholipids (PL) in stead of triacylglycerols (TAG, Sæle et al., ubpublished), while requirements for PL in A. halibut juveniles are not known.

The main objective of this study is to investigate lipid composition in intestinal, liver and muscle tissue as a function of dietary PL/TG and post-prandial time. We know that high dietary PL gives better growth in larvae and juveniles of a range of fish species, and we have also shown that dietary PL can affect intestinal transport and TG in Ballan wrasse (Sæle et al, unpublished). The hypothesis is therefor that fish fed the low PL/TG diet will have more total lipid and in particular TG in intestinal tissue. We are also curious to what the dietary ratio of PL/TG does to lipid metabolism in liver and muscle. Therefore, samples of these tissues, taken 24 h post feeding, were included in the study.

Materials and methods

Approximately 5000 Atlantic halibut larvae were transferred from a yolk sac larvae incubator (silo) to a standard 1.5-m diameter 0.8-m depth first feeding tank. The larvae were fed *Artemia* nauplii, short time enriched with MultiGain, from 1 until day 48 dpff. Thereafter they were weaned to dry feed (Otohime) and

fed until day 82. The larvae were then transferred to 15, 50-l tanks and acclimated to the new environment for 5 days before the experiment started. Each tank had continuous water supply of 10 l / hour, central aeration and a belt feeder. Clay was added to the tanks three times a day to create turbidity. Larvae were fed formulated feeds continuously, using belt feeders, and were also hand fed three times during a day. In the morning before hand-feeding and clay addition and in the evening after hand-feeding.

When juvenile fish reached 0.92 ± 0,42 g with a total length of 46.32 ± 7.13 mm, they were fed diets with graded levels of PL by increasing dietary soya lecithin inclusion. The experiment lasted for two months and was a regression design with 3 replicates and 5 levels. Diets were produced by Skretting-ARC (SARC) (**Table 11.5.1**).

Lipid class composition of the diets was analysed using high-performance TLC, as described by Bell et al., (1993) and Jordal et al., (2007) (**Table 11.5.2**). The plates (20 x 10 cm) were developed at 5 cm in methyl acetate – isopropanol – chloroform – methanol–0·25% (w/v) aqueous KCl (25:25:25:10:9, by vol.) to separate PL classes from neutral lipids running at the solvent front (Vitiello and Zanetta, 1978). After drying, the plates were developed fully in isohexane – diethyl ether – acetic acid (80:20:1.5, by vol.) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160˚C for 15min after spraying with 3% copper acetate (w/v) in 8 % (v/v) phosphoric acid and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS- Planar Chromatography, version 1.2.0).

All larvae were euthanized with an overdose metacaine (MS-222TM; Norsk medisinaldepot AS, Bergen Norway). Total length (TL) and weight were registered before intestine, liver and muscle tissues were dissected out and snap frozen on liquid nitrogen for lipidomics analysis. Lipids were extracted from tissue samples using dichloromethane and methanol in a modified Bligh-Dyer extraction in the presence of internal standards with the lower, organic, phase being used for analysis. The extracts were concentrated under nitrogen and reconstituted in 0.25mL of dichloromethane:methanol (50:50) containing 10mM ammonium acetate. The extracts were placed in vials for infusion-MS analyses, performed on a SelexION equipped Sciex 5500 QTRAP using both positive and negative mode electrospray. Each sample was subjected to 2 analyses, with IMS-MS conditions optimized for lipid classes monitored in each analysis. The 5500 QTRAP was operated in MRM mode to monitor the transitions for over 1,100 lipids from up to 14 lipid classes. Individual lipid species were quantified based on the ratio of signal intensity for target compounds to the signal intensity for an assigned internal standard of known concentration. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of individual fatty acids within each class.

Results and Discussion

After two months, the halibut had grown from 0.92 ± 0,42 g to 10.12 ± 3,84 g and from a total length of 4.63 ± 0.71 cm to 9.85 ± 1.30 cm, however there were no differences in growth between the diet groups (**Figure 11.5.1**). Growth in halibut, this size, did not benefit from dietary PLs increasing from 9 to 30 % of total lipid. In Atlantic salmon increased PL/TAG ratio led to better growth in juveniles up to 2.5 g. However this growth effect disappeared after the fish had reached 2.5 g (Taylor and Grosell, 2006).

Results of lipidomics and gene expression are still under statistical analyses.

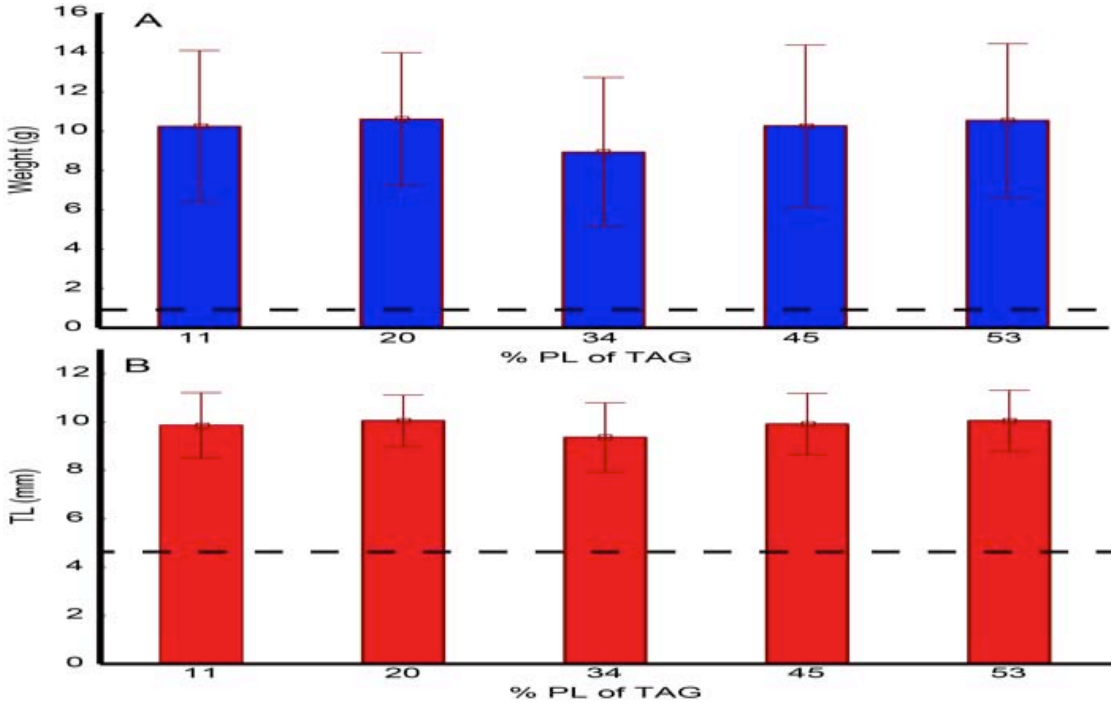

Figure 11.5.1. Final weight (A) and length (B) of halibut. Stippled line shows start weight and length

Table 11.5.1a. Diet compositions in %

FM North-Atlantic 12C: fish meal from the North Atlantic, cpsp 90 ntc 12149: is pre-digested fish meal

Diet	1	2	3	4	5
Water	-3.36	-3.36	-3.36	-3.36	-3.36
Soy oil	6.00	4.50	3.00	1.50	0.00
Soy lecitin	0	1.50	3.00	4.50	6.00
krill meal	2.50	2.50	2.50	2.50	2.50
Wheat	15.37	15.37	15.37	15.37	15.37
FM North-Atlantic 12C	74.63	74.63	74.63	74.63	74.63
cpsp 90 ntc 12149	2.50	2.50	2.50	2.50	2.50
Fishoil North-Atlantic	2.06	2.06	2.06	2.06	2.06
vitamin premix	0.10	0.10	0.10	0.10	0.10
Yttrium premix	0.10	0.10	0.10	0.10	0.10
Mineral premix	0.10	0.10	0.10	0.10	0.10

Table 11.5.1.b. Calculated proximate composition

Diet	1	2	3	4	5
Dry matter	93.00	93.00	93.00	93.00	93.00
Moisture	7.00	7.00	7.00	7.00	7.00
Protein	57.00	57.00	57.00	57.00	57.00
Lipid	18.00	18.00	18.00	18.00	18.00
Ash	10.56	10.56	10.56	10.56	10.56
Starch	8.76	8.76	8.76	8.76	8.76

Table 11.5.2. Lipid class (mg/g) analysis (HPTLC) of diets 1 to 5

Feed	1	2	3	4	5
LysoPC	4.1	6.3	8.4	10.5	9.7
Sphingomyelin	1.4	1.5	1.7	1.9	2
Phosphatidylcholine	8.1	13.3	19	23.9	29.1
Phosphatidylserine	1.2	3.2	7.6	9.8	11.6
Phosphatidylinositol	0	2.3	5.6	7.1	8.3
Cardiolipin	0.1	0.2	0.4	0.4	0.5
Phosphatidylethanolamine	2	5.2	9.4	11.5	13.1
Diacylglycerol	1	1.3	1.7	1.5	1.3
Cholesterol	6.9	7.9	7.8	7.6	7.9
Free fatty acid	10.5	13	15.2	15.2	16.5
Triacylglycerol	149	161	152	144	139
Cholesteryl ester	nd	nd	nd	nd	nd
Sum Phospholipids	16.9	31.9	52.2	65	74.1
Sum Neutral lipids	167	183	177	169	164
Sum Lipids	184	215	229	234	238

References

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Deviations from Annex I and their impact:

Regarding Task 11.5, we have received the analytical results of lipidomics from Metabolon, but we found a mistake in the data that has now been corrected. However, we have not had time to analyse the data again. Therefore, we have asked for **Deliverable 11.5 Report on the effect of dietary phospholipids on Atlantic halibut juveniles,** which was due in Mo 48, to be postponed. We expect to deliver in August 2018.

WP 12 Nutrition – wreckfish

WP No:	12	**WP Lead beneficiary:**		P19. CMRM
WP Title (from DOW):	Nutrition – wreckfish			
Other beneficiaries (from DOW):	P2. FCPCT	P8. IEO		
Lead Scientist preparing the Report (WP leader):	Fatima Linares			
Other Scientists participating:	José Luis Rodriguez (P19), Blanca Álvarez-Blázquez (P8), Evaristo Pérez (P8), Antonio Vilar (P32), Gema Pazos (P19) Javier Roo (P2) and Marisol Izquierdo (P2)			

Objectives

1. Test the effectiveness of live prey and influence of enrichment on wreckfish larvae,
2. Determine the influence of broodstock feeds on fecundity and spawning quality.

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the first year of the project the work done in this WP was related to wild fish composition and the feeding of wreckfish broodstock to allow the formulation of the broodstock diets. Wild wreckfish were sampled from February to October 2014 and the stage of the reproductive development was evaluated. Fish dissection and sample collection of muscle, liver and gonads were collected to carry out biochemical analysis to know the nutritional status of wild fish. The first analysis showed a high amount of proteins (82% DW) and a low lipid content (6% DW). A high variability was observed in liver and gonad composition. With reference to fatty acids, muscle polyunsaturated (PUFA), saturated (SFA) and monounsaturated (MUFA) fatty acids were 36-46%, 28-30% and 25-33%, respectively and n-3 PUFA content reached 32-40% in the muscle. Liver fatty acid profiles showed a broader variability with a lesser content of EPA, DHA and ARA than muscle.

Additionally histological analysis of gonads were performed and showed that of 33 gonads examined, 15 were males and 18 were females and no evidence of hermaphroditism was obtained. With respect to the influence of the broodstock food composition on the reproductive development, some samples of semi-moist diet supplied to the P8. IEO broodstock were collected at different times of freezing to perform the biochemical analysis and no differences were found between samples taken at different times and with different freezing times.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the second period of the project the highlights were:

1. Comparisons of wild and reared wreckfish composition showed that fish from intensive culture have more lipids in muscle (27,5%DW) and liver (62%) than those obtained in wild fish with 7% in muscle and 40% in liver. In contrast, protein content is higher in muscle of wild wreckfish than in reared fish and some differences were also observed in the fatty acid profile with higher values of PUFA and n-3 PUFA in wild than in reared wreckfish. DHA values represent 11% in reared fish and 26% in wild fish.

Results from wild fish were very useful to formulate specific dry food for wreckfish broodstock.

2. First results of fatty acid profile of wreckfish larvae show that PUFA, SAFA and MUFA content (% of total fatty acids) have a little variation in the first 10 days of life.
3. Regarding wreckfish broodstock feeding regimes, results obtained from first experiments showed that most of commercial dry food has too much fat for wreckfish.
   First results with dry food 1 demonstrated that it should be increased the amount of proteins and decreased the level of fat. Furthermore, dry food for wreckfish must contain a big amount of n-3 PUFA and the EPA/ARA rate must be around 1.5 similar to that obtained previously in wild wreckfish.
4. A clear relationship between fatty acid profile of oocytes and broodstock diet was found. Samples of oocytes were obtained from females fed with semimoist diet and dry food. Furthermore, some differences were observed in fatty acid profile of oocytes from females of different wreckfish broodstock showing that there is a relationship between fatty acid content and oocytes development
5.

Summary of progress towards objectives (31-48 Mo):

During the second period of the project the highlights were:

1. Enrichment products for living prey (rotifers and *Artemia*) were designed. Two levels of ARA content were used for enrichment product for rotifer and one level of ARA for *Artemia* and the effect of the new enrichment products on the biochemical composition of rotifers and *Artemia* was evaluated.
2. First data of fatty acid profile were obtained from 1dph until 26 dph of larvae to complete the data obtained previously until 10dph.
3. A clear relationship between fatty acid profile of broodstock diet (semi-moisture, dry food and a mixture of hake and squid) and fatty acid profile of oocytes and eggs from females fed with the different diet was found.
4. Results obtained with dry food 2 demonstrated that the wreckfish diet must contain a big amount of proteins, low level of lipids, a high amount of n-3 PUFA and the EPA/ARA ratio must be similar to that obtained in wild females gonads (about 1-1.5). Nevertheless the diet with a mixture of hake/squid (half and a half) seems to be a diet with good quality because of the protein content and the big amount of n-3 PUFA (EPA and DHA) although the EPA/ARA obtained in oocytes and eggs from females fed with this diet is high comparing with the one obtained in wild females gonads.
5. First data of fatty acid profile of sperm from wreckfish males of different broodstock were obtained.

Details for each Task

Task 12.1. Live preys and enrichments for wreckfish larvae. (led by Fátima Linares (P19), Javier Roo (P2) & Marisol Izquierdo (P2))

Partners involved:

Fátima Linares (P19) , Javier Roo (P2), Blanca Álvarez -Blázquez (P8), José Luis Rodríguez (P19), Evaristo Pérez (P8), Marisol Izquierdo (P2)

Some new enrichment products for live food were developed. Data of biochemical analyses performed at CIMA (CMRM) of gonads from wild wreckfish females and eggs and larvae obtained from reared fish were sent from CMRM to FCPCT (P2) to develop some live food enrichment products for larval wreckfish.

Three experimental enrichment products were formulated to meet the EPA&DHA and ARA levels obtained from tissues of wild-catch wreckfish. For experimental enrichment preparation a combination of different

products based on microalgae were use: Chlorella meal powder (>1% Fat, > 12% Protein; Shaanxi Pioneer Biotech Co., Ltd, Xi'an, China), Microalgae DHA Powder (>40% DHA; Shaanxi Pioneer Biotech Co., Ltd, Xi'an, China), and ARA Powder (>40% ARA); Shaanxi Pioneer Biotech Co., Ltd, Xi'an, China). **Table 12.1.1.**

The experimental enrichments for rotifer were formulated using two different levels of ARA (3 and 10%) and in the case of *Artemia* only one level of ARA (9%) was used.

Table 12.1.1. Experimental enrichment ingredients.

Experimental Enrichment	*DHA- Rot*	*ARA-Rot*	*ARA-Art*
	Ingredients (g kg^{-1}diet)		
Chlorella powder	*500*	*500*	*400*
Microalgae DHA Powder	*400*	*400*	*500*
ARA Powder	*20*	*100*	*100*

The analysis of the experimental products attained higher levels of ARA & EPA& DHA than reference values form samples (**Table 12.1.2**).

Table 12.1.2. Proximate (% dry matter) and fatty acids composition (%TFA) of experimental enrichment.

	DHA- Rot	*ARA-Rot*	*ARA-Art*
	Proximate analysis (% dry matter)		
Lipids	9.34±0.14	9.34±0.24	9.23±0.34
Proteins	25.58±0.21	30.08±0.13	22.54±0.09
Ash	4.34±0.03	5.13±0.10	6.11±1.85
	Fatty acid content (%TFA)		
Saturated	19.39	17.61	21.93
Monoenoics	8.30	8.76	8.35
n-3	48.82	45.72	45.14
n-6	20.87	25.43	24.26
n-9	4.55	5.65	4.88
Total n-3HUFA	44.20	41.42	44.62
14:0	0.40	0.47	0.41
16:0	17.10	13.49	17.90
16:1 n-7	0.85	0.52	1.12
18:0	1.30	2.93	2.91
18:1 n-9	3.90	5.03	4.23
18:1 n-7	0.54	0.50	0.46
18:2 n-6	12.99	11.66	7.88
18:3 n-3	4.24	4.00	0.19
20:1 n-9	0.09	0.08	0.08
20:4n-6 (ARA)	3.10	9.86	9.03
20:5n-3 (EPA)	6.33	5.87	6.40
22:6n-3 (DHA)	33.06	31.16	33.55
EPA/ARA	2.04	0.60	0.71
DHA/EPA	5.22	5.31	5.24
DHA/ARA	10.66	3.16	3.71
Oleic/DHA	0.12	0.16	0.13
Oleic/n-3HUFA	0.09	0.12	0.09
n-3/n-6	2.34	1.80	1.86

HUFA. highly unsaturated fatty acid; ARA. arachidonic acid; DHA. docosahexaenoic acid; EPA. eicosapentaenoic acid.

Rotifer enrichment:

L-type rotifer, *Brachionus plicatilis,* growth on commercial baker yeast was used. Rotifers were enriched in conical 500 L tanks (water volume 400 L) at a density of 1000 rotifers mL−1 in an air conditioned illuminated room (12 L:12 D, from 8:00 am to 8:00 pm). The water was kept at a salinity of 37 g L−1 and 20 ± 0.01 ◦C. Rotifers were enriched twice 6 and 3 hours prior to being collected (2:00 am and 5:00am) with 0.3 g/Million rotifers enriched. After 6h the rotifers were filtered cleaned and sampled for biochemical analysis. The analysis of rotifer enriched with the different enrichment products (*Rot-DHA and Rot-ARA*) comparing with rotifer no enriched (*Rot-Yeast*) are shown in **Table 12.1.3.**

Table 12.1.3. Proximate (% dry matter) and fatty acids composition (%TFA) of rotifers enriched with different experimental enrichments

	Rot-Yeast	*Rot-DHA*	*Rot-ARA*
	Proximate analysis (% dry matter)		
Lipids	10.21±0.06	12.76±0.43	13.08±0.85
Proteins	58.20±0.37	60.66±0.03	60.91±0.41
Ash	11.94±0.71	10.24±0.69	9.34±0.30
Moisture	92.17±0.05	91.46±0.04	91.43±0.13
	Fatty acid content (%TFA)		
Saturated	23.29	20.73	18.67
Monoenoics	53.48	40.41	38.71
n-3	5.45	16.50	18.37
n-6	13.42	16.45	18.55
n-9	24.24	20.54	20.10
Total n-3HUFA	3.68	13.52	15.27
14:0	2.43	1.59	1.41
16:0	14.40	13.39	11.69
16:1 n-7	17.40	13.35	12.51
18:0	5.36	4.30	4.17
18:1 n-9	21.64	16.01	15.67
18:1 n-7	4.72	3.43	3.23
18:2 n-6	10.48	12.52	12.58
18:3 n-3	1.62	2.80	2.96
20:1 n-9	0.89	0.72	0.71
20:4n-6 (ARA)	**1.15**	**1.35**	**3.53**
20:5n-3 (EPA)	**1.58**	**3.12**	**3.23**
22:6n-3 (DHA)	**0.68**	**8.00**	**9.49**
EPA/ARA	1.37	2.31	0.91
DHA/EPA	0.43	2.56	2.94
DHA/ARA	0.59	5.92	2.69
Oleic/DHA	31.88	2.00	1.65
Oleic/n-3HUFA	5.88	1.18	1.03
n-3/n-6	0.41	1.00	0.99

HUFA. highly unsaturated fatty acid; ARA. arachidonic acid; DHA. docosahexaenoic acid; EPA. eicosapentaenoic acid.

Artemia Enrichment:

Artemia salina (EG Inve) was used. Newly hatched *Artemia* was enriched in one conical 150 L tanks (water volume 100 L) at a density of 500 Arte/mL−1 in an air conditioned illuminated room (12 L:12 D, from 8:00 am to 8:00 pm). The water was kept at a salinity of 37 g L−1 and 24 ± 0.01 ◦C. *Artemia* was enriched in a single dose 12 hours prior to being collected (20:00 pm) with 0.4g/L. After this period *Artemia* was sampled for biochemical analysis. The analysis of *Artemia* enriched with the enrichment product (*Art-ARA*) comparing with *Art-non-enriched* are shown in **Table 12.1.4.**

Table 12.1.4. Proximate (% dry matter) and fatty acid composition (%TFA) of *Artemia* enriched with experimental enrichment

	Art-Non enrich	*Art-ARA*
Proximate analysis (% dry matter)		
Lipids	21.42±0.18	19.48±0.24
Proteins	54.59±0.87	68.53±2.21
Ash	7.19±0.15	10.03±0.02
Moisture	86.37±0.11	92.07±0.08
Fatty acid content (%TFA)		
Saturated	21.41	20.73
Monoenoics	32.35	40.41
n-3	35.56	16.50
n-6	8.14	16.45
n-9	21.55	20.54
Total n-3HUFA	3.33	13.52
14:0	1.00	1.59
16:0	13.71	13.39
16:1 n-7	2.23	13.35
18:0	6.26	4.30
18:1 n-9	20.66	16.01
18:1 n-7	6.36	3.43
18:2 n-6	6.35	12.52
18:3 n-3	27.93	2.80
20:1 n-9	0.02	0.72
20:4n-6 (ARA)	**0.49**	**1.35**
20:5n-3 (EPA)	**0.76**	**3.12**
22:6n-3 (DHA)	**0.61**	**8.00**
EPA/ARA	1.56	2.31
DHA/EPA	0.81	2.56
DHA/ARA	1.26	5.92
Oleic/DHA	33.90	2.00
Oleic/n-3HUFA	6.20	1.18
n-3/n-6	4.37	1.00

HUFA. highly unsaturated fatty acid; ARA. arachidonic acid; DHA. docosahexaenoic acid; EPA. eicosapentaenoic acid.

The influence of different enrichments products for live food on wreckfish larvae could not be tested because the amount and the survival of larvae obtained was not sufficient to perform the experiments.

Although the larval mortality was 100% at 26dph, preliminary data of fatty acid profile (**Fig. 12.1.1**) of wreckfish were obtained from 1dph to 26 dph to complete the data obtained previously (until 10 dph. 2nd Report). The data obtained show a decrease of all the groups of fatty acids (expressed in ng/µg DW) with the larval development, while in percentage of total fatty acids, only little differences are observed, saturates present an increase from 21% (1dph) to 31% (26 dph), while n-3 PUFA values decrease from 35.6 to 29.5%, and DHA content decrease from 25.1 to 20.6% at the same period of time.

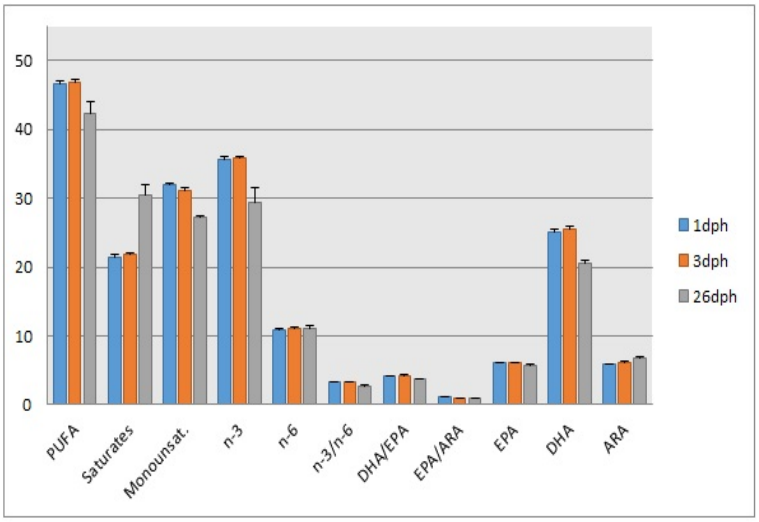

Figure 12.1.1. Fatty acids of wreckfish larvae (%of total fatty acids from 1 to 26 dph).

Task 12.2 Influence of broodstock feeding regimes for fecundity and spawn quality. (Led by Fátima Linares (P19), Blanca Álvarez-Blázquez (P8) & Marisol Izquierdo (P2))

Partners involved: Fátima Linares (P19), Blanca Álvarez-Blázquez (P8), José Luis Rodríguez (P19), Evaristo Pérez (P8), Gema Pazos (P19), Antonio Vilar (P32) &Marisol Izquierdo (P2)

Regarding wreckfish broodstock feeding regimes, a specific dry food for wreckfish broodstock (Dry food 2) was formulated. The results obtained so far demonstrated that most of commercial dry food has too much fat for wreckfish broodstock, so the level of fat should be much lower than in commercial food containing a large amount of n-3 HUFA and the EPA/ARA ratio must be around 1.5, similar to that obtained previously in wild fish. The different diets supplied to wreckfish broodstock from the beginning of the project are shown in **Table 12.2.1.** Following the experiments done before with semi-moist diet and Dry food 1, the effect of three different feeding broodstock regimes, Semi-moist diet, Dry food 2 and Hake/Squid (half and a half) on fatty acid composition of oocytes and eggs from females fed with these diets was checked. Semi-moist diet has been supplied to S1 tank of IEO broodstock; dry food 2 has been supplied to S2 tank of IEO broodstock since February 2016. IGAFA broodstock was feeding only with squid (until December 2016) and with a mixture of hake/squid (half and a half) from that time until now. The feeding was ad libitum. The MC2 broodstock is not used for the feeding experiments since it is placed in an Aquarium and the feeding control became more difficult.

Table 12.2.1. Type of diets used for wreckfish broodstocks feeding

	2014	*2015*	*2016*	*2017*
Stock IEO Tank S1 n = 5	Semi-moist diet	Semi-moist diet	Semi-moist diet	Semi-moist diet
Stock IEO Tank S2 n = 6	Semi-moist diet	Dry food 1	Dry food 2	Dry food 2
Stock IGAFA n = 10	Vitalis Repro/Vitalis Cal	Squid	Squid	Hake/Squid
Stock MC2 n = 17	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M

The semi-moisture diet was a mixture of 14.8% white fish, 14.8% of oily fish, 18% mussels, 17.6% squid and 34.8% fishmeal. The ingredients of dry food 2 are shown in **Table 12.2.2** and consisting of 25% fish meal, 34% squid meal, 7,5% krill etc. Crude protein was 68,2%DW and the lipid content was 12,5% (data from Sparos).

Table 12.2.2. Ingredients of Dry food 2 formulated for wreckfish broodstock

Ingredients	*Dry food 2 %*
Fishmeal 70 LT FF Skagen	25.000
CPSP 90	10.000
Squid meal	34.200
Krill meal (Aker Biomarine)	7.500
Wheat Gluten	7.000
Wheat Meal	7.250
Tuna oil	1.000
Algatrium 70% DHA	0.200
Incromega DHA 500TG	1.000
VEVODAR	1.300
Vit & Min Premix PV01	2.000
Lutavit E50	0.050
Soy lecithin - Powder	1.500
Macroalgae mix	1.000
Antioxidant powder (Paramega)	0.200
Antioxidant liquid (Naturax)	0.200
SelPlex - Se yeast	0.020
Carophyll Pink 10% - astaxanthin	0.050
Nucleotides (Nucleoforce)	0.030
L – Taurine	0.500
Total	100.000

Samples of diets were taken out for biochemical analysis. The values of proteins, total lipids and fatty acids of different diets supplied to IEO and IGAFA broodstocks during 2016 and 2017 are shown in **Tables 12.2.3 and 12.2.4.**

Table 12.2.3. Protein and lipid composition (% of dry weight) of broodstock wreckfish diets

Diets	*Proteins*	*Lipids*
Semi-moist diet	64.66	17.35 ± 2.45
Dry food 2	68.2	12.50 ± 0.53
Hake	82.5 ± 4.65	5.54 ± 0.46
Squid	57.54 ± 1.66	10.79 ± 0.38
Hake/Squid	62.93 ± 4.23	7.89 ± 4.23

Table 12.2.4. Fatty acid profile of diets (% of Total FA)

	Semi-moist diet	*Dry food 2*	*Hake*	*Squid*	*Hake/Squid*
14:0	4.81 ± 0.59	4.07 ± 0.05	5.65 ± 0.99	3.42 ± 0.13	2.48 ± 0.11
16:0	19.51 ± 0.93	17.61 ± 0.04	21.40 ± 0.43	23.28 ± 0.48	21.32 ± 0.40
17:0	1.01 ± 0.18	0.88 ± 0.01	0.96 ± 0.10	0.81 ± 0.01	0.69 ± 0.00
18:0	4.05 ± 0.30	4.14 ± 0.15	4.03 ± 0.24	5.39 ± 0.13	5.03 ± 0.06
Saturated (SAFA´s)	29.96 ± 01.19	27.1 ± 0.21	32.14 ± 1.08	33.45 ± 0.37	29.92 ± 0.43
16:1n-7	4.79 ± 0.51	3.45 ± 0.03	3.31 ± 0.50	2.30 ± 0.31	2.45 ± 0.23
18:1n-9	12.21 ± 1.46	12.33 ± 0.11	8.34 ± 0.54	3.56 ± 0.22	6.02 ± 0.46
18:1n-7	3.82 ± 0.35	3.44 ± 0.01	4.28 ± 0.24	2.20 ± 0.12	2.71 ± 0.13
20:1n-9	3.55 ± 0.64	2.68 ± 0.02	0.47 ± 0.05	2.99 ± 0.08	1.98 ± 0.06
22:1n-11	3.53 ± 1.03	1.79 ± 0.05	0.01 ± 0.02	0.24 ± 0.04	0.06 ± 0.01
Monoenoics (MUFA´s)	31.55 ± 0.97	26.01 ± 0.11	19.98 ± 1.94	13.94 ± 0.48	15.25 ± 0.78
18:2n-6	7.03 ± 0.64	9.35 ± 0.09	1.51 ± 0.10	0.35 ± 0.04	0.85 ± 0.07
18:3n-3	1.16 ± 0.14	1.4 ± 0.04	0.83 ± 0.15	0.20 ± 0.03	0.46 ± 0.05
18:4n-3	1.66 ± 0.26	1.39 ± 0.05	1.78 ± 0.36	0.55 ± 0.11	0.88 ± 0.09
20:4n-6	1.25 ± 0.28	6.9 ± 0.12	0.97 ± 0.06	4.11 ± 0.29	2.73 ± 0.07
20:5n-3	8.84 ± 0.48	8.19 ± 0.11	10.32 ± 0.14	16.94 ± 0.31	14.27 ± 0.23
22:5n-3	1.29 ± 0.24	1.01 ± 0.14	0.83 ± 0.03	0.98 ± 0.07	0.97± 0.06
22:6n-3	15.91 ± 1.16	17.94 ± 0.05	31.02 ± 1.88	28.35 ± 0.90	32.55 ± 1.10
Polyunsaturated (PUFA´S)	38.49 ± 1.65	46.89 ± 0.1	47.88 ± 1.47	52.61 ± 0.40	54.83 ± 0.97
Σn-3	29.40 ± 1.57	30.31 ± 0.14	45.10 ± 1.63	47.40 ± 0.62	49.44 ± 1.00
Σn-6	8.28 ± 0.56	16.25 ± 0.06	2.48 ± 0.13	4.45 ± 0.26	3.58 ± 0.01
n-3/n-6	3.56 ± 0.23	1.87 ± 0.01	18.20 ± 1.53	10.67 ± 0.72	13.81 ± 0.31
DHA/EPA	1.43 ± 0.66	2.19 ± 0.02	3.00 ± 0.15	1.67 ± 0.08	2.28 ± 0.09
EPA/ARA	1.86 ± 0.73	1.19 ± 0.04	10.67 ± 0.80	4.14 ± 0.29	5.22 ± 0.17

Semi-moist diet presents 65% of proteins and 17% of lipids. Dry food 2 has 68 % of proteins and 12% of lipids. Hake has the highest level of proteins (82%) and the lowest amount of lipids (5.5%), squid has 57.5% proteins and 11% of lipids and the mixture of hake and squid (half and a half) has 63%of proteins and 8% of lipids.

PUFA content is higher in hake/squid diet (55%TFA) than in dry food 2 (47%) and semi-moist diet (38%), n-3PUFA values represent the 49% of total fatty acids in hake /squid and 30% and 29% in dry food 2 and semi-moist diets respectively, while n-6 PUFA content reaches a high value in dry food 2 (16%) followed by semi-moist diet (8%) and hake/squid 4%. The diet with a highest level of EPA is hake/squid (14%). Dry food 2 has the highest level of ARA (7%) while in semi-moist diet and hake/squid represent the 1% and 3% of total fatty acids. The EPA/ARA ratio is 5 in hake/squid and 1 in dry food 2, which is similar to the one previously obtained in tissue samples of wild fish.

Samples of oocytes (Ø>700µ) were obtained by gonadal biopsies of females fed with the three diets, which were described above. All the samples were frozen at -80ºC, and freeze dried to be analysed.

Results of oocytes fatty acid composition from females of IEO and IGAFA broodstocks are shown in **Table 12.2.5.**

Table 12.2.5. Fatty acid profile of oocytes (Ø >700 µ) from females fed with different diets (%TFA)

Diets oocytes	*Semi-moist diet OSM 0.710-2.033 µ*	*Dry food 2 ODF 0.778-2.118µ*	*Hake/Squid OHS 0.831-1.388µ*
14:00	1.55 ± 0.11a	1.72 ± 0.03a	1.24 ± 0.13b
16:00	15.09 ± 0.63	14.68 ± 1.50	14.46 ± 0.85
17:00	1.00 ± 0.05a	0.75 ± 0.11b	0.62 ± 0.11b
18:00	5.02 ± 0.19	4.77 ± 0.57	4.70 ± 0.26
Saturated (SAFA´s)	22.97 ± 0.57	22.17 ± 2.07	21.22 ± 1.15
16:1n-9	1.31 ± 0.04	0.98 ± 0.22	1.16 ± 0.20
16:1n-7	4.46 ± 0.39	4.49 ± 0.49	4.39 ± 0.32
18:1n-9	17.83 ± 0.34	19.41 ± 4.21	17.08 ± 1.83
18:1n-7	5.36 ± 0.38	4.78 ± 0.74	6.00 ± 0.77
20:1n-9	1.95 ± 0.27	1.94 ± 0.33	1.63 ± 0.11
Monoenoics (MUFA´s)	33.58 ± 1.10	33.91 ± 4.33	32.35 ±2.89
18:2n-6	4.45 ± 0.56b	5.58 ± 0.16a	3.25 ± 0.47c
20:4n-6	1.88 ± 0.36b	6.74 ± 1.25a	1.61± 0.99b
20:5n-3	7.57 ± 0.19b	5.49 ± 0.35c	10.72 ± 1.13a
22:5n-3	2.80 ± 0.26	2.76 ± 1.40	3.63 ± 0.33
22:6n-3	24.60 ± 1.53ab	21.32 ± 1.90b	25.48 ±1.16a
Polyunsaturated (PUFA´s)	43.46 ± 1.34	43.92 ± 4.10	46.42 ± 2.29
Σn-3	36.87 ± 1.59ab	31.35 ± 3.31b	41.31 ± 2.15a
Σn-6	6.34 ± 0.84b	12.32 ± 1.21a	4.86 ± 0.50b
n-3/n-6	5.90 ± 0.93b	2.58 ± 0.20c	8.63 ± 0.90a
DHA/EPA	3.25 ± 0.15b	3.91 ± 0.27a	2.40 ± 0.18c
EPA/ARA	4.16 ± 0.59b	0.86 ± 0.10c	6.71 ± 0.38a

There are not significant differences in SAFAS's, MUFA's and PUFA's of oocytes from females fed with semi-moist diet (OSM), dry food 2 (ODF) and the mixture of hake and squid (OHS) with values of SAFA´s between 21-23%, MUFA 32-34% and PUFA 43-46%.

The highest content of n-3 was observed in OHS (41%), while OSM had 37% and ODF 31% of total fatty acids. In the case of n-6 PUFA the values are much higher (12%) in ODF than in OHS and OSM (5-6%).

In individual fatty acids the most important saturates are 16:0 and 18:0, which represent 14-15% and 5% respectively of total in the three kinds of oocytes. In the case of MUFA, 18:1n-9 represents the 17-19% of TFA, 18:1n-7, 5-6% and 16:1n-7 the 4% in the three types of oocytes.

Regarding n-3PUFA, the values of 20:5n-3 (EPA) and 22:6n-3 (DHA) are significant differences in the oocytes from females fed with the three diets. EPA has the highest value in OHS, 11% of total, 8% in OSM and 5% in ODF. DHA values have significant differences between OHS and ODF (25 and 21% respectively) and there are not differences between OSM and ODF and OSM and OHS.

In the case of n-6 PUFA, the 20:4n-6 (ARA) level is the highest in ODF representing the 7% of total fatty acids in comparison with OSM and OHS with 2%. The 18:2n-6 presents values of 6% (ODF) , 4% (OSM) and 3% in OHS.

The n-3/n-6 ratio is high in OHS (9) because of the high amount of EPA and has values of 6 and 3 in OSM and ODF respectively. DHA/EPA ratio have values of 4, 3 and 2 in ODF, OSM and OHS respectively, while the EPA/ARA ratio are higher in OHS (7) than in OSM (4) and a much lower value is obtained in ODF (1) because of the high value of ARA.

On the other hand, as mentioned above, some samples of viable eggs of females from IEO broodstock and IGAFA broodstock were taken out for biochemical and fatty acid composition analyses. Lipid (%DW) and fatty acid composition of eggs are shown in the **Fig 12.2.1.**

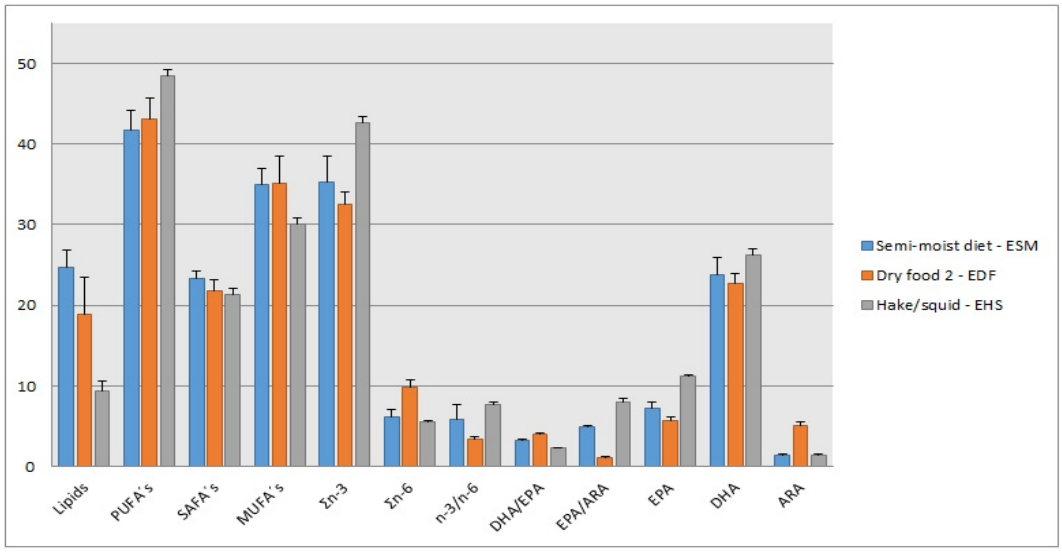

Figure 12.2.1. Lipid (%DW) and fatty acid composition (%TFA) of eggs from females fed with different diets

The total amount of lipids is higher (significant differences) in eggs from females of the IEO broodstock fed with semi-moist diet, ESM, and with dry food, EDF, (25 and 19%DW respectively) than in eggs from

females from the IGAFA broodstock fed with hake and squid (half and a half), EHS, with 9% of lipids which is clearly influenced by the food (hake and squid).

With respect fatty acid composition, the total amount of PUFA's is higher in EHS (48%TFA) than in EDF and ESM (41-43%), on the contrary MUFA's have the lowest level in EHS (30%) while EDS and EDF have 35%. n-3 PUFA are higher in EHS (43%) than in ESM (35%) and EDF (32.5%) while n-6 PUFA have higher values in EDF (10%) than in the rest of eggs (6%). The DHA content is a little higher in EHS with values of 26%TFA than in the rest of the eggs. EHS eggs have more amount of EPA (11% of total) than EDF and EHS (6-7%). In the case of ARA the highest values were found in EDF (5%) while ESM and EHS have values about 1-1.5%.

The tendency of these results is similar to the one obtained previously in oocytes from females fed with the same diets. A correlation was found between the fatty acid profile of wreckfish broodstock feeding and eggs fatty acid profile.

On the other hand some samples of sperm were taken out from males of different broodstock (Stock IGAFA, stock IEO and stock AF) to be characterized (velocity and motility of sperm) and to perform the fatty acid profiles. As it was reported previously (2nd Periodic Report and D6.1, wreckfish males produced a high volume of sperm with a high percentage of motile cells and this high speed was associated with a long swimming duration compared to other marine fish. The fatty acid profile of sperm from males of different stocks is shown in **Table 12.2.6.**

Table12.2.6. Fatty acid composition (mean values, %TFA) of sperm from males from different wreckfish broodstocks

Stocks sperm	*Stock IGAFA*	*Stock IEO*	*Stock AF*
Saturated (SAFA´s)	32.02 ± 1.92	32.56 ± 0.19	31.71 ± 1.96
Monoenoics (MUFA´s)	10.82 ± 1.80	9.35 ± 0.79	9.81 ± 0.35
Polyunsaturated (PUFA´s)	57.15 ± 0.60	58.10 ± 0.63	56.43 ± 1.93
Σn-3	51.50 ± 0.44	48.92 ± 4.54	49.86 ± 1.49
Σn-6	5.52 ± 0.62b	8.99 ± 3.97a	6.44 ± 0.45b
n-3/n-6	10.15 ± 1.96a	6.22 ± 2.62b	7.85 ± 0.28b
DHA	36.67 ± 1.96	36.89 ± 2.96	36.47 ± 1.74
EPA	10.81 ± 1.12a	8.78 ± 1.76b	9.91 ± 0.36ª
ARA	4.94 ± 0.75b	7.84 ± 3.85a	5.91 ± 0.40b
DHA/EPA	3.45 ± 0.53b	4.37 ± 0.78a	3.71 ± 0.29b
EPA/ARA	2.49 ± 0.70a	1.35 ± 0.69b	1.70 ± 0.16b

There are not significant differences between the main groups of fatty acids in the sperm of males from the different stocks, with values of SAFA`s between 32-33%, MUFA`s 9-11% and PUFA´s 56-58% of the total

fatty acids. The n-3 PUFA content varies between 49-51% of the TFA and the n-6 PUFA content is higher in sperm from IEO stock (9%TFA) than in stock IGAFA (5%) and AF (6%).

Deviations from Annex I and their impact:

Task 12.1.The influence of different enrichments products for live food on wreckfish larvae could not be tested because the amount and the survival of larvae obtained were not sufficient to perform the experiments. It will be necessary to obtain batches of larvae that allow the necessary quality to conduct the experiments. As indicated in the WP18 report, changes are being made in husbandry and larval culture to achieve greater larval survival. There were not deviations in Task 12.2.

WP 13 Nutrition – grey mullet

WP No:	13	**WP Lead beneficiary:**		P19. IOLR
WP Title (from DOW):	Nutrition – grey mullet			
Other beneficiaries (from DOW):	P2. FCPCT	P3. IRTA	P13. UNIBA	P18. CTAQUA
Lead Scientist preparing the Report (WP leader):	William (Bill) Koven			
Other Scientists participating:	Marisol Izquierdo (P2), Alicia Estevez (P3), Aldo Corriero (P13), Rocio Robles (P18)			

Objectives

1. Improve enrichment products, weaning, grow out and broodstock diets,
2. Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Studies on the taurine requirement at different stages of development during the larval rearing of grey mullet showed a significant ($P<0.05$) effect of dietary taurine on larval growth and survival. This effect is strongest during rotifer feeding compared to *Artemia* feeding which also significantly ($P<0.05$) influences growth in later stages of larval development. Nevertheless, the results indicated that larvae fed both high taurine enriched rotifers and *Artemia* survived and grew (length) significantly better and these protocols have been recommended for larval rearing.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The larval and juvenile taurine studies were successfully carried out reinforcing the conclusion that rotifer taurine has a far reaching and significant effect on larval and juvenile growth from 12 to 44 dph. As un-enriched *Artemia* have considerable levels of taurine, there appears to be no added benefit of feeding taurine enriched *Artemia* on larval weight. However, fish that had fed on the high taurine rotifers or both high taurine rotifers and Artemia were markedly ($P<0.05$) longer than fish in the rest of the treatments. In fact, larvae that were fed the high taurine diets from 2-19 dph survived significantly ($P<0.05$) better than the rest of the treatments.

Dietary taurine during rotifer feeding (4.4 and 6.4 mg taurine g -1 DW) had a prolonged effect at 44 dph, which was weeks after rotifer feeding had ceased. Consequently, the continuity of taurine influence during juvenile growth was monitored. The fish from the taurine (4.4 and 6.4 mg g -1 DW) treatments were significantly larger than the low taurine control. This meant that fish from each treatment were maintained with similar sized cohorts.

Summary of progress towards objectives (31-48 Mo):

In **Sub-task 13.1.1** the effect of dietary DHA on larval performance was investigated. This study found no dietary DHA effect on larval performance, in terms of wet weight gain and rotifer consumption rate. This suggests that 5.5% DHA in the commercial enrichment "Red Pepper" is sufficient for the growing of this species, although DHA levels below this weren't tested. Interestingly, the 5.5% DHA DW diet level resulted in significantly improved larval survival over the other higher DHA treatments at 40 dph or 15 days after the rotifer treatments had ceased. This emphasizes the importance of feeding an effective level of DHA at the rotifer stage on survival in later development stages. In **Sub-task 13.2.1** preliminary results suggested that the hepatic CSD pathway for taurine synthesis, in the absence of dietary taurine, is still active but that the expression of this key gene increases with increased levels of dietary taurine until 1% where its expression drops at the high dietary taurine level of 2%. Similarly, the gene for ADO, which is a key enzyme in another less dominant pathway in taurine synthesis, was highly expressed in the 1 % taurine fish livers and then dropped in fish feeding on the 2% taurine diet. Preliminary findings in **Sub-task 13.2.2** also show a taurine dose dependent response on the gene expression of hepatic CYP7A1 in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of this enzyme in fish consuming the highest dietary taurine level (2% DW diet). In **Sub-task 13.3.1** the effect of dietary DHA on juvenile mullet wet weight gain found no treatment effect on growth and that variability in weight gain was mostly attributed to age (dph). In addition, all treatment fish exhibited generally similar size distribution in their respective populations and as well as excellent survival (92.4, 88.8 and 97.6% in the 0.4, 0.8 and 1.2% DHA DW diets, respectively). In **Sub-task 13.3.2** the effect of four levels of Tau supplementation on best performing DHA treatment using non-fish meal grow out diet from 13.3.1. The results showed that grey mullet juveniles have a minimum 0.5% taurine requirement**,** which is within the range of taurine requirements measured in a variety of marine teleosts. However, there was no taurine effect on size distribution. In **Sub-task 13.4.1** Notable differences in the fatty acid profiles when comparing the gonads from wild and domestic adult grey mullet broodstock were found in saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids of the n-3 and n-6 groups. Differences were particularly marked in EPA in both sexes and DHA in wild males compared to captive cohorts. This was primary rational to investigate the effect of replacing soybean oil (VO) with fish oil (FO) in mullet broodstock diets. The percent hatching of eggs from the FO broodstock was significantly ($P<0.05$) higher than eggs from the VO broodstock, while survival in larvae in the two treatments at the end of 0 dph were not significantly different ($P>0.05$) from each other, although the FO was higher. Larvae from the FO broodstock, regardless of salinity exposure, demonstrated 100% swim bladder inflation by 5 dph, where there was no swim bladder inflation in fish from the VO broodstock during the course of the food deprivation study. In **Sub-task 13.4.2** adult wild grey mullet showed large ripe ovaries with late vitellogenic (Vgs) oocytes as the most advanced oocyte stage while hatchery-produced grey mullets showed extensive alpha and beta atresia of vitellogenic follicles, a sign of cessation of the reproductive activity. The highest relative levels of Vgs mRNA were observed in adult wild specimens, which are indicative of a residual Vtg transcription activity in specimens at the end of vitellogenesis. The domestic mullet brooders showed very low Vgs expression levels corresponding to a negligible Vgs transcription activity agreeing with spent condition (extensive atresia of vitellogenic follicles)

Details for each Task

Task 13.1 Improvement of larval and juvenile performance (led by IOLR; Bill Koven).

Sub-task 13.1.1 The effect of dietary DHA on larval and juvenile performance

Introduction

A number of authors have shown that the long chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid (DHA; 22:6n-3) promotes growth more effectively than the other LCPUFAs; eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) in marine fish larvae (Watanabe et al., 1989; Takeuchi et al., 1990; Toyota et al., 1991; Watanabe, 1993, Koven et al., 1993, Wu et al., 2002). The contribution of DHA to weight gain lies in its contribution to membrane fluidity and function in the neural membranes of the

eyes and brain as well as its involvement in immune function and gene expression (Izquierdo and Koven, 2011; Arts et al., 2009). Optimum DHA levels in larval feeds to promote growth and survival range from 0.5 % for *Acanthochromys poliacanthus* (Southgate and Kavanagh, 1999) to 2.5 for Atlantic halibut, *Hippoglossus hippoglossus* (Hamre and Harboe, 2008). When first feeding larvae are fed a diet deficient in DHA, high mortalities and reduced growth rates are likely to occur in only 10-15 days (Izquierdo et al., 1989; Sargent et al., 1997), while DHA will improve larval performance in a dose dependent manner. The essential fatty acid requirements of larval grey mullet have not been clearly defined although analysis of eggs and carcass content has been reported (Tamaru et al. 1992). In addition, there is evidence for Δ6 desaturase activity (Argyropoulou et al. 1992), which could modulate the essential fatty acid requirement. However, marine fish larvae are strict carnivores and its natural marine environment would be rich in n-3 LCPUFAs suggesting a dietary need for essential fatty acids such as DHA. The aim of the larval study in this task investigated the effect of dietary rotifer DHA on larval performance, in terms of growth (length, weight), survival and whole fatty acid composition.

Methods and Materials

The experimental system consisted of twelve 400 l tanks where UV treated, filtered (10 µm) ambient sea water (40 ‰) at 25 ^{o}C (computer controlled; Gavish, Israel) entered the bottom of the tanks and exited near the top through a 500 µm filter. Three rotifer DHA enrichment treatments were tested where their enrichment preparations contained 5.5, 12 and 20% DHA of total fatty acids (TFA). This allowed the testing of each of these treatments in replicates of 4 tanks/treatment. 2-15 dph larvae were fed twice daily the rotifer treatments (10 rotifers/ml) together with adding $0.5x10^6$ cells/ml of *Nannochloropsis oculata* to "green" the tanks. Rotifer samples were taken on at least 4 days and larvae were sampled for weight gain and fatty acid content on 15 dph. After 15 dph, all larvae were fed *Artemia* nauplii enriched on the routine essential fatty acid enrichment preparation "Red Pepper (Bernaqua, Belgium), which had 5.5% DHA of TFA to 20 dph followed by offering a weaning diet (caviar, Bernaqua, Belgium) until 40 dph (100-300 µm).

Results and Discussion

The relevance of DHA in diets for marine fish larvae has been well documented (Watanabe et al., 1989; Izquierdo, 1996; Sargent et al., 1999) and its positive effect on survival has been related to its important role in stress control (Watanabe et al., 1993; Izquierdo, 2005, Ganga et al., 2006), immune system development (Montero et al., 2003) and improvement of health and bacterial resistance in fish larvae (Brandsden et al., 2003). However, apart from DHA's contribution to improved survival and stress resistance during early larval development, DHA, more than other EFAs, appears to be the most effective larval growth promoter (Watanabe et al., 1989; Watanabe and Kiron, 1994) and necessary for normal behavior (Masuda et al., 1999).

However, in the present study, there was no dietary DHA effect on larval performance in terms of wet weight gain (**Figure 13.1.1)** and rotifer consumption (**Figure 13.1.2**). In fact, the variability in these parameters was significantly ($P<0.0001$) due to age (dph) and not dietary DHA, which suggests that 5.5% DHA in the commercial enrichment "Red Pepper" is sufficient. On the other hand, it is still unknown if dietary DHA levels lower than this would still provide good larval performance and/or if the larvae have any capability to synthesize this essential fatty acid. The production of Δ6 and Δ5 desaturases is suggested in current studies on juvenile grey mullet conducted at the IOLR. Interestingly, the low dietary DHA treatment resulted in highly significant improved larval survival over the other higher DHA treatments on 40 dph (**Figure 13.1.3**), which is 25 days after the rotifer treatments have ceased. This emphasizes the importance of feeding DHA at the rotifer stage on survival in later development stages despite the fact that the growth rates are much higher during *Artemia* and weaning diet feeding. A clearer picture will emerge once all fatty acid analyses and dry weight results (40 dph) have been completed.

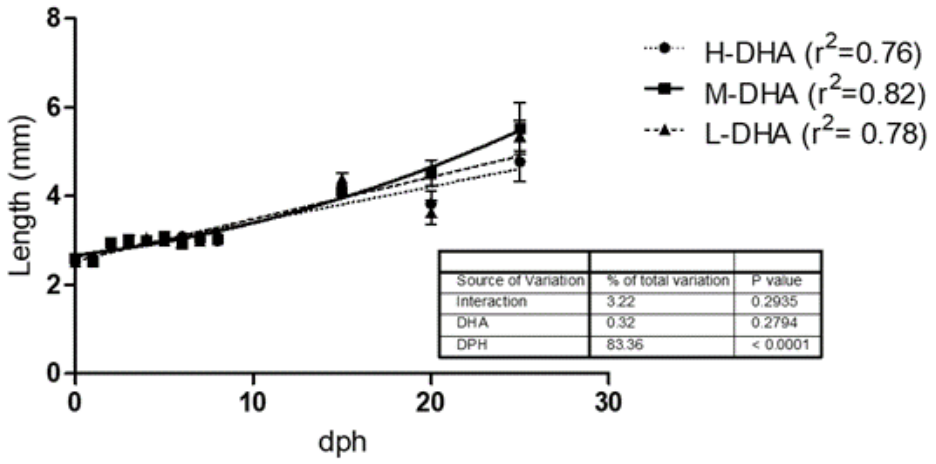

Figure 13.1.1. The effect of feeding the rotifer DHA treatments on the standard length of 2-15 dph mullet larvae and on 20 and 25 dph fish, which were 5 and 10 days, respectively, after the rotifer treatments had stopped (n=4). Akaike's Information Criteria (AIC) found the linear model best represented the data in the high (probability =75.6%) and low DHA (probability=60.23%) treatments whereas a quadratic model was more suitable (probability=85.21%) to describe feeding the moderate DHA rotifers. The r^2 values for the high, medium and low rotifer DHA treatments were 0.76, 0.82 and 0.78, respectively. Two-way ANOVA analysis demonstrated that the source of variation was very significantly (P<0.0001) due to dph and not DHA level.

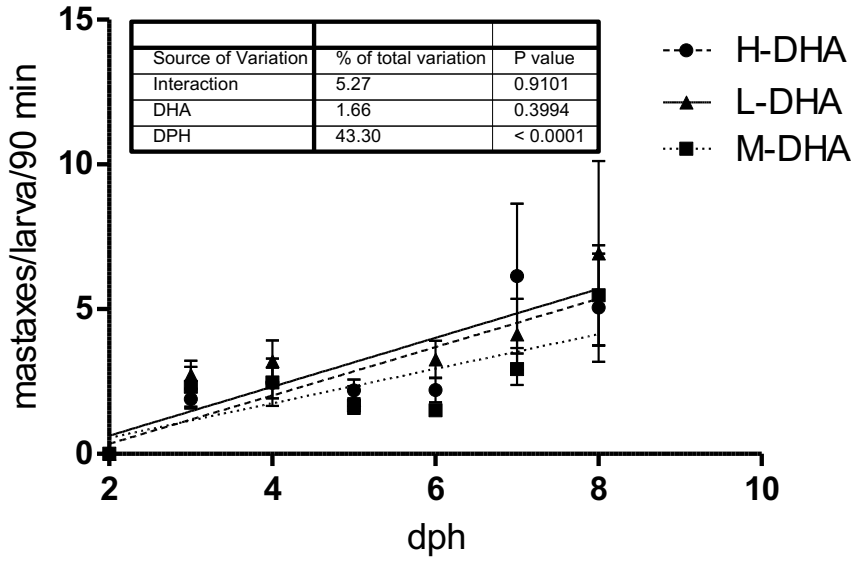

Figure 13.1.2 The effect of feeding the rotifer DHA treatments on rotifer consumption (average mastaxes/larva) in 2-8 dph larvae 90 minutes after the first feeding (n=4). Akaike's Information Criteria (AIC) found the linear model best represented the data in the high, moderate and low DHA treatments (probability=79.28, 67.55, 78.78%, respectively). Two-way ANOVA analysis demonstrated that the source of variation was very significantly (P<0.0001) due to dph and not DHA level (P>0.05).

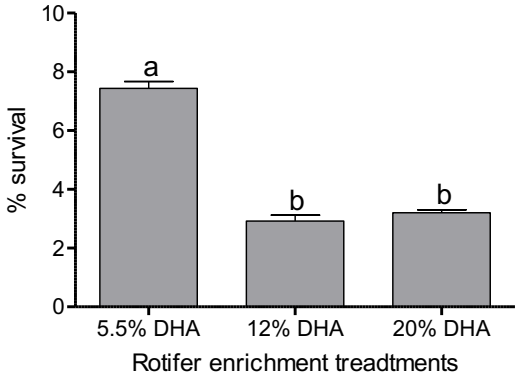

Figure 13.1.3 The effect of rotifer DHA enrichment treatments (5.5, 12, 20% DHA of TFA) on survival at 40 dph, which is 25 days after the rotifer treatments had stopped (n=4). Percentage values were arsine transformed and then analyzed by one way ANOVA which demonstrated that fish fed the low rotifer DHA treatment (5.5% of TFA) survived significantly better ($P<0.0001$) than cohorts fed the higher DHA rotifers.

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Sub-task 13.1.2 Using the most effective DHA-taurine diet from (13.1) to investigate the effect of supplemental ArA on larval growth, survival, presence of urinary crystals, as well as synchrony in "silvering" during metamorphosis.

Results from studies on dietary DHA in larval grey mullet are currently being tabulated and analyzed. A photoperiod spawning is expected in January and the effect of ArA in rotifers enriched as well on the most effective levels of DHA and taurine on grey mullet larval performance will be performed. If January spawning is unsuccessful or does not provide sufficient numbers of good quality fertilized eggs, then the experiment will be carried out next Fall.

Task 13.2. Determining mullet nutritional needs for improved weaning to a dry diet (led by IOLR, Bill Koven).

Sub-task 13.2.1. Determine expression of the taurine rate limiting enzyme; cysteine sulfinate decarboxylase (CSD) at various stages (larval and grow out)

Introduction

An essential nutrient in the diet of the grey mullet is the β-amino sulfonic acid taurine, which is not incorporated into proteins, but plays an array of critical roles in its free form. These include involvement in bile salt synthesis, anti-oxidative defence, cellular osmoregulation, as well as contributing to visual, neural and muscular function (Fang et al., 2002; Omura and Inagaki, 2000). Taurine cannot be synthesized in carnivorous teleosts and therefore must be provided in the diet. The yellowtail (*Seriola quinqueradiata*), bluefin (*Thunnus thynnus*) and skipjack (*Katsuwonus pelamis*) tunas as well as the Japanese flounder

(*Paralichthys olivaceus*) were found deficient in cysteine sulfinate decarboxylase (CSD), a key enzyme in the taurine synthesis pathway (Yokoyama et al., 2001; Goto et al., 2003; Chen et al., 2005; Takagi et al. 2008). However, it is unclear if herbivorous and omnivorous fish, where vegetation is a major component of their diet and is taurine poor, would have taurine synthesis capability. It has been reported that common carp (*Cyprinus carpio*) do not have a dietary taurine requirement and no significant expression of CSD but retain taurine in the tissues suggesting that this species synthesizes taurine through a different pathway (Kim et al., 2008).

Taurine is synthesized either from the oxidation of cysteine via cysteine dioxygenase (CDO), which generates cysteine sulfinate that is decarboxylated by cysteine sulfinic acid decarboxylase (CSD), or from the oxidation of cysteamine by cysteamine (2-aminoethanethiol) dioxygenase (ADO). Both pathways generate hypotaurine, which is oxidized to taurine. This ability may attenuate or possibility eliminates the need for dietary taurine in some species. Although, studies carried out on juvenile grey mullet at the IOLR (P4), which is omnivorous, demonstrated a significant dietary taurine requirement (see 13.3.2), at the 0.5% DW dietary level, it is unclear if this species is able to synthesize taurine through the CSD and/or ADO pathways.

As a result, fish from the different taurine treatments were taken at the end of the 13.3.2 study and their livers removed and analysed for the gene expression of ADO and CSD. These analyses are presently being completed. However, the initial results are presented here which represent clear trends that are expected to be reinforced once all samples are analysed.

Methods and Materials

Total RNA was extracted from *Mugil Cephalus* liver tissue by the guanidiniumthiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) using Bio-Tri RNA reagent (Bio Lab Ltd., Jerusalem, Israel). The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (Synergy HT, BioTek). The purity of each sample was assessed for proteins by the 260 vs. 280 nm ratio. After residual DNA removal, qScript™ cDNA Synthesis Kit (Quanta) was used for the synthesis of cDNA from RNA samples according to manufacturer's protocol. Degenerate primers were designed in order to sequence the desired genes. The PCRs were carried out in a final volume of 25 µl using the GoTaq® Green Master Mix (Promega, Madison, WI) and 25 pmol of each Primer. PCR products were purified using QIAquick PCR Purification Kit (QIAGENE, Hilden, Germany) and sequenced by Hy- Labs (Rehovot, Israel). Gene identity was confirmed by comparing the obtained sequences with those available at the Genebank (http://www.ncbi.nlm.nih.gov/Genbank/). Real time PCR was performed using PerfeCTa® SYBR® Green FastMix®, Low ROX™ (Quanta Biosciences, Inc. MD, USA). Gene specific primers (Table X) were designed and synthesized by Agenktek (Tel-Aviv, Israel).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed (in triplicates) in a total reaction volume of 10 µl, consisting of the respective primer set (300 nM), cDNA template and PerfeCTa® SYBR® Green FastMix®, Low ROX™ (QuantaBioSciences, Inc. MD). Since the fluorophore, Fast SYBR Green®, binds in a nonspecific manner to double strand DNA, it is necessary to ensure that the amplified PCR product is homogenic and unified. Hence, the presence of a single amplicon was verified at the end of each run via a dissociation analysis (Melting curve), by which fluorescence was quantified in regard to temperature rise. When temperature increases, DNA strands separate and the DNA bounded fluorophore releases. A single peak in a gauss curve, which outlines the change in fluorescence as a function of temperature (-dF/dT), will indicate a homogenous PCR product.

To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR experiments included minus-reverse transcriptase controls (i.e., PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = $2^{-\Delta\Delta Ct}$, Ct – threshold cycle (Livak and Schmittgen, 2001).

Results

The preliminary results show a taurine dose dependent response in the gene expression of both cysteine sulfinic acid decarboxylase (CSD) and cysteamine (2-aminoethanethiol) dioxygenase (ADO) in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of these enzymes in fish fed the highest dietary taurine level (2% DW diet). The RQs of CSD and ADO (**Figure 13.2.1**) increased 9.2 and 59.7 times, respectively from the control to 1% taurine. Moreover, the results show considerable CSD synthesis when the fish are not consuming any taurine although the ADO pathway is not stimulated until fish are ingesting 1%.

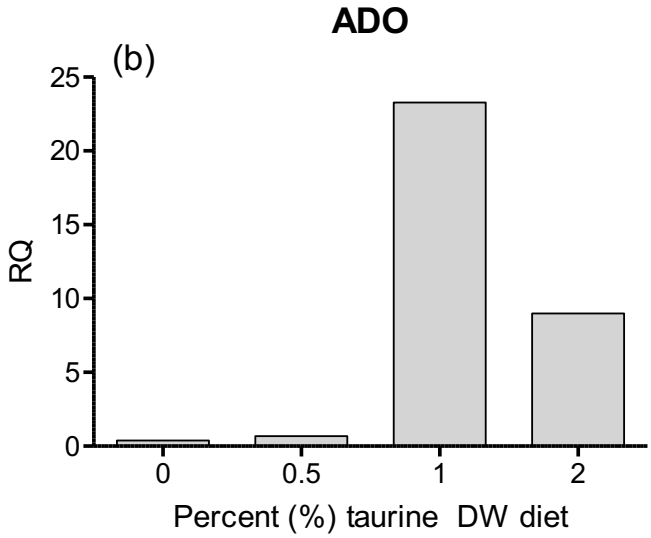

Figure 13.2.1. The effect of dietary taurine (0, 0.5, 1.0, 2.0% DW diet) on the gene expression of cysteine sulfinic acid decarboxylase (CSD) and cysteamine (2-aminoethanethiol) dioxygenase (ADO) in juvenile fish. Statistics will be performed once all samples are analysed.

Discussion

The preliminary results suggest not only that the CSD pathway is active in the main taurine synthesizing pathway in the absence of dietary taurine but that the expression of this key gene increases with increased levels of dietary taurine until 1% where CSD expression drops at the high dietary taurine level. The synthesis of taurine in the liver when levels of this nutrient are increasing in the diet seems counter intuitive. However, taurine can function as an osmolyte to maintain cell volume. Conceivably, increased taurine in the blood circulation of the liver, due to higher dietary taurine, may stimulate increased synthesis within liver cells to reduce osmotic pressure across the membrane in order to prevent cell shrinkage and changes in intracellular hydro-mineral balance. The cell taurine content of an astrocyte primary culture varied with extracellular taurine concentration through endogenous synthesis via the CSD pathway (Reymond et al. 1996). However, Bitoun and Tappaz (2000) argued that taurine synthesis would not be sufficient to explain taurine intracellular content, which was likely controlled by synthesis of the TauT transporter that allowed more extracellular taurine to enter the cell. On the other hand, the capacity of endogenous taurine synthesis in liver cells may be greater than brain astocyte cells investigated in that study. Moreover, the increased RQ of almost 60 times of the ADO pathway when fish were consuming 1% DW diet suggest added biosynthesis ability to cope with increasing extracellular taurine. Taking this one step further, it is possible to speculate that at very high levels of dietary taurine, intracellular biosynthesis shuts down and possibly, in parallel, TauT transporter synthesis increases.

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Sub-task 13.2.2 (IOLR) Determine expression of rate limiting enzyme of bile salt synthesis, cholesterol 7α-hydroxylase (CYP7A1) at various stages (larval and grow-out)

Introduction

One of the major roles for taurine is to conjugate with bile acids such as cholic acid or chenodeoxycholic acid in the liver, which is then stored in the gall bladder. Conjugated bile acids, when released into the lumen of the intestine after feeding, emulsify fats to make them more accessible for digestion and absorption. In fish, bile acid is conjugated not with glycine but with taurine by the bile acid- coenzyme A (CoA)–amino acid N-acyltransferase in the liver and implies that taurine is critical in fish for lipid digestion and absorption (Huxtable, 1992). Moreover, 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in bile salt synthesis (Fukuda et al., 2011) and has been associated with dietary taurine and its growth promoting properties. Fish liver samples taken in 13.3.2 for analysis of the gene expression of CSD and ADO, were also analysed for CYP7A1 in order to determine if dietary taurine up regulated bile salt synthesis. Fatty acid profiles of the eyes, liver and muscle are still being determined and will complement the initial CYP7A1 results presented here (Fig. 13.2.2).

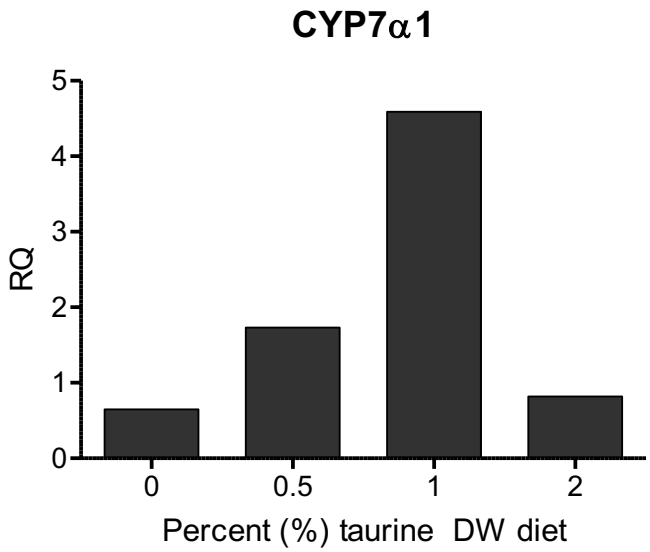

Figure 13.2.2. The effect of dietary taurine (0, 0.5, 1.0, 2.0% DW diet) on the gene expression of CYP7A1 in juvenile fish. Statistics will be performed once all samples are analysed.

Methods and Materials (see 13.2.1)

Results

The preliminary results show a taurine dose dependent response in the gene expression of CYP7A1 in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of this enzyme in fish consuming the highest dietary taurine level (2% DW diet). The RQ of CYP7A1 increased 7 times from the control to 1% taurine and then decreased to similar expression levels of the control diet.

Discussion

The results suggest that taurine is being used to synthesize bile acid and maybe one of the reasons how this nutrient promoted significant growth in fish fed ≥ 0.5% taurine in the diet. Taurine dietary supplementation of a number of species resulted in increased bile salt production (Kim et al. 2007, 2015). This was supported by results from Yu et al. (2012), who found a correlation between dietary taurine and CYP7a1 synthesis in juvenile turbot, *Scophthalmus maximus*. In addition, a taurine deficiency in fish has been associated with green liver syndrome caused by decrease in production of bile pigments (Sakai et al. 1990). Results from the fatty acid analyses of the eyes, liver and muscle, which are currently being performed, might reinforce the conclusion that dietary taurine increases bile salt production by showing increased fatty acid assimilation into specific or all of these tissues as a function of dietary taurine at level from 0 to 1.0% DW of diet. Koven et al. (2016) reported on selective tissue assimilation of fatty acids when taurine was supplemented to the diet of white grouper (*Epinephelus aeneus*).

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Task 13.3 Determining grey mullet nutritional needs for a more cost-effective production (led by IOLR, Bill Koven).

Sub-task 13.3.1 Effect of DHA/EPA/ARA ratio in non-fish meal grow-out diets on fish performance.

Introduction

The seawater servicing 200 l v-tank experimental system is only filtered and not as yet UV treated as originally planned and is prone to bacterial infection. As a result the DHA and arachidonic acid studies will be performed separately and not in parallel in the 400 l v-tank hatchery system, which has filtered (10 µm) and UV treated seawater that is also temperature and salinity controlled (Gavish, Israel).

Although the DHA requirement during the juvenile stage generally decreases compared to larval development, the necessity for this essential fatty acid is still considerable, particularly if the fish remains strictly carnivorous. In fish that become omnivorous/herbivorous after metamorphosis and/or inhabit freshwater or brackish water, such as the grey mullet, may have some capability to produce Δ5 and Δ 6 desaturases and elongases to synthesize DHA, EPA and ArA from shorter chain 18 carbon n-3 and n-6 polyunsaturated fatty acid precursors. An indigenous production of essential LCPUFAs may result in a reduced dietary requirement for them. In fact, Zouiten et al., (2008) reported that mullet have desaturase activity as they begin to feed on plant and detritus as juveniles seek out less saline environments. The question remains what is the dietary DHA requirement and the contribution of this essential fatty acid to growth and body composition.

Methods and Materials

A study on the effect of dietary DHA on juvenile mullet performance, in terms of growth and survival was carried out in the late spring of 2016 on fish reared from hatching (26/1/16) at the ARDAG hatchery in Eilat, Israel and delivered to the IOLR when fish were 70 dph (2.7 ± 0.15 g) and maintained until the beginning of the experiment. The experimental system consisted of fifteen, 400 l V-tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25 ^{o}C entered the bottom of the tanks and exited near the top through a 500 µm filter. Fish aged 138 dph (3.61 g ± 0.01 g) were stocked (45 fish/tank) in each of the tanks allowing the testing of three DHA treatments (0.4, 0.8 and 1.2 % DW diet) in replicates of 5 tanks per treatment until 222 dph (12 week experiment). Before stocking, 5 fish were sacrificed as a representative example at time 0 (T0) and samples of the liver, muscle and eyes taken for fatty acid analysis. The fish were fed 1 mm pellets produced by Sparos Ltd (Faro, Portugal) using the IOLR mullet closed formula at 5% of estimated tank biomass distributed over 5 rations per day.

At approximately monthly intervals a subsample of fish were individually live weighed and returned to the tank. The study continued until the fish had grown at least 200%. At the end of this part of the experiment and at the end of the study, the fish were weighed and fish samples taken from each tank for fatty acid analysis of the liver, eyes and muscle. The analysis of these samples has been delayed due to technical problems with the gas chromatograph and has recently resumed.

Results

In **Figure 13.3.1** wet weight gain was almost exclusively (86.34 % of variation) the result of age and not dietary DHA. All diets showed linear growth (linear was compared with non-linear growth using AIC analysis) with relatively high r^2 values while the DHA effect on size distribution was negligible (0.18%). In addition, all treatment fish exhibited generally similar size distribution in their respective populations (**Figure 13.3.2**). All DHA treatments demonstrated very high percentage of survival which were 92.4, 88.8 and 97.6% in the 0.4, 0.8 and 1.2% DHA DW diets, respectively (**Figure 13.3.3**).

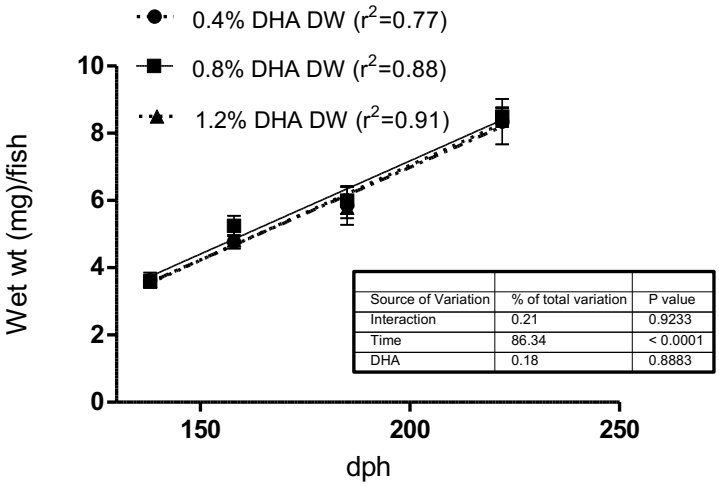

Figure 13.3.1. The effect of dietary DHA on wet weight with days post hatching (DPH) (n=4). Akaike's Information Criteria (AIC) found the linear model best described the results correlating 0.4, 0.8 and 1.2% DHA treatments with age (probability = 80.1, 82.6, 62.7%, respectively). Two-way ANOVA found that wet weight significantly increased with time (DPH) ($P<0.0001$) which was not markedly affected by dietary DHA ($P>0.05$).

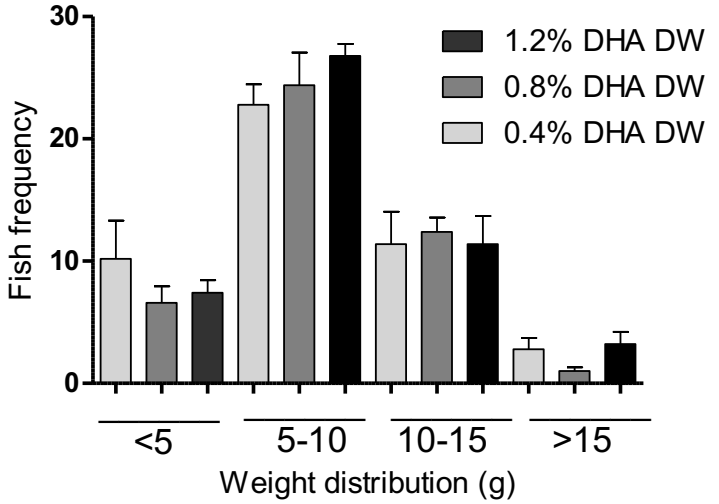

Figure 13.3.2 The effect of dietary DHA on weight distribution in 222 dph fish fed the 0.4, 0.8 and 1.2% dietary DHA treatments. (n=4). ANOVA was not significant ($P>0.05$).

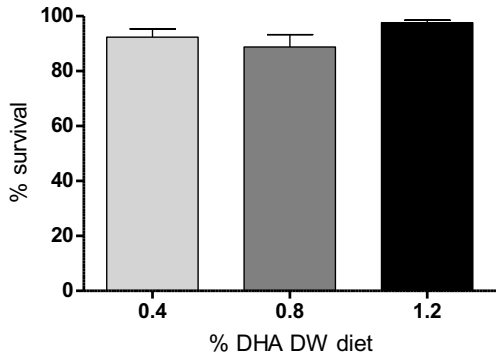

Figure 13.3.3. The effect of DHA diets (0.4, 0.8 and 1.2 % DHA DW diet) on percent (%) survival at the end of the experiment (n=4). ANOVA was not significant (P>0.05).

Discussion

The DHA requirement for juvenile fish, although generally less than during the larval stages, can still be considerably high. However, fish living in fresh water and/or omnivorous or herbivorous after metamorphosis may have varying capability to produce sufficient Δ6 and Δ5 desaturases and elongases and therefore synthesize DHA, EPA and ArA from their shorter chain precursors linolenic (18:3n-3) and linoleic (18:2n-6) fatty acids if they are present in the diet (Yu and Sinhuber, 1975). In contrast, marine species have very limited elongase and desaturase capability, requiring the ingestion of fully formed EFA for good growth and survival (Watanabe, 1982; Sargent and Henderson, 1995; Izquierdo, 1996). Grey mullet larvae, as in all teleosts at this developmental stage, are strict carnivores but incrementally transit to an omnivorous/herbivorous mode of feeding after metamorphosis. As they search out the less saline waters of estuaries, their DHA requirement as well as their capability to elongate and desaturate shorter chain precursors to LCPUFAs may change. Indeed, preliminary results from Deliverable 13.1 show an increasing gene expression of Δ6 desaturase and elongases that are salinity dependent.

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Sub-task 13.3.2 Effect of four levels of Tau supplementation to best performing DHA/EPA/ArA non-fish meal grow out diet from 13.3.1 on fish performance

Introduction

The β-amino sulfonic acid taurine plays an array of critical roles that include involvement in bile salt synthesis, anti-oxidative defense, cellular osmoregulation, as well as contributing to visual, neural and muscular function (Fang et al., 2002; Omura and Inagaki, 2000). Taurine is not found in terrestrial plant protein sources but is well represented in the natural diet of many marine species where fishmeal is particularly taurine rich (5–7 mg/ g DM) (Yamamoto et al., 1998). The taurine requirement in juvenile fish varies widely and is species dependent. For example the taurine requirements in juvenile Florida pompano (*Tranchinotus carolinus*) was 0.54-0.65% (Salze et al., 2014), 0.32-1.5% in California yellowtail (*Seriola lalandi*) (Jirsa et al., 2014), 1.15% in turbot (Qi et al.. 2012), 0.5% in cobia *Rachycentron canadum* (Lunger et al. 2007), 0.5% in dentex (*Dentex dentex*) (Chatzifotis et al. 2008), 0.6-1.6% in Japanese flounder, *Paralichthys olivaceus* (Kim et al 2007) and 0.5% in red sea bream, *Pagrus major* (Matsunari et al. 2008a,b). On the other hand, there was no reported taurine requirement in herbivores/omnivores such as the Red hybrid tilapia (Divakaran et al. 1992), channel catfish, *Ictalurus punctatus* (Robinson et al. 1978) and common carp, *Cyprinus carpio*, (Kim et al. 2008). As the grey mullet transits from carnivorous larvae in the sea to omnivorous juveniles in lower saline estuarine waters, the aim of the present study was to determine the taurine requirement in juvenile fish of this species that may have the capacity for taurine synthesis.

Methods and Materials

The experimental system consisted of sixteen, 400 l V-tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25 ^{o}C (computer controlled; Gavish, Israel) entered the bottom of the tanks and exited near the top through a 500 µm filter at a rate of 7 tank exchanges per day. This allowed the testing of four taurine 1 mm pelleted diets (0, 0.5, 1.0 and 2.0% DW diet) (Sparos Inc., Faro, Portugal) in replicates of 4 tanks per treatment. The experimental system was exposed to a light intensity of 500 lux with a photoperiod of 11 L/13 D. Each tank was stocked with 38 fish (126 dph) and fed their respective diets at 4% of tank biomass per day distributed over 5 rations. The experiment continued for 58 days (184 dph) where the fish had grown at least 100%. At the end of this period the weight of each fish and length was measured while 4 fish from each tank were sampled for fatty acid and taurine analyses of their eyes, muscle and liver. The digestive tract (DT) from 16 fish per tank was dissected out. Eight DTs were frozen at -80 ^{o}C for RNA extraction (Pept1 gene expression) while the other 8 fish were placed in buffered formalin for histology. Five fish from each tank were frozen at -20 ^{o}C for proximate analysis.

Results and discussion

The results showed that grey mullet juveniles have a minimum 0.5% requirement for dietary taurine (**Figure 13.3.4**), which is within the range of taurine requirements measured in a variety of marine species such as the Florida pompano (Salze et al., 2014), California yellowtail (Jirsa et al., 2014b), cobia *Rachycentron canadum* (Lunger et al. 2007), dentex, *Dentex dentex*), (Chatzifotis et al. 2008), Japanese flounder, *Paralichthys olivaceus* (Kim et al 2007) and red sea bream, *Pagrus major* (Matsunari et al. 2008a,b). Although these fish were grown in 40 ‰ and showed a taurine requirement, they still exhibited taurine synthesis capability (see preliminary results in Sub-task 13.2.1), which increased in both the CSD and ADO pathways up to 1% taurine DW diet but then decreased substantially in the highest taurine diet (2% DW diet). This suggests that the overall taurine requirement might be higher than 0.5% as part of the taurine requirement as there appears to be endogenous synthesis of this nutrient. On the other hand, fish fed the 2% taurine diet may be ingesting excessive levels of taurine resulting in decreased production of endogenous taurine.

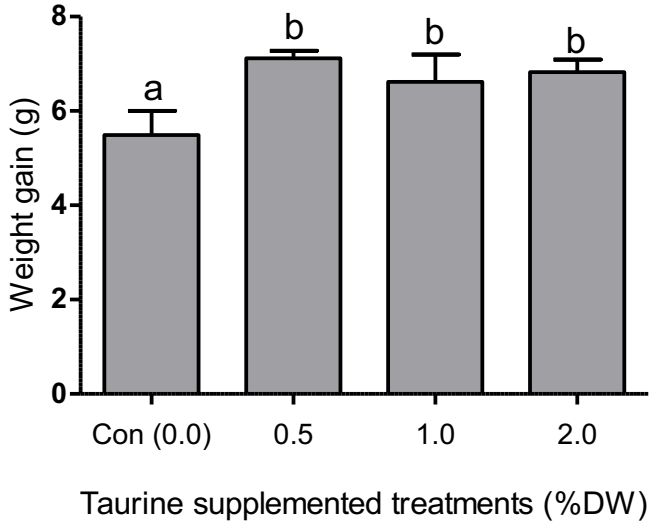

Figure 13.3.4. The effect of the taurine diets (control-0, 0.5, 1.0 and 2.0% DW diet) on average weight gain per fish per treatment (n=4). ANOVA was significant (P<0.05) where fish fed all diets containing taurine exhibited superior weight gain compared to the control fish.

Although there was a tendency for diets from 0 to 1% taurine to produce less smaller and larger fish, overall there was no significant (P>0.05) dietary taurine effect on size distribution in the population (**Figure 13.3.5**). Once all the analyses are completed, the results should demonstrate a more comprehensive picture of the effect of dietary taurine on juvenile grey mullet.

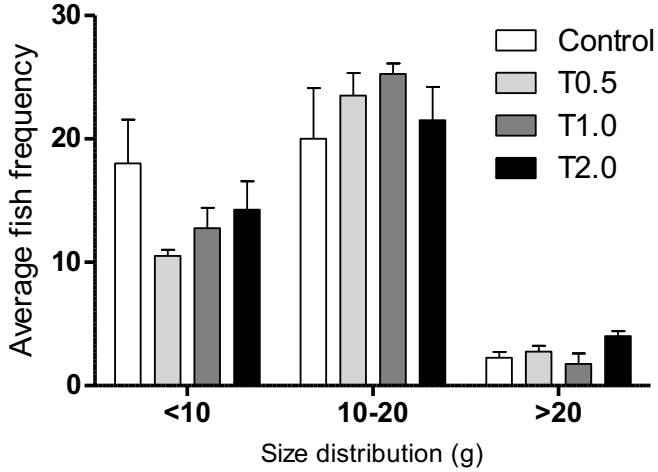

Figure 13.3.5. The effect of the taurine diets (control-0, 0.5, 1.0 and 2.0% DW diet) on size distribution. ANOVA analysis was not significant.

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Sub-task 13.3.3 (led by IOLR; Bill Koven) Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.

This study will compare the effect of two types of soybean protein meals to be used in the IOLR grow out diet for grey mullet. Experiment planned in the spring

Sub-task 13.3.4 (led by IOLR; Bill Koven) The selected feed from Sub-tasks 13.3.1, 13.3.2, 13.3.3 will be compared to the current feed on the market used for mullet culture and fed to

adult mullet until gonadal maturation. At the end of the study fish performance on these two feeds will be evaluated in terms of growth, FCR, PER as well as flesh and bottarga quality.

This experiment is currently on-going.

Sub-task 13.3.5 (led by CTAQUA; Rocio Robles) Comparison of vegetable oil-no fish meal grow out diet with a n-3 HUFA rich fish meal finishing diet on the nutritional and organoleptic values of fish flesh and bottarga quality.

It is planned to carry out this study during Summer-Fall spawning season of 2018.

Task 13.4 Design adequate feeding regimes for brood stock to optimize reproduction success (led by UNIBA, Aldo Corriero).

Sub-task 13.4.1 (IOLR) Broodstock dietary effects on mullet reproduction (e.g., natural pigments, DHA/EPA/ARA ratio, Tau) on egg quality, in terms of fecundity, hatching success, and larval first feeding.

Introduction

Fish reared in captivity may exhibit reproductive dysfunction, such as the inhibition of final oocyte maturation and spawning for females and the production of poor quality sperm by males (Mylonas et al., 2010). These dysfunctions can be overcome by hormonal therapies and modulation of environmental parameters (Mylonas et al., 2010) as well as providing an effective broodstock diet which should mimic body composition of brooders in the wild (Izquierdo et al., 2001; Rodríguez-Barreto et al., 2014).

During vitellogenesis significant quantities of proteins must be made available for transfer to the developing oocytes as well as lipids and carotenoids. Lipids that are mobilized are particularly rich in both saturated and monounsaturated fatty acids for energy provision as well as long chain polyunsaturated fatty acids (LCPUFA) primarily represented by the essential fatty acids (EFA) eicosapentaenoic (EPA; 20:5n-3), arachidonic (ARA; 20:4n-6) and docosahexaenoic (DHA; 22:6n-3) acids. EPA and ARA are precursors of eicosanoids, a group of active compounds with very important physiological functions such as reproduction (Tocher 2003). ARA derived eicosanoids have also shown to be involved in pheromone attraction, steroidogenesis, and oocytes maturation (Henrotte et al., 2011; Sorbera et al., 2001). Thus, supplying proper levels and ratios of EFA in broodstock diets is vital not only to produce eggs with the suitable contents of these fatty acids to ensure embryo and larvae development (Sargent et al., 2002; Tocher 2010), but also in the regulation of reproductive physiology (Henrotte et al., 2011; Sorbera et al., 2001). N-3 LCPUFA, which are derived directly from the dietary input of broodstock (Sargent, 1995) as well as from body reserves during the period of gonadogenesis, are crucial to female fecundity and to embryo and early larval development, growth and survival (Sargent, 1995; Rodríguez-Barreto et al., 2014).

The supplementation of fish oil and the essential n-3 LCPUFAs to broodstock diets and its benefit to egg and larval quality has been well established (Watanabe et al. 1984a,b) in a number of commercially farmed teleosts (Watanabe et al., 1984a,b,c, 1985a,b; Fernandez-Palacios et al., 1995; Navas et al., 1997). Tandler et al. (1995) reported that growth survival and swimbladder inflation in gilthead seabream (*Sparus aurata*) larvae were improved when fish oil was used instead of soybean oil in broodstock diets. On the other hand, the herbivorous Nile tilapia, *Oreochromis niloticus*, demonstrated higher fry per spawning as well as total fry production when fed a diet supplemented with soybean oil relatively rich in n-6 PUFA (Watanabe, 1982). Nevertheless, the most effective level of n-3 LCPUFA in brood stock diets is species specific and an excess of these fatty acids can be detrimental (Fernandez-Palacios et al., 1995). In the case of sparids, the n-3 LCPUFA requirement reportedly ranged from 1.5 to 2.0% n-3 LCPUFA DW diet (Izquierdo, 1996). Levels above this caused yolk sac hypertrophy and a decrease in larval survival (Fernandez-Palacios et al., 1995). In contrast to the carnivorous gilthead sea bream and European sea bass (*Dicentrarchus labrax*), the grey mullet is omnivorous following metamorphosis, which suggests a diet relatively low in n-3 LCPUFA. However, grey mullet bottarga or intact roe is a highly prized delicacy in Japan and around the Mediterranean and is a

rich source of n-3 LCPUFA (Scano et al., 2010). This suggests that grow out diets may not be suitable and that there is a dietary requirement for n-3 LCPUFA in the brood stock feed.

Methods and Materials

Sampling

Eight hatchery-produced 6-year old grey mullet stock were sampled by P4. IOLR during early October (3 females) and November (3 females and 2 males) 2016. In parallel, 16 wild specimens (10 females and 6 males), caught by traditional trap nets (lavoriera) in the Schiapparo Channel (Apulia, Italy) during their migration from the Lesina Lagoon to the spawning grounds of the Adriatic Sea, were sampled by P13. UNIBA in early September 2016. The age of wild grey mullets sampled by UNIBA was estimated through the analysis of the scales (Meunier, 2002). Gonads from wild and captive males and females individuals were taken, immediately frozen and kept at -80ºC, until analysis.

Proximal and main fatty acids composition analyses of diets and gonads from mature grey mullet (P15. ULL)

Moisture contents were determined in approximately 500 mg samples by thermal drying in an oven at 110°C, until constant weight. Total lipid (TL) was extracted by homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (Christie, 1982) and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic acid (90:10: 1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m 9 0.32 mm I.D. 9 0.25 lm; Sigma-Aldrich, Madrid, Spain). Helium was used as the carrier gas and temperature programming was 50–150°C at 40°C min^{-1} slope, then from 150 to 200°C at 2°C min^{-1} , to 214 °C at 1°C min^{-1} and, finally, to 230°C at 40°C min^{-1}. Individual FAME and DMA were identified by reference to authentic standards, and further confirmation of FAMEs and DMAs identity was carried out by GC-MS (DSQ II; Thermo Electron Corp).

Fish holding and experimental design

Based on the results of proximal and main fatty acid (FA) composition in gonads from wild and domesticated mullets (Table 1), the initial and present study to improve the broodstock diet for grey mullet focused on the increased supplementation of the n-3 LCPUFA through the addition of more fish oil to the diet. During the onset of the reproductive season (early July 2017), 6-year old captive grey mullet broodstocks were divided into two groups, that were fed with either a mullet grow out diet containing soybean oil (VO) that was previously developed by P4. IOLR, or with diet containing fish oil (FO), which is relatively rich in n-3 LCPUFA. The experiment was conducted in triplicates. Fish were maintained in 4 m^3 tanks supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40‰ salinity and subjected to natural fluctuations of light and temperature. Food was provided at the rate of 1-1.5% of their body weight. Fish were conditioned for spawning using protocols developed by P4. IOLR (Aisen et al. 2005) with some modifications elaborated in WP7.

Larval rearing trials

Fertilized and spawned eggs from each of the broodstock groups (VO and FO) were stocked in eight 400 l V-tanks (200 eggs/l) or four tanks per treatment for the food deprivation experiment. VO and FO eggs were simultaneously stocked in 3 plastic plates per treatment where each plate contained 12 five ml wells that was stocked with one egg per well. Hatching rate was determined 24 h after stocking and survival of the hatched larvae at the end of 0 dph. Tanks in the food deprivation experiment were supplied with temperature controlled (Gavish, Israel; 24-25 ºC), filtered (10 µm) and UV treated sea water (40 ‰) that entered the tanks from the bottom and excited through a 500 µm filter at an exchange rate of 300% per day. In two tanks from each broodstock treatment set, the salinity was decreased to 25 ‰ at 2 dph over the course of one day

so that 3-7 dph larvae were exposed to only 25 ‰ in these tanks. This means duplicate tanks were used for each of the four treatments (VO-25, VO-40, FO-25, FO-40). All larvae were not exposed to direct lighting during the course of the food deprivation experiment in order not to stimulate feeding activity. Larval samples from each of the tanks (average of 220 larvae/sample) were taken daily for DW determination and fatty acid analysis, which are still being processed. However, fatty acid analyses of the gonads of females and males of both wild and captive broodstock fish were carried out (P15.ULL).

Statistics

One-way ANOVA and regression analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. ANOVA analyses and Barlett's test for equal variances were carried out simultaneously.

If significance ($P < 0.05$) was found for ANOVA while Barlett's test was not significant ($P > 0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P < 0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P < 0.05$) differences among treatments. Percentage values were first arsine transformed before analyses. All data are presented as mean ± SEM.

Results

Proximal and main FA composition of TL of P4 IOLR diet and gonads from wild and domestic adult grey mullet broodstock are compared in **Table 13.4.1.** Notable differences between these profiles are; (1) total saturated fatty acids (SFA) were much higher in wild females and males compared to captive females and males, while (2) captive male gonads are much higher in monounsaturated fatty acids (MUFA) than their male counterparts in nature. (3) Captive females and males have much higher levels of n-6 polyunsaturated fatty acids (PUFA) than their wild cohorts, particularly linoleic acid (18:2n-6), while there are (4) considerably more total n-3 PUFA in wild female and male gonads compared to the gonads in captive fish. Differences were particularly marked in EPA in both sexes and DHA in wild males compared to captive ones.

Based on these results, the effects of fish oil supplementation in the broodstock diet of grey mullet on gonadal development in and between sexes, occurrence of successful spawning giving rise to fertilized eggs, hatching success, larval survival during food deprivation and swim bladder inflation rate, were tested.

Nonetheless, some diet effects could be perceived during the larval growth. Although in **Figure 13.4.1.** the rates of decline of weight loss with age (dph) during food deprivation in the four treatments were not significant ($P>0.05$) from each other, there was an observed pattern where the highest rate of dry weight (DW) decline was in the VO larvae where all fish were dead at 5 dph or 3 days after the capability of exogenous feeding.

In contrast, larvae from the FO broodstock demonstrated a slower DW decline, particularly in the 25‰ treatment, and were still alive at 7 dph, which was 5 days after the onset of the ability to consume live prey.

Interestingly, the 2 dph larvae from the FO treatment were larger than the 2 dph fish from the VO treatment, although not significantly ($P>0.05$). Nevertheless, the percent hatching of eggs from the FO broodstock was significantly ($P<0.05$) higher than eggs from the VO broodstock (**Figure 13.4.2**), while survival in larvae in the two treatments at the end of 0 dph were not significantly different ($P>0.05$) from each other, although the FO was higher (**Figure 13.4.2**).

There was a very significant effect ($P<0.0001$) of broodstock treatment on swimbladder inflation in the food deprivation experiment (**Figure 13.4.3**). Larvae from the FO broodstock, regardless of salinity exposure, demonstrated 100% swim bladder inflation by 5 dph, where there was no swim bladder inflation at all in fish from the VO broodstock during the course of the food deprivation study.

Table 13.4.1. FA composition (% of total FA) of TL of diets and gonads from *Mugil cephalus* broodstock

	DIET	WILD		CAPTIVE		
	NCM	Female	Male	Female regr.	Female mat.	Male
Moisture (%)	11.29 ± 0.08	55.69 ± 5.65	83.23 ± 0.68	81.02 ± 0.01	52.33 ± 0.63	79.85
Total lipid (% DM)	10.85 ± 0.04	30.52 ± 3.32	13.08 ± 0.58	10.09 ± 6.09	30.63 ± 2.69	13.07
Crude protein (% DM)	35.04 ± 1.55	54.43 ± 3.29	86.17 ± 2.73	76.72 ± 6.99	52.43 ± 1.93	69.65
Fatty acids						
Total SFA	16.70 ± 0.14	20.60 ± 1.27	33.17 ± 1.69	23.06 ± 8.16	12.12 ± 0.23	25.21
14:00	1.14 ± 0.03	1.81 ± 0.59	1.07 ± 0.32	0.49 ± 0.18	0.35 ± 0.01	0.78
16:00	11.40 ± 0.12	13.83 ± 1.20	23.65 ± 1.30	15.00 ± 4.49	8.48 ± 0.26	18.29
18:00	3.13 ± 0.02	3.89 ± 0.23	6.74 ± 0.38	7.25 ± 3.31	3.12 ± 0.11	5.71
Total MUFA	38.70 ± 0.34	43.84 ± 3.95	15.04 ± 1.51	25.63 ± 4.72	41.50 ± 2.80	28.8
16:01	2.13 ± 0.06	16.42 ± 2.33	3.43 ± 0.60	3.34 ± 0.79	7.07 ± 1.02	3.87
18:01	32.85 ± 0.35	25.47 ± 3.59	10.50 ± 1.23	20.18 ± 4.08	33.00 ± 1.81	20.74
20:01	1.96 ± 0.07	0.35 ± 0.27	0.28 ± 0.22	1.38 ± 0.08	1.04 ± 0.16	2.69
22:01	1.52 ± 0.00	nd	nd	nd	0.07 ± 0.12	nd
Total n-6 PUFA	34.25 ± 0.06	6.03 ± 2.31	8.84 ± 3.13	24.87 ± 4.42	26.86 ± 2.79	26.65
18:02	33.70 ± 0.04	2.27 ± 0.86	0.85 ± 0.22	12.37 ± 1.76	22.67 ± 3.13	16.37
20:02	0.39 ± 0.00	nd	0.14 ± 0.21	1.70 ± 0.44	0.79 ± 0.12	2.92
20:03	nd	0.38 ± 0.17	0.16 ± 0.18	2.64 ± 0.44	1.57 ± 0.46	1.69
20:04	nd	1.93 ± 0.85	4.99 ± 1.86	6.16 ± 3.13	0.51 ± 0.03	4.32
Total n-3 PUFA	9.15 ± 0.26	22.58 ± 6.32	39.29 ± 3.49	18.67 ± 6.42	14.47 ± 0.43	16.29
18:03	5.37 ± 0.09	1.42 ± 0.63	0.26 ± 0.23	0.54 ± 0.07	1.20 ± 0.24	0.8
18:04	0.51 ± 0.07	1.36 ± 0.45	0.33 ± 0.22	0.09 ± 0.12	0.23 ± 0.06	nd
20:04	0.26 ± 0.00	2.06 ± 1.00	0.56 ± 0.20	0.27 ± 0.06	0.51 ± 0.03	0.33
20:05	1.04 ± 0.05	3.91 ± 1.39	4.63 ± 0.69	1.31 ± 0.23	1.67 ± 0.31	0.84
22:05	0.42 ± 0.01	3.74 ± 1.67	7.81 ± 1.06	2.20 ± 0.50	2.13 ± 0.02	2.66
22:06	1.39 ± 0.03	9.37 ± 3.77	25.51 ± 3.03	14.05 ± 6.09	8.35 ± 0.25	11.34
n-3/n-6	0.27 ± 0.01	4.08 ± 1.25	5.06 ± 2.12	0.79 ± 0.40	0.54 ± 0.04	0.61
Total n-3 HUFA	3.28 ± 0.10	19.34 ± 5.92	38.70 ± 3.28	18.04 ± 6.37	13.03 ± 0.15	15.49

Results are given as mean ±SD for a variable number of samples (n=10 for wild females and n=6 for wild males; n=2 for regressed females, n=3 for fully mature females and n=1 for captive male). nd, non-detected.

Table 13.4.2. Summary of grey mullet broodstock body weight (BW) and reproductive performance in two different diets distinguished by their oil source: fish oil (FO), soybean oil (VO).

	Treatment groups	
	FO	VO
Av. BW females (g)	1660.36±67.8	1753.67±75.5
Av. BW males (g)	987.86±46.06	905.77±48.11
Post vitellogenic females (%)	71.86±5.9	69.86±9.4
Spermiating males (%)	28.97±16.8	13.09 ±7.2
Fertilized spawns (%)	50	66
Fecundity	2.12±0.1	2.89±0.9

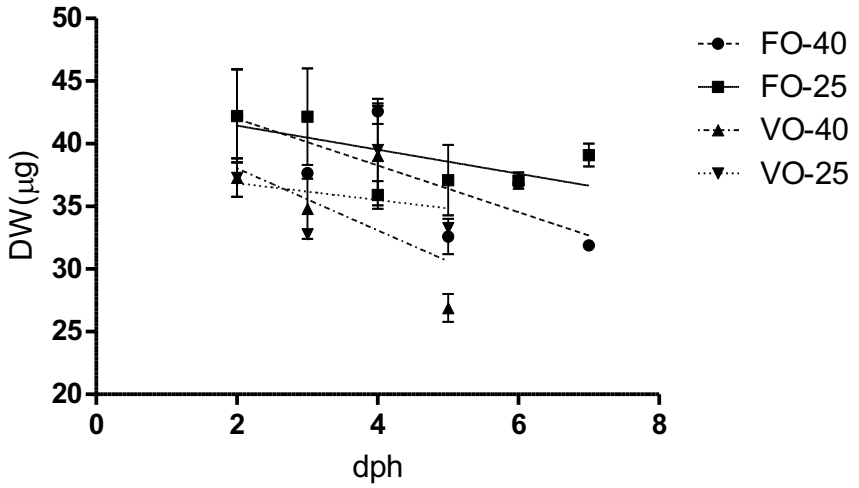

Figure 13.4.1. The rate of decline in 2-7 dph larval dry weight (DW) during food deprivation. Akaike's Information Criteria (AIC) found the linear model best represented the regressed DW data of larvae maintained in 25 or 40 ‰ from brood stock fed fish oil (FO) or vegetable oil (VO) diet treatments. Slopes of lines were not significantly different from each other (P=0.5754)

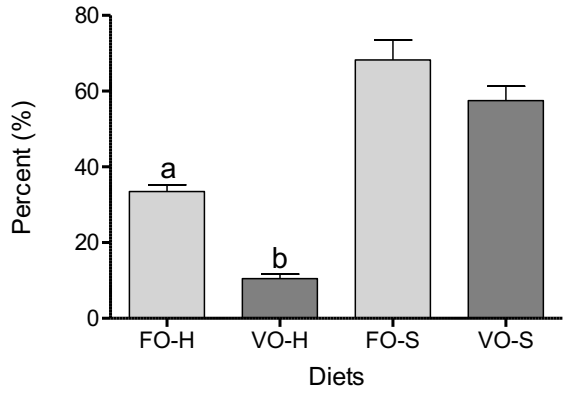

Figure 13.4.2. The effect of broodstock diets FO (Fish oil) and VO (vegetable oil) on percent hatching (H) and survival (S) at the end of the day of hatching (T0). Percent values having different letters were significantly different (P<0.05).

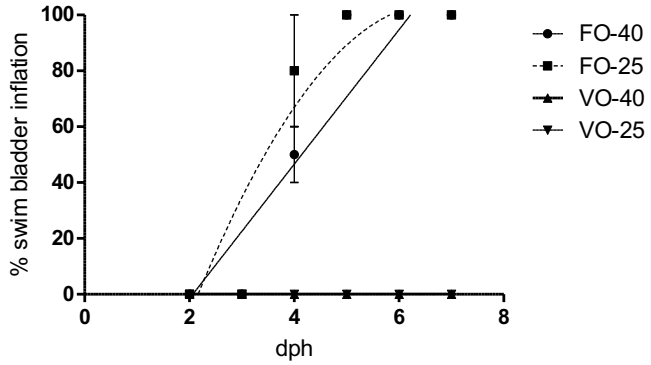

Figure 13.4.3. The rate of % swim bladder increase in 2-6 dph larvae during food deprivation. Akaike's Information Criteria (AIC) analyzing regressed percent data of swim bladder inflation rate in larvae from broodstock fed the FO diet found the linear model (61.9%) best represented larvae in 40 ‰ sea water while a second order polynomial model (74.4%) best represented larvae in 25 ‰. There was no swim bladder inflation in larvae from the VO broodstock diet, irrespective of rearing salinity. The regression lines of swim bladder inflation in the FO larvae were highly significantly different (P<0.0001) than the regression lines of the VO larvae but not significantly (P>0.05) different from each other.

Discussion

The rearing conditions including diet may affect the pattern of energy usage and reserves that are mobilized towards reproduction. In this sense, artificial diets and confinement conditions have been reported to increase tissue fat composition (Rodríguez-Barreto et al., 2012, 2014, 2015). However, in this study the mobilization of energy reserves in terms of lipids and proteins is quite similar between wild and captive mature females (Table 13.4.1.1). When comparing the fatty acid profiles of female and male gonads from wild and captive fish, there were notable differences in SFA, MUFA and n-6 PUFA. Nevertheless, this initial study on improving the mullet broodstock diet focused on the marked differences in n-3 LCPUFAs; EPA and DHA, which are well represented in fish oil.

The use of fish oil in broodstock diets of mullet leading to better hatchability, tolerance of food deprivation and improved swim bladder inflation is largely supported in the literature. Navas et al. (1997) found that egg quality and hatching rates were improved in seabass (*Dicentrarchus labrax*) by feeding appropriate amounts of n-3 LCPUFA during the vitellogenin period. The importance of species specific effective levels of n-3 LCPUFA was also reported by Li et al. (2005) who determined that levels of these essential fatty acids below 2.40 or above 3.7% DW diet in crescent sweetlips, *Plectorhynchus cinctus*, had a negative effect, while between these values resulted in good egg quality and larval performance. Zakeri et al. (2011) showed that replacing soybean oil with increasing levels of fish oil in the broodstock of yellowfin sea bream, *Acanthopagrus latus,* improved relative fecundity, percentage of buoyant eggs, hatchability, survival rate of larvae at 3 dph and higher starvation tolerance. On the other hand, there are no definitive results tying n-3 LCPUFA in the broodstock diet with swim bladder inflation. In support of this, Koven et al. (1990) argued that there is no compelling evidence that n-3 LCPUFA markedly affected swim bladder inflation in gilthead sea bream. In contrast, Tandler et al. (1995) observed that a broodstock diet high in these essential fatty acids was associated with over 80% of the resulting larvae having a functional swim bladder compared to only 55% in the progeny of broodstock consuming a low n-3 having a functional swim bladder. Fish oil components, such as vitamin A and E as well as pigments may also effect egg quality and larval performance (Izquierdo and Koven, 2011). Alternatively, the fact that larvae from the FO diet demonstrated a slower DW decline and survived considerably longer than their VO cohorts may suggest that they were more developed. This might have led to a more functional rete mirabilis and pneumatic duct compared to the VO larvae, which are critical to successful swim bladder inflation.

Sub-task 13.4.2 Definition of specific requirements of protein, TAU, ARA, DHA and carotenoid sources to optimize spawn quality in mullet. Analysis of liver Vtg gene expresssion, oocyte Vtg receptor gene expression and yolk accumulation under different dietary conditions.

For the study of vitellogenesis, three hatchery-produced females belonging to a 6-year old stock were sampled by IOLR in early November 2016 and 16 wild specimens, caught by traditional trap nets (lavoriera) in the Schiapparo Channel (Apulia, Italy) during their migration from the Lesina Lagoon to the spawning grounds of the Adriatic Sea, were sampled by P13. UNIBA in early September 2016. The age of wild grey mullets sampled by UNIBA was estimated through the analysis of the scales (Meunier, 2002) (**Fig. 13.4.4**).

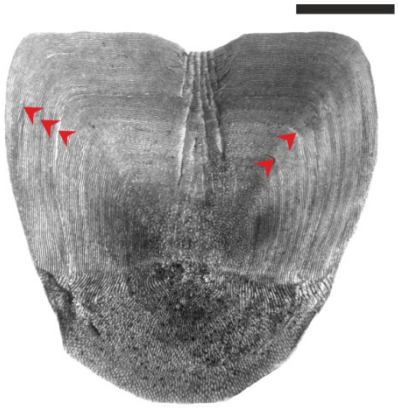

Figure 13.4.4. Scale from a 5-year old wild grey mullet. Bar = 3 mm. Arrowheads point to annuli.

From each fish, biometric data (Total Length, TL, in cm; Body Mass, BM, in g; Liver Mass, LM, in g) were recorded and gonads and liver were sampled. A list of all the grey mullet females sampled for the vitellogenesis study is provided in **Table 13.4.3.**

Table 13.4.3. List of adult wild and hatchery-produced grey mullet females

Fish Origin	Sampling area	Sampling Date	Total Length (TL, cm)	Body mass (BM, g)	Gonad mass (GM, g)	Liver mass (LM, g)	Age (year)
Wild	Schiapparo channel (Italy)	09/09/2016	38.0	574.9	111.3	6.1	4
		09/09/2016	40.0	602.5	96.8	10.0	4
		09/09/2016	42.0	864.9	9.3	13.9	5
		09/09/2016	47.0	1071.5	232.6	14.2	6
		09/09/2016	50.0	1332.0	280.4	12.3	6
		13/09/2016	46.0	843.0	150.0	11.0	5
		13/09/2016	49.0	1132.0	245.0	15.8	6
		14/09/2016	40.0	595.0	108.0	9.7	4
		14/09/2016	43.0	807.2	166.7	7.5	5
		14/09/2016	45.0	798.0	154.9	9.1	5
Hatchery-produced	P7. IOLR	03/11/2016	47.1	1240.0	128.2	21.8	6
		03/11/2016	50.3	1480.0	8.1	32.7	6
		03/11/2016	54.4	1970.0	34.4	34.6	6

Adult wild grey mullets sampled during their migration towards the spawning ground of the Adriatic Sea showed large ripe ovaries with late vitellogenic oocytes as the most advanced oocyte stage (**Fig. 13.4.5a**) and no signs of recent spawning (post-ovulatory follicles). The ovaries of the three age 6 hatchery-produced grey mullets showed extensive alpha and beta atresia of vitellogenic follicles (**13.4.5b**), a sign of cessation of the reproductive activity.

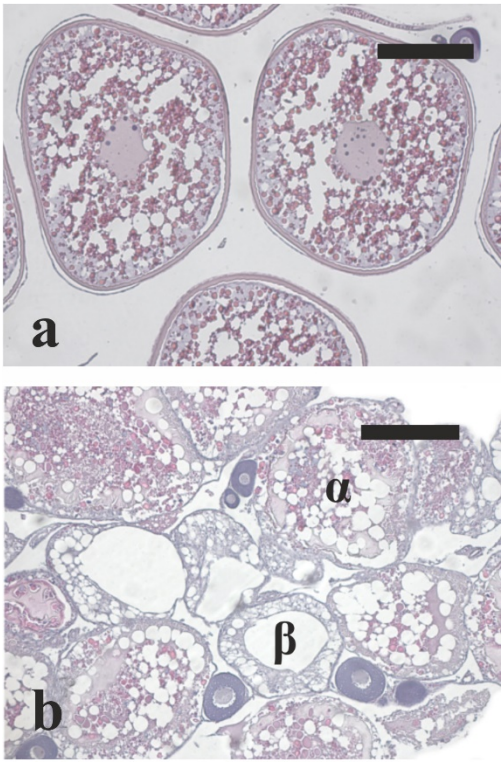

Figure 13.4.5. Micrographs of grey mullet ovary sections a) Late vitellogenic oocytes. b) α and β atretic vitellogenic follicles. Haematoxylin-eosin staining. Magnification bars = 200 μm.

Total RNA was obtained from gonad and liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 μl of RNase free water and stored at –80°C. The cDNA was prepared from 1.2 μg total RNA. Random hexamer primers were used for the cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen).

The partial cDNA sequences of vitellogenin receptor (VgR) and β-actin were amplified from total cDNA by means of several overlapping PCR reactions (**Fig. 13.4.6**). For that purpose, degenerate primer pairs were designed against conserved sequences from various Perciform species (Pousis et al., 2011; Pousis et al. 2012). All PCRs were performed on a PCR Sprint Thermal Cycler using ~120 ng cDNA, 10 pmoles of each oligonucleotide primer, 0.2 mM dNTP mix, 10X Taq polymerase buffer and 1.5 unit Taq Polymerase (Eppendorf). The amplification products were analyzed for size on 1.2% agarose gels containing ethidium bromide. The band of interest was excised from the gel, purified using Nucleo Spin extract II (Macherey-Nagel) and ligated into the pCR 2.1 TOPO cloning vector (TOPO TA cloning kit; Invitrogen). *Escherichia coli* competent cells (Invitrogen) were used for transformation. Approximately 20 μl of the recombinant plasmid was sent to the Primm Sequence Service (Primm Srl, Italy) for sequencing with M13 reverse and M13 forward primers.

The *in silico* analysis showed that grey mullet VgR, like other piscine VgRs, is highly homologous to amphibian and bird VgRs and to mammal LDLR/VLDLR. The comparative analysis of VgR expression in ovary samples from wild and hatchery-produced grey mullets will be performed during the fourth reporting period.

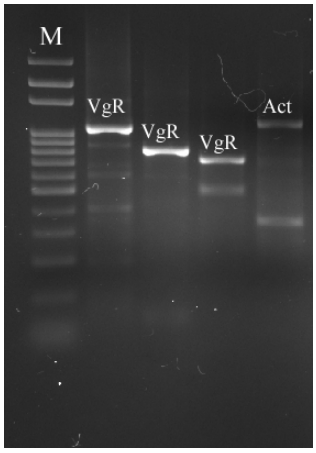

Figure 13.4.6. Agarose gel electrophoretic analysis of β-actin and Vitellogenin Receptor (VgR) RT-PCR products. mRNA at 125 ng from grey mullet ovary was used as template for reverse transcription, followed by amplification with specific primers pairs. Aliquots of 8 µl were loaded onto the agarose gel together with 1 kbp DNA marker (M).

Vitellogenin (VgA, VgB and VgC) primers were designed (**Table 13.4.4**) against the relevant grey mullet Vgs sequences reported in GenBank (accession number AB288932, AB288933, AB288934) using the Primer3 software (Rozen & Skaletsky, 2000) and their specificity was checked with both in silico (the UCSC "In-Silico PCR" and the NCBI Primer-BLAST tools) and by means of agarose gel electrophoresis.

It was confirmed that the VgA primer set did not produce any amplified fragments using as a template VgB or VgC cDNA, and vice versa. Total RNA was obtained from pituitary and liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Reverse transcription of 1000 ng of total RNA was performed using SuperScript III Reverse Transcriptase (Invitrogen®) and diluted cDNA (1:10) was used in all following qPCR reactions. For gene expression analysis qRT-PCR experiments were carried out in triplicate using the QuantStudio™ 7 Flex System (Applied Biosystems®, Thermo Fisher SCIENTIFIC) using 1µl of diluted cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). No template controls were included as negative controls for each primer pair.

The quantification of the β-actin gene was used as the endogenous control. Amplification parameters were as follows: hot start at 95°C for 15 min; 40 amplification cycles (95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec). Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied Biosystems®, Thermo Fisher SCIENTIFIC) and analyzed with the DART-PCR Excel workbook (Peirson et al., 2003). Actual amplification efficiency values (E) for each amplicon were used to correct Cq values before analyzing these data by the ΔCq method to compare relative expression results. Gene expression levels were calculated by: relative expression = $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001).

Table 13.4.2.3. Primers for greater amberjack real-time PCR

VgA FOR	GCAGTAGACTCAGCTCTTCAG
VgA REV	CAGCCTGGGAGGAGTGAGC
VgB FOR	ATCCCCGCTGACCTGTCAAG
VgB REV	TGACTGGTCCAGCTGGGGC
VgC FOR	CCACAGTGAGATGTGTTTACAC
VgC REV	TCTCCATTGGCCCGAACGTG
β-actin FOR	CCTTCTACAACGAGCTGAGAG
β-actin rev	CGTCATGGACTCCGGTGATG

Geometric mean values of Vgs mRNA expression levels relative to β-actin are reported in **Table 13.4.5**. The highest relative levels of Vgs mRNA were observed in adult wild specimens caught in Italy. These expression values are comparable to those found in greater amberjack during the spawning season (Pousis et al., 2017) and they are indicative of a residual Vtg transcription activity in specimens at the end of vitellogenesis. Hatchery-produced grey mullets showed very low Vgs expression levels (VgC expression was even below that of β actin), corresponding to a negligible Vgs transcription activity, which is in agreement with the fact that these specimens were in spent condition (extensive atresia of vitellogenic follicles).

During the fourth reporting period, the study of the vitellogenic process will be extended to sexually mature grey mullets fed different diets and will also include the analysis of vitellogenin receptor gene expression.

Table 13.4.5. Geometric mean values of vitellogenin A (VgA), vitellogenin B (VgB) and vitellogenin C (VgC) expression levels relative to β-actin.

	VgA	VgB	VgC
Hatchery-6Y	1.1	22.4	-1.6
Wild	152.6	548.4	13.3

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Deviations from Annex I and their impact:

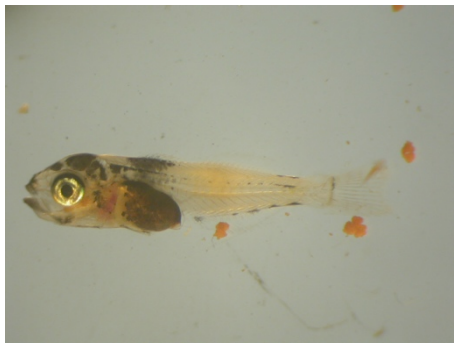
Sub-task 13.1.1

The use of the 17 l aquarium experimental system for the tasks in 13.1, which was written in the DOW, was reconsidered as the fish had high mortality in this system. Instead, the 400 l experimental system was used for larval and juvenile experiments where the survival was much better but the number of experimental units was reduced. This meant that taurine, DHA and ArA studies have been or will be tested in tandem as individual experiments. However, the aim of determining the most effective levels of these nutrients for good larval and juvenile performance and as a basis to improve enrichment protocols and weaning diets was performed and analyses are pending and will be completed at a later date. As mullet are naturally fall spawners (with a possible photoperiod induced spawning in January 2018), analyses are still currently being performed at the writing of this report.

The deliverable D13.1 "*Determine changes in the essential fatty acid requirement as a function of developmental stage and ambient salinity in grey mullet*" will be submitted in February 2018; was due in M18. Deliverable D13.2 "Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey Mullet" was due in M18 and will be submitted in January 2018. Deliverable D13.3 "Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression accumulate will be submitted in January 2018, and was due in M36. Deliverable D13.4 "Determine the effects of essential fatty acids and Tau in non-fish meal feeds on flesh and bottarga quality in grey mullet" will be submitted in September 2018, and was due in M48.

Group Work Packages

Larval husbandry



In the 2nd periodic report of meagre larval husbandry, **Task 14.1** compared the control group fish, which were weaned onto dry feed from 20 dph and fed to 30 dph, with the treatment fish, which were weaned from 10 dph and grown to 23 dph. There was significantly ($P<0.05$) lower mortality and higher growth in the control fish compared to the treatment larvae. Pancreatic enzyme activity in the control and treatment fish were similar although pepsin was higher in the control but early weaning of the larvae did not have any effect on total skeletal deformities. This study concluded that meagre larvae can be weaned from live feed to an artificial diet as early as 10 dph, although key parameters in larval performance may be reduced. This study was submitted as deliverable ***D14.1. Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles***. In the 3rd periodic report and these results were published in Aquaculture Research; "Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A. 2017. Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research, 48: 5289-5299"

In the greater amberjack larval studies, **Task 15.1** showed that rotifers enriched with marine lecithin supplemented with 20% Echium oil showed the best results. **Sub-task 15.2.1** found that the gene expression of GHRH, GH, IGF-I and II and IGFBPs were not affected by semi-intensive or intensive larval rearing while in **Sub-task 15.2.2** the optimum egg stocking density, in terms of larval performance, was between 25 and 50 eggs l^{-1}. In **Sub-task 15.2.3** the study of the ontogeny of the digestive system showed that enzyme activity measured for a particular age range is independent of the larval geographical origin and environmental rearing conditions. Nevertheless, amberjack larvae seem to efficiently digest dietary protein from 20-30 dph. **Sub-task 15.3.1** compared tank hydrodynamics in the semi-intensive tanks (2000 l) and mesocosm tanks (40,000 l) and found that the semi-intensive tanks had higher current velocity (cm s^{-1}) at all depth layers than the mesocosm tanks. In **Sub-task 15.3.2**, the photoperiods of 24L:00D and 18L:6D did not generally affect larval growth or mRNA expression levels of IGF, GH and GnRH proteins. There were no marked differences in larval growth as a function of tank color (black, green, white) although white tank larvae exhibited the highest survival rate and expression of IGF-1 and GH.

In the greater amberjack larval studies, the main objectives were: (1) Effects of different feeding strategies on larval performance in intensive systems, (2) Development of feeding protocol and rearing system in mesocosm semi-intensive systems, (3) Development of industrial protocol for larval rearing. **Task 15.1** showed that rotifers enriched with marine lecithin supplemented with 20% Echium oil showed the best results, although they were not significant. **Sub-task 15.2.1** found that the gene expression of GHRH, GH, IGF-I and II and IGFBPs were not affected by semi-intensive or intensive larval rearing while in **Sub-task 15.2.2** the optimum egg stocking density, in terms of larval performance, was between 25 and 50 eggs l^{-1}. In **Sub-task 15.2.3** the study of the ontogeny of the digestive system showed that enzyme activity measured for a particular age range is independent of the larval geographical origin and environmental rearing conditions. Nevertheless, amberjack larvae seem to efficiently digest dietary protein from 20-30 dph. The full description of the work and results is provided in ***D15.2 Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing***.

Sub-task 15.3.1 compared tank hydrodynamics in the semi-intensive tanks (2000 l) and mesocosm tanks (40,000 l) and found that the semi-intensive tanks had higher current velocity (cm s^{-1}) at all depth layers than the mesocosm tanks. The results of deformity evaluation showed a marked appearance of different types of

skeletal anomalies in all treatments throughout the larval stages In **Sub-task 15.3.2**, the photoperiods of 24L:00D and 18L:6D did not generally affect larval growth or mRNA expression levels of IGF, GH and GnRH proteins. There were no marked differences in larval growth as a function of tank color (black, green, white) although white tank larvae exhibited the highest survival rate and expression of IGF-1 and GH while these measurements were the lowest in fish reared in the green tanks. The work done is fully described in **D15.3**.

In **Task 15.4**, *Development and validation of the industrial protocol* was carried out during this period. A preliminary assay of semi-intensive mesocosm larval rearing was performed in preparation for experiments in the following years. Samples of larvae from hatching to end of metamorphosis were collected to evaluate ossification pattern and staining protocols. Information from the following deliverables were implemented; ***D15.1 Effective greater amberjack larval stocking densities, D15.2 Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing, D15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing*** *and* ***D15.4 Ontogeny of greater amberjack larval visual and digestive system.*** Following the hatchery phase, individuals were transferred for weaning and size selected. The final number of juveniles transferred for pre-growing was approximately 15,000 that were classified in 4 size-classes between 0.3 and 2.5 g.

NIREUS Larval rearing trials were carried out during 2017 which received 4 batches of eggs from Argosaronikos farm (1.0, 1.2, 0.65 and 0.5 million eggs, respectively). The eggs were directly incubated in the rearing tanks and the rearing temperature was set at 24.5 to 25.0 ºC. Larval rearing was performed following the standard protocol of the hatchery and the feeding was based on enriched rotifers, instar I and enriched instar II *Artemia* nauplii followed by artificial diets. The light conditions in the tanks were modified where possible in order to increase the light intensity on the surface of the tanks at >1000 lux. This resulted in significant higher survival in the tanks, which modified light conditions applied. Furthermore, following 20 dph fish were selected in size and grouped accordingly. The result of the project modifications was a significant improvement in the performance of the larvae and in particular their survival rate. The hatchery finally transferred in cages 48,300 juveniles of 25-50 g. Part of these juveniles are now used for the implementation of **Task 21.1.1.**

In the **Task 16.1** optimal combinations of factors to improve pikeperch larval rearing were tested. The results related to the experiments 2 (effects of feeding-related factors) and 3 (effects of population parameters) are presented. In experiment 2 it was demonstrated that weaned juveniles of 1.0-1.5 g mean body weight can be produced in 53 days, with relatively good survival (3.6-13.1%). A longer weaning duration increased mean swim bladder inflation (18% *vs* 67%) and final biomass increase. In addition, discontinuous feeding increased the final biomass produced in tanks while co-feeding (6 days) and the onset of the weaning period (10 or 16 days dph) had no significant effect on the final biomass and the percent of inflated swim bladders, while the method of food distribution only affected the rate of swim bladder inflation. During the course of the experiment, the mean specific growth rate (SGR) was 15.6% day^{-1}. There was a strong effect of the interaction between the onset of weaning (10 *vs* 16 dph) and its duration (3 *vs* 9 days) on the mean larval size and weight measured at 25 and 53 dph, which were higher when fish were weaned later with a longer weaning duration. Pikeperch larvae growth was also influenced by the interaction between the method of food distribution and whether or not co-feeding was implemented. In fact, when co-feeding was applied, no effect of the method of food distribution was observed, whereas in the absence of co-feeding, the larvae were heavier and larger with continuous feeding. On the other hand, this effect was no longer observed after 25 dph suggesting that this interaction is effective only during the weaning period. In conclusion, our results suggest that a later onset and longer duration of weaning followed by discontinuous feeding improved larval survival, growth and reduced deformities in pikeperch populations.

In experiment 3 (effect of population parameters), it was demonstrated that the production of juveniles of 1.8-1.9 g mean body weight can occur in 52 days as well as high levels of swim bladder inflation and tank biomass which is a marked improvement of pikeperch juvenile production in RAS conditions. Final biomass correlated with a higher initial larval density (100 larvae l^{-1}) and the use of larvae supplied by bigger females. In the comparison of the predatory behaviour of cannibals vs non-cannibals**,** predation tests revealed that cannibals show less predatory behaviour than non-cannibals, but they were significantly more efficient in prey capture. In the comparison of the digestive enzymatic activity of predators vs non-predators, results showed that trypsin and amylase activity values were higher in non-predator larvae than in predator larvae. Furthermore, pepsin activity values were lower in non-predators than in predator larvae. These results indicate that predatory larvae have a more developed digestive system development (higher levels of acid proteases in comparison to alkaline proteases) at the same age of non-predator larvae.

In **Task 17** on halibut showed that larvae in the RAS had better growth and survival compared to the larvae in the FT system. In **Sub-task 17.2** metagenomic studies aiming to identify probiotic candidates in the systems are underway. In **Task 17.3** production of on-grown *Artemia* has been completed and the full description of the work can be found in **D11.1** *Report on the nutrient profile of Artemia nauplii and on-grown Artemia.* **Sub Task 17.4** has been completed and the full description of the work can be found in ***D17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance***.

In wreckfish, important advances have been made in the understanding of ontogeny and larval development (**D18.**1) as well as the initial stages to develop an adequate larval feeding protocol. Most of the organs (except for the maxillary teeth at the upper jaw that became visible at 19 dph) appeared by 8 dph. The formation of intestinal villi started between 7-8 dph and increased in size until 16 dph. Following this, their size was reduced and at 23 dph where no more micro villi found in the intestinal area. At the day of hatching (0 dph) the retina appeared as a simple hemispherical sheet of undifferentiated neural epithelium (UNE) enclosing the lens, which was comprised of a spiral of unspecialized cells. The first differentiation in the different layers was visible at 3 dph. The pigment epithelium (PE) was not formed by this day. The ontogenesis of the organs related to the digestive and the vision system was not completed until 23 dph. Major structures like the gastric glands or the pyloric caeca appeared when larvae were 5.5 mm, and characterizes the time when the development of the digestive system is completed. No food was detected in the digestive tract, which means there was complete dependence on endogenous feeding resulting in limited growth performance. The maximum period that the larvae survived never exceeded 27 days post hatching (dph). The optimal incubation temperature in this trials was shown to be 16±0.8°C.

In grey mullet, the objectives were; (1) investigating environmental and nutritional factors that affect larval rearing. (2) Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production. (3) Determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding. The second objective will be carried out in 2018. In **Sub-task 19.1.1**, which determined the effect of algal type and concentration in rearing tanks on larval performance demonstrated that the higher turbidity (1.2 NTU) increased rotifer consumption independently of algal type although common biochemical factors between *Nannochloropsis oculata* and *Isochrysis galbana* may still influence larval performance. The significant ($P<0.05$) effect of turbidity level on rotifer consumption in 5 dph larvae was markedly similar to the treatment effect on survival in 51 dph fish despite that more than three weeks had elapsed since the fish were exposed to the algal treatments suggesting the long-term effect and importance of rotifer feeding. The results of this study were described in the submitted deliverable **D19.1** *Determine most effective type and concentration of algae used in grey mullet larval rearing.*

Sub-task 19.1.2 determined if the benefit of algal addition to rearing tanks was due to background lighting or other factors that contribute to larval performance. The results verified that high *N. oculata* turbidity (1.2 NTU) improved larval performance over the low *N. oculata* turbidity treatment (0.8 NTU). Moreover, larvae in the high *N. oculata* turbidity treatment significantly consumed more rotifers, as well as displaying better

growth and survival than larvae exposed to the same turbidity derived from clay. This suggests a further advantage that live algae provides over its ability to produce turbidity in the larval rearing of grey mullet.

Task 19.2 Compared the selected microalgae type and protocol (**Task 19.1**) with a lyophilized substitute demonstrated that larvae exposed to lyophilized and live *Nannochloropsis oculata* , which gave a turbidity of *ca* 1.2 NTU, in the rearing tanks resulted in very similar larval performance in terms of rotifer ingestion rate, swim bladder inflation, growth and survival. These results suggest that lyophilized algae, which are available commercial product, can be used to replace live algae. This would lead to a significant saving in time, labour and infrastructure. Worthy of note is that the advantage of algae over clay is not lost during the lyophilisation process. Consequently, the results of the present study recommends the use of lyophilized *Nannochloropsis oculata* in the larval rearing of grey mullet.

Task 19.4 Determined when to wean larvae and which weaning diet to use according to DT maturation and the shift from carnivorous to omnivorous feeding. The results showed that mullet juveniles grew significantly less, in terms of length and final weight, when fed only an ulva based herbivorous diet compared to a commercial carnivorous feed, while fish fed the 1:1 omnivorous mix of ulva and the commercial feed exhibited markedly ($P<0.05$) superior growth than all the treatments. Fish fed the herbivorous diet demonstrated significantly higher numbers of smaller fish (<100 mg), than the carnivorous and omnivorous diet fish and, in general, exhibited a population skewed to slower growing individuals. Conversely, 200-300 mg carnivorous and omnivorous treatment fish represented a significantly ($P<0.05$) higher percentage of the population than the herbivorous diet fed fish. Mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch rich microalgae and macroalgae, as well as benthic protein rich organisms characterizing the lower salinity estuarine waters they move into at this developmental stage. Furthermore, the high amylase and maltase activity in the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing resulting in improved growth. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds.

WP 14 Larval husbandry – meagre

WP No:	14	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Larval husbandry - meagre			
Other beneficiaries (from DOW):	P15. ULL			
Lead Scientist preparing the Report (WP leader):	Alicia Estevez			
Other Scientists participating:	Enric Gisbert (P3), Covadonga Rodriguez (P15), Jose Antonio Perez (P15)			

Objectives

1. To reduce costs by early weaning in meagre larvae and improve growth, survival and larval quality.

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the first 12 month period (year 2014) of the project one experiment was carried out using 4 different feeding schedules

- Group **A:** Weaning on dry feed started from 20 dph and completed on 30 dph, (control group)
- Group **B:** Weaning started from 20 dph and completed on 30 dph (same as the control but using half the amount of *Artemia* metanauplii)
- Group **C:** Weaning started from 15 and completed on 25 dph
- Group **D:** Weaning started from 12 dph and completed on 23 dph, with three replicates each.

Samples were taken periodically for biochemical analyses (lipids and fatty acid composition of larvae and live prey), digestive and antioxidant enzyme analyses, growth (length and weight) and skeletal deformation analysis.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The task was finished in 2015 and the deliverable already delivered in May 2016. Two trials were carried out, one in 2014 (already described in the first periodic report) and another in 2015. In both trials different feeding regimes using the standard protocol for larval rearing and weaning at day 20 or early weaning at day 12 or day 15 post hatch, using half of the amount of the Artemia provided in the standard protocol. Although early weaning in meagre can be carried out, care should be taken to avoid cannibalism (very high especially if weaning is carried out very early -12 dph- and using low quantity of Artemia). Thus, it was recommended to use low light intensity (200 lux), increase the number of feeding doses along the day, and grade the fish separating big larvae (cannibals) from the tank to allow small larvae to continue growing. In the trials a commercial feeding (Gemma micro, Skretting) was used, the formulation of this type of microdiets has been improved in the last years and probably in the future the big differences in microdiet acceptance and larval growth will be improved.

The results obtained in the study also showed that the larvae were able to digest the microdiets in an effective way and no severe skeletal deformations could be detected, early weaning had not any significant effect on the incidence of total skeletal deformities in the juveniles of meagre.

The results of the trials have been published in:

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A. 2017. Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research, 48: 5289-5299.

Summary of progress towards objectives (31-48 Mo):

In the 4th periodic report the results reported in the deliverable ***D14.1 Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles*** (2nd periodic report) was published in Aquaculture Research; "Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A. 2017. Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research, 48: 5289-5299"

Details for each Task

Task 14.1 Determining the earliest and most cost effective weaning period (led by IRTA, Alicia Estevez and Enric Gisbert and ULL, Covadonga Rodriguez and Jose Perez).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D14.1 Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles***.

WP 15 Larval husbandry – greater amberjack

WP No:	15	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Larval husbandry – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P8. IEO	P15. ULL	P27. FORKYS
Lead Scientist preparing the Report (WP leader):	Carmen María Hernández Cruz (P2)			
Other Scientists participating:	Nikos Papandroulakis (P1), Jerez Salvador (P8), Covadonga Rodríguez (P15), Popi Tsakoniti (P40)			

Objectives

1. Effects of different feeding strategies on larval performance in intensive systems,
2. Development of feeding protocol and rearing system in mesocosm semi-intensive systems,
3. Development of industrial protocol for larval rearing.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Task 15.1 Effect of feeding regime and probiotics. To achieve the objectives proposed in this task a first trial of rotifer enrichment was performed. Different proportions of *Echium* oil were used to enrich the rotifers considering 4 different enrichment periods (3, 6, 10 and 24 hours). The best results obtained, density and frequency supply of enriched prey will be assayed on amberjack larval rearing. **Task 15.2 Comparison of semi-intensive and intensive rearing.** During the reporting period preliminary trials were performed in order to establish the larval rearing methodologies in the two rearing systems at the P2. FCPCT. Three different larval rearing densities will be evaluated: 25, 50 and 75 eggs l^{-1} in triplicate tanks for a period of 30 days in two experiments. In all experiments, severe cannibalism and dispersion of total length was observed. Furthermore, during the reporting period a second trial was implemented for comparing the semi-intensive and the intensive methodologies at HCMR.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The main objective of the studies during this period was to improve the survival, growth and performance of greater amberjack larvae by improving the feeding regime, culture density and larval culture conditions. Even though the complete analysis is pending, the results are significant because the achieved very high survival rates are reported for the first time in greater amberjack, indicating a significant technological step in the larval rearing of this species, which will enable its commercial production.

Summary of progress towards objectives (31-48 Mo):

The main objective of the present studies was the development of an industrial protocol for the larval rearing of greater amberjack based on the results of the previous tasks. To achieve this objective, the trials carried out were based on the information collected in the previous reports. Survival obtained differed widely between the different trials, ranging from < 1% to ~ 10%. With all the available information, general

guidelines for larval culture were established. To evaluate ossification pattern and staining protocols, samples of larvae, from 3.38 ± 0.15 mm to 18.52 ± 0.73 mm standard length, were studied. Staining protocols were made point. It was observed that the ossification begins in the skull when the larva has a size of 3.38 ± 0.15 mm and the larva is completely ossified when it has a size of 13.03 mm ± 0.09. For the evaluation of the developed protocols some trials were tested in two Greek hatcheries: Galaxidi Marine and NIREUS Aquaculture hatchery in collaboration with HCMR. Following the hatchery phase, individuals were transferred for weaning and selected in size. The result of the adaptations was a significant improvement in the performance of the larvae and in particular their survival rate. A total of 63.300 juveniles were finally transferred to cages.

Task 15.1. Effect of feeding regime and probiotics. In this study different rotifer enrichment treatments were tested. They included commercial enrichments; LC60/20:4n-6/10ppm carotenoids, LC60/20:4n-6/10ppm carotenoids and 20% *Echium* oil, and T4 LC60/20:4n-6/10ppm carotenoids+ 20% Black cumin oil. The study showed that the rotifers enriched for a short period (3 h) with marine lecithin supplemented with 20% *Echium* oil showed the best results compared to the other commercial treatments although it was not significant. The results of the present trial suggest the positive effect of experimental live prey enriching emulsions supplemented with immune modulators such as *Echium* oil and black cumin oil compared to commercial emulsions on larval performance of *Seriola dumerili*.

Task 15.2. Comparison of semi-intensive and intensive rearing. Following the first trial with the very low overall survival in the two systems, a second experiment was organised in 2016. The survival in the case of the mesocosm was 18.7±0.8% while it was 8.2±3.1% for the intensive tanks. The gene expression of IGF-I and some of the IGFBPs were affected by the rearing method while the GHRH, GH, IGF-II were not.

Task 15.3 Effect of environmental parameters during rearing. The hydrodynamic field was estimated in tanks of 2,000 and 40,000 l, and the results showed higher survival at the end of the experiment in 2,000 l tanks, independent of egg stocking density, compared to the 40,000 l mesocosm tanks. The results of deformity evaluation showed a marked appearance of different types of skeletal anomalies in all treatments throughout the larval stages The photoperiod study of (24L:00D vs 18L:6D), according to the results, photoperiod did not affect the mRNA expression of any of the IGF binding proteins studied except IGF-BP1, which was higher in fish reared under the condition of 24L:00D. The study of the effect of tank color showed no differences in larval growth in terms of total length and body weight between the different tank colors, but differences were observed in the survival rates among the different groups. The gene expression analysis revealed significant differences among the treatments. Tank colour appeared to have an effect at the mRNA expression levels of GH at 17 dph with fish reared with a white background exhibiting the highest levels of expression while the lowest was in fish reared in the green tanks. The work done is fully described in deliverable 15.3.

Task 15.4. Development of industrial protocol. During this period a preliminary assay of semi-intensive mesocosm larval rearing was performed in preparation for experiments in the following years. Samples of larvae from hatching to end of metamorphosis were collected to evaluate ossification pattern and staining protocols.

Details for each Task

Task 15.1. Effect of feeding regime and probiotics (led by IEO Jerez Salvador).

Task 15.1. Effect of feeding regime and probiotics (IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez).

A feeding strategy for the larval rearing of greater amberjack (*Seriola dumerili*) was developed. This included defining the (a) prey concentration and supply frequency and (b) use of immune modulators substances. The results of the performed trials were evaluated in terms of survival, growth, larval nutritional condition (RNA/DNA ratio), physiological parameters (oxidative stress and immune system) and ontogeny

of the digestive enzymes. The deliverable improved the protocol of larval husbandry by determining efficient prey density and supply frequency and knowledge gained at using immune modulators.

The full description of the work and results is provided in Deliverable 15.2 "*Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing*".

Different trials were conducted to determine the effect of the enrichment products supplemented with immune modulators substances for live prey (rotifers) to be fed to greater amberjack larvae at different prey density and supply frequency.

1. Effects of enrichment products combined with immune modulators substances in live prey (rotifers) culture.

A first rotifer enrichment trial was performed in order to select products and period of enrichment. In this assay, rotifers were enriched with a polar lipid rich emulsion containing a marine phospholipid and arachidonic acid (AA, 20:4n-6) combined with 10 ppm of carotenoids (esterified astaxanthin). Different concentrations of *Echium* oil were then added as a probiotic/immunostimulant, given its role as modulator of the stress response in fish. From these preliminary results, the enrichment protocol based on a 6g/100l of the marine lecithin/20:4n-6/10 ppm carotenoids supplemented with 20% *Echium* oil for a short period (3 hours) was selected.

2. Effects of selected enrichment products combined with immune modulators substances and prey density (rotifers) in the larval rearing of greater amberjack.

Experimental conditions

The effect of the selected enrichment protocol (T3) was assessed on greater amberjack larval performance. In addition to *Echium* oil as probiotic/immune-stimulant, black cumin oil (*Nigella sativa*) was also tested, since black cumin seeds have been recently shown to also enhance growth performance and immunity in fish (Awad et al., 2013). Furthermore, the selected protocol was tested at two different prey densities. To this end, the rotifer enrichment commercial protocol (S. presso®, Inve Aquaculture, Dendermonde, Belgium) (T1) was compared with three experimental treatments (now T2, T3 and T4) added at 6g/100l concentration for 3 h to the rotifer enrichment tanks under the same rearing conditions stated previously. T2 consisted of the LC60/20:4n-6/10ppm carotenoids basic emulsion, whereas T3 and T4 consisted of this lipid emulsion combined with 20% *Echium* oil and 20% black cumin oil, respectively.

Greater amberjack newly hatched larvae, at an initial density of 100 larvae l^{-1} (mean total length 3.62±0.14 mm), were randomly distributed into 18 experimental tanks of 100 l capacity. Two prey concentrations were used, 5 (Low prey density) and 10 (High prey density) rotifers ml^{-1}. Rotifers enriched with one of the four test lipid emulsions were added to the larval rearing tanks twice a day (8:00 and 16:00). Larvae were randomly sampled at 1, 7 and 12 dph from the experimental tanks. At the end of the trial (12 dph) larvae of each tank were counted and the percentage of survival calculated. Total length and percentage of larvae with inflated swim bladder were also determined. Daily prey intake was estimated by the differences between added and remaining rotifers in the larval rearing tanks.

Results

Larval growth (total length, TL) significantly (P<0.05) increased from 1 dph (3.502 ± 0.156 mm) to 7 dph (3.751 ± 0.318 mm) and 12 dph (4.510 ± 0.424 mm), irrespective of the enrichment treatment. At each age, no differences were encountered in the TL of larvae fed at Low (5 rot ml^{-1}) or High (10 rot ml^{-1}) prey density for each enrichment treatment. However, at 7 dph, larval growth was significantly (P<0.05) lower in larvae fed the commercial treatment (T1) **(Fig. 15.1.1.a).** At 12 dph, all T1 fed larvae died **(Fig. 15.1.1.b).**

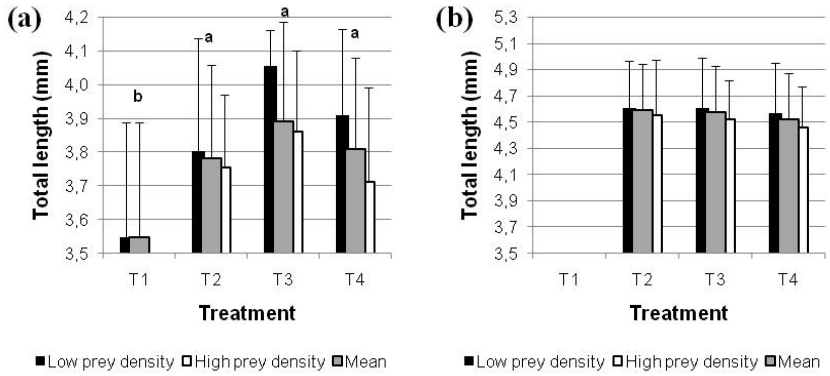


Figure 15.1.1.a.b Total length (mm) of 7 dph (a) and 12 dph (b) greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, P <0.05).

No significant (P>0.05) differences were found in the percentage of inflated swim bladder in larvae fed at low or high prey density for each enrichment treatment at 7 dph and 12 dph. However, at 12 dph, the percentage of larvae with inflated swim bladders was significantly (P<0.05) lower in T2 larvae compared to the T3 and T4 cohorts.

Fish survival was very low at the end of the feeding period independently of dietary regime and prey density treatment. On the other hand, there was a clear but non-significant (P>0.05) trend of increased survival in T3 and T4 treatments compared to T1 fish which didn't survive past 12 dph.

Regardless of dietary regime, the density of rotifers (5 or 10 rots ml-1) in the larval culture tank did not significantly affect fish growth performance and feeding behavior although larvae receiving the commercial treatment (T1) showed the worst results. Comparatively results obtained show a positive effect of the experimental emulsions used to enrich the rotifers, and particularly those where immune substances were added suggesting that prey density had no effect on larval performance.

3. Effects of selected enrichment products combined with immune modulators substances and feeding frequency in the larval rearing of greater amberjack

Experimental conditions

A set of experiments were carried out in order to test the combined effect of enrichment products containing immune-stimulants (PUFA-rich lipids, carotenoids and *Echium* oil or black cumin oil, *Nigella sativa*) and the feeding frequency on *S. dumerili* larval performance in terms of digestive enzymes activities as well as immunity and oxidative stress status.

Two experiments were carried out (Trial 1 and Trial 2) in order to ensure availability of larval samples for analysis, and confirm the agreement of growth and survival results from two different egg sources and broodstock groups. In trial 1, the eggs were obtained from IEO facilities while in trial 2, the eggs used were produced at FCPCT facilities.

Rotifers enriched with one of four treatments previously described were added to the larval rearing tanks twice (10:30 h and 20:30 h) or three times (10:30 h, 15:30 h and 20:30 h) day^{-1}. The enrichment period of rotifers given the commercial treatment (S. presso®, Inve Aquaculture, Dendermonde, Belgium) (T1) was 8 hours before being added to the larval tanks while the period of enrichment of the three experimental treatments (T2, T3 and T4) was 3 hours.

Larval sampling (7 and 12 dph) was carried out randomly from the experimental tanks. Total length, swim bladder inflation percentage and volume, eye diameter and daily prey intakes were determined. At the end of the trial, larvae of each tank were counted and survival calculated. Larvae were also be examined for oxidative stress in terms antioxidant enzymes catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) and humoral parameters of the immune system (activity of peroxidase, proteases and anti-proteases, anti-bactericidal). The ontogeny of the digestive enzymes of amberjack larvae focusing on total protease, lipase, and amylase was also performed.

Results

Average larval total length significantly increased from 1 dph to 7 dph and 12 dph irrespective to dietary treatment. Larvae fed with black cumin oil treatment 3 times day^{-1} were higher those fed 2 times day^{-1} at 12 dph. At 7 dph, larval growth was significantly higher ($P < 0.05$) in fish fed with cumin oil with respect to those receiving control treatment. The best survival was demonstrated at 12 dph when the larval amberjack were fed 3 times day^{-1} compared to only 2 times day^{-1}. Supplementing *N. sativa* oil during rotifer enrichment also resulted in earlier swim bladder inflation and higher swim bladder volume at 12 dph.

The digestive enzyme activities were higher in fish feeding on black cumin supplemented rotifers where significantly higher lipase and protease alkaline activities were observed. Presumably the increase in protease and lipase activities resulted in better digestion and assimilation of dietary protein and lipid promoting feed efficiency. However, amylase activity, the major enzyme associated with carbohydrate digestion was not increased by the assayed immune-stimulants, which is not surprising as this fish, at all developmental stages, are strict carnivores.

Regarding antioxidant defense enzyme activities and lipid peroxidation products in response to immunostimulants, the results showed that age was an important factor to consider determining the biochemical responses to oxidative stress. An increase in some of the antioxidant activities was observed from 7 to 12 dph larvae independent of treatment. The activities of the SOD and GST enzymes were affected by immunostimulants differently depending on the age of the larvae. The activities of these two enzymes were reduced by *Echium* oil and cumin oil at 12 dph but only SOD was reduced by cumin oil at 7 dph. The effects of immunostimulants on the peroxidation status of the larvae were not evident at 7dph although altered levels of lipid peroxidation products, showing higher values of TBARS, were observed in *Echium* supplemented larvae at 12 dph.

Larvae fed with the control or T2 diet (PUFA-rich lipids and carotenoids containing diet) had similar levels of humoral innate activities. However the larvae fed with the *Echium* oil containing diet showed lower levels of protease activity at 7dph, although similar levels than control larvae were observed at 12 dph. In the black cumin oil treatment, a surprising inhibition of peroxidase and bactericidal activities were observed at both 7 and 12 dph, while protease activity was inhibited at 7 dph.

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.2. Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing***.

Task 15.2 Comparison of semi-intensive and intensive rearing (led by HCMR, Nikos Papandroulakis).

Sub-task 15.2.1 (by HCMR, Nikos Papandroulakis).

The objective of this particular sub task is the comparison between intensive (in RAS with 500 l tanks) and semi-intensive (Mesocosm with 40,000 l tanks) larval rearing. The evaluation will be based on a comparative study of the:

(i) ontogeny of the visual system of the larvae, (influenced by feeding) through histological procedures,

(ii) larval oxidative stress through the activity of specific enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione S-transferase and the concentration of glutathione).

(iii) investigating the larval somatotropic axis (consisting of the growth hormone-releasing hormone (GHRH), growth hormone (GH), insulin-like growth factors (IGF-I and II), associated carrier proteins (IGFBPs) and receptors that represents the endocrine and autocrine regulator for skeletal muscle growth.

During the reporting period a final trial was performed in order to compare the larval performance in the two rearing systems given the pure results obtained during the previous trials.

The implementation of this work was achieved with the participation of the following group of HCMR personnel: N. Papandroulakis, A. Tsalafouta, N. Mitrizakis, S. Stefanakis, P. Anastasiadis, M. Vassilakis, Y. Strakantounas E. Sfakaki, N. Kopidakis.

Semi-intensive Mesocosm Larval rearing and Intensive rearing in closed water recirculation system

The methodology followed was similar to the one described in the previous report without any changes

Controlled parameters

The growth of the individuals was estimated with regular measurements of total length and wet weight from a representative sample of larvae per tank. At the end of rearing period (~30 dph) populations were counted and transferred for pre-growing.

Furthermore, for comparison purpose with the results of the 2015 trial, samples were collected for investigating the larval somatotropic axis (consisting of the growth hormone-releasing hormone (GHRH), growth hormone (GH), insulin-like growth factors (IGF-I and II), associated carrier proteins (IGFBPs) and receptors) that represents the endocrine and autocrine regulator for skeletal muscle growth.

qPCR experiments

RNA purification and cDNA synthesis

Primers were available from previous work. Samples of pre-larvae and larvae were let to thaw on ice, disrupted and homogenized using the TissueRuptor (Qiagen, Hilden, Germany) for 20 s in 600 µl RLT plus buffer (RNeasy Plus Mini Kit Qiagen, Valencia, USA). Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Valencia, USA). RNA yield and purity was determined by measuring the absorbance at 260 and 280 nm using the Nanodrop® ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany), and its integrity was tested by electrophoresis in 1% agarose gels. Reverse transcription (RT) was carried out using 1 µg RNA with QuantiTect Reverse transcription kit (Qiagen).

Quantitative real-time PCR (qPCR)

The mRNA expression of genes encoding for Growth Hormone (GH), Growth Hormone Releasing Hormone (GHRH), Insulin-like Growth factor I & II (IGF-I & IGF-II), Insulin-like Growth factor Binding Proteins 1, 2, 3 & 5 (IGF-BP1, IGF-BP2, IGF-BP3, IGF-BP5) was determined with quantitative polymerase chain reaction (qPCR) assays using the *KAPA SYBR® FAST* qPCR Kit (Kapa Biosystems). Reactions were cycled and the resulting fluorescence was detected with MJ Mini Thermal Cycler (Bio-Rad) under the following cycling parameters: 95 °C for 3 min (HotStarTaq DNA Polymerase activation step), 94 °C for 15 s (denaturation step), 60 °C for 30 s (annealing step), 72 °C for 20 s (extension step), 40 cycles (step 2–step 4). Levels of Growth Hormone (GH), Growth Hormone Releasing Hormone (GHRH), Insulin-like Growth factor I & II (IGF-I & IGF-II), Insulin-like Growth factor Binding Proteins 1, 2, 3 & 5 (IGF-BP1, IGF-BP2, IGF-BP3, IGF-BP5)] mRNA were normalized based on the reference genes *18S* and b-actin. A relative standard curve was constructed for each gene, using 4 serial dilutions (1:5) of a pool of all cDNA samples. We also performed geNORM analysis (Vandesompele *et al*., 2002) in order to validate which are the most suitable reference genes to serve as an internal control and we concluded to *eEF1a* and *18S*.

Statistical analysis

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). All data are presented as means ± standard deviation (SD). Data were initially screened for normality and homogeneity. Statistical comparisons of temporal patterns of gene expression between the different developmental stages and the various rearing conditions were made using two-way ANOVA. Holm-Sidak's honestly significant difference

test for multiple comparisons was used to determine significant differences among groups. The significant level used was $P < 0.05$.

Sample collection

During the 2016 trial, 5 pooled samples both from the mesocosm and intensive reared fish were taken at 3, 5, 17, 25, and 30 days post hatch (dph) and were used for expression analysis of Growth Hormone (GH), Growth Hormone Releasing Hormone (GHRH), Insulin-like Growth factor I & II (IGF-I & IGF-II), Insulin-like Growth factor Binding Proteins 1, 2, 3 & 5 (IGF-BP1, IGF-BP2, IGF-BP3, IGF-BP5).

Results

Rearing was performed without significant problems and no pathologies were presented. The overall survival in all cases was significantly improved. In the case of the Mesocosm survival was 19.29 and 18.11% in the two tanks used during the reporting period while it was 10.4 and 6.0% for the 500 l tanks. The results of the growth performance are shown in **Table 15.2.1.1**. Larval growth (total length) represented in **Figure 15.2.1.3.**

Table 15.2.1.1 Growth rate (exponential) of greater amberjack larvae in terms of total length and wet weight during the rearing

Total Length	**2015**
40.000 l	0.065 d^{-1}
500 l	0.047 d^{-1}
Wet weight	
40.000 l	0.27 d^{-1}
500 l	0,24 d^{-1}

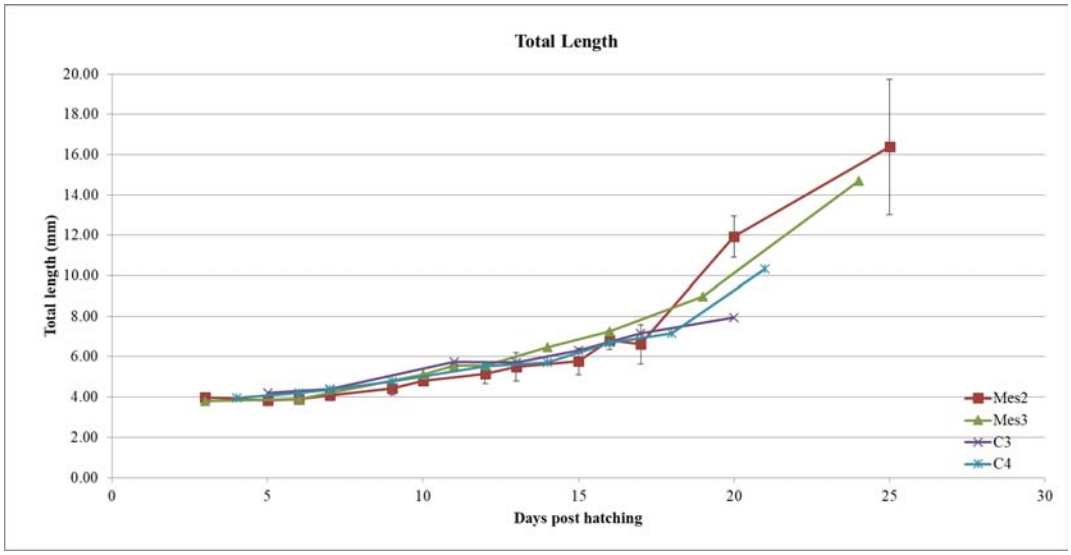


Figure 15.2.1.3: Evolution of the total length of greater amberjack larvae reared under intensive or semi-intensive conditions (Mean and standard deviation).

The rearing method (mesocosm vs intensive) appear to affect the mRNA expression levels of IGF-I. There was a gradual increase in mRNA levels as development proceeds with significant differences observed ($P < 0.05$) between the two conditions tested at 30 dph (**Figure 15.2.1.4**).

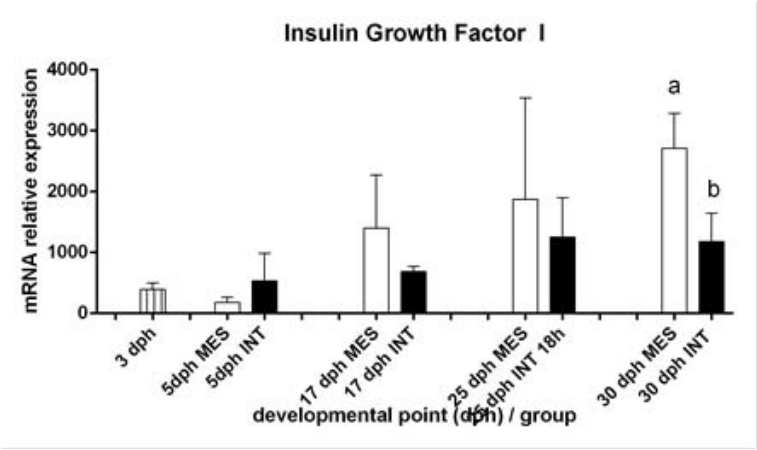


Figure 15.2.1.4. mRNA relative expression levels of IGF-I between the different rearing methods during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

The mRNA expression levels of IGF-II were not altered based on the rearing method (**Figure 15.2.1.5**).

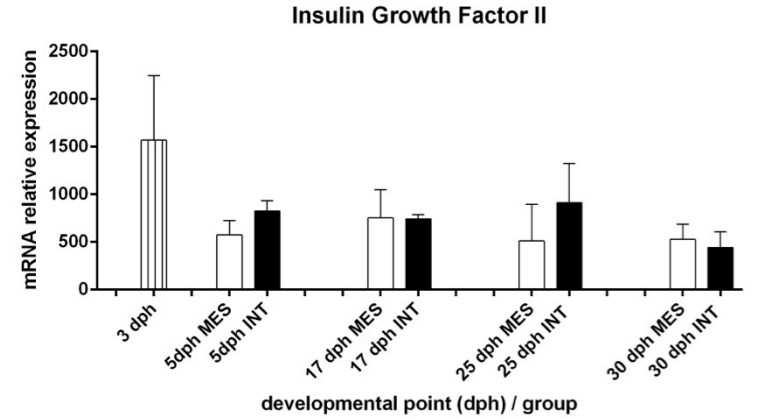


Figure 15.2.1.5. mRNA relative expression levels of IGF-II between the different rearing methods during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

The expression of GH was not affected by the rearing method used but higher mRNA levels were observed at 17 dph (**Figure 15.2.1.6**). GHRH expression levels were not affected by the rearing method used but throughout development remained generally at lower levels but increased at 25 dph in the intensive rearing (**Figure 15.2.1.7**).

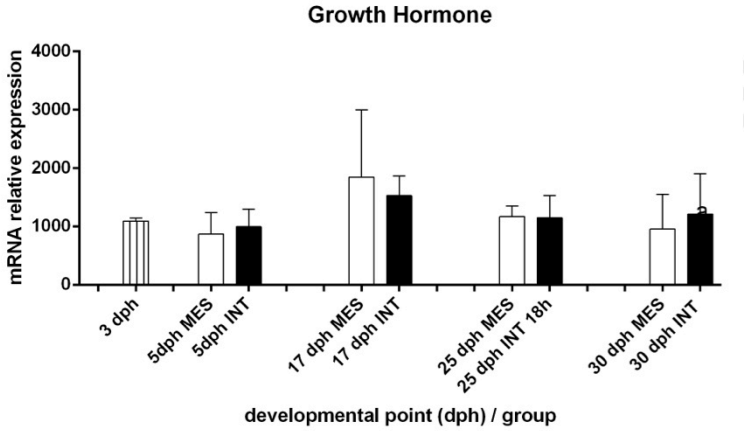


Figure 15.2.1.6. mRNA relative expression levels of GH between the different rearing methods during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

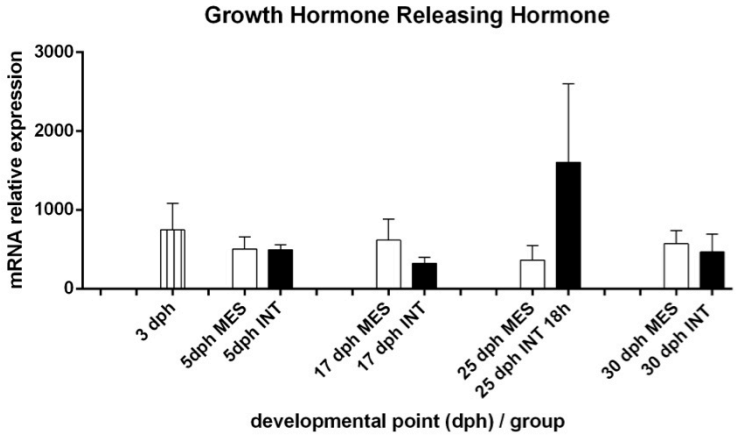


Figure 15.2.1.7. mRNA relative expression levels of GHRH between the different rearing methods during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

The rearing method applied affect the mRNA expression of the IGF binding proteins 1 and 5 (**Figure 15.2.1.8a**) For the first, the higher levels ($P < 0.001$) were observed in the mesocosm reared fish compared to the intensive reared fish from 25 dph to 30 dph.

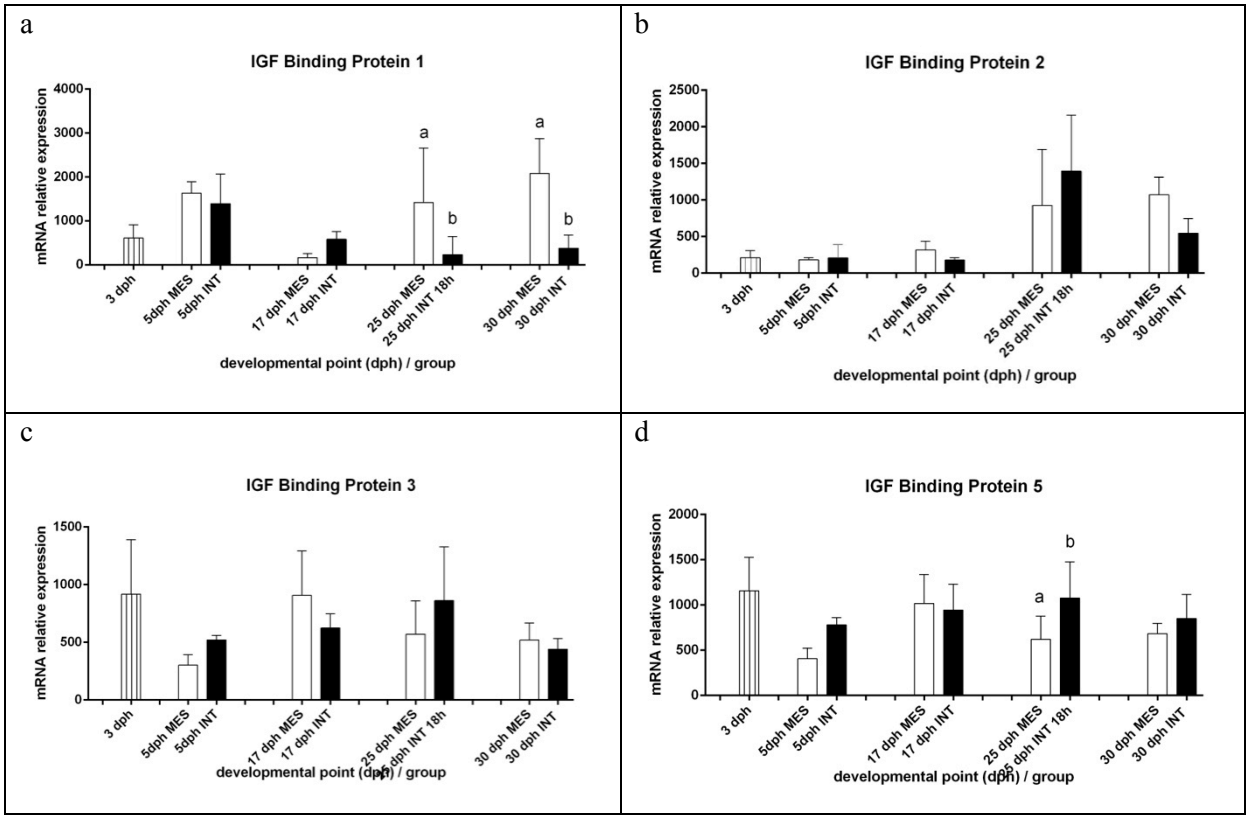


Figure 15.2.1.8. mRNA relative expression levels of IGF-I binding proteins between the different rearing methods during early ontogeny of *Seriola dumerili*: (a) IGF-BP1; (b) IGF-BP2; (c) IGF-BP3; (d) IGF-BP5. Values are means ± standard deviation (n = 5). Different letters indicate significant differences between the sampling points during ontogeny whereas asterisks indicate differences between mesocosm and intensive reared fish ($P < 0.05$).

The mRNA expression levels of IGF-BP2 appeared low at the beginning of development until the 25 dph were there was a increase ($P < 0.05$) and remained high until the 30 dph (**Figure 15.2.1.8b**). No differences were observed in the case of IGF-BP3 (**Figure 15.2.1.8c**), However in the case of IGF-BP5 the mRNA expression levels remained generally stable with the exception of 25 dph where peak values ($P < 0.05$) were observed for the larvae at intensive conditions (**Figure 15.2.1.8d**).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.1. Effective greater amberjack larval stocking densities***, and ***Deliverable 15.2. Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing***.

Task 15.3 Effect of environmental parameters during rearing (led by FCPCT, Carmen Maria Hernández Cruz).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing.***

Task 15.4 Development of industrial protocol (led by IEO, Jerez Salvador).

Sub-task 15.4.1 Development of an industrial protocol for larval rearing based on the results of the previous tasks (IEO, Salvador Jerez, Virginia Martín, Eduardo Almansa).

To achieve this objective, the trials carried out were based on the information collected in the previous reports:

D15.1 Effective greater amberjack larval stocking densities

D15.2 Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing

D15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing

D15.4 Ontogeny of greater amberjack larval visual and digestive system

Moreover, during the 2nd Reporting Period, preliminary trials were performed in order to obtain additional information in our facilities.

With all the available information, general guidelines for larval culture were established.

Material and methods

Experimental conditions

Spawned eggs obtained from F1 greater amberjack broodstock at the IEO-COC facilities were collected with a passive egg collector placed in the outflow of the spawning tank, rinsed and placed in 90 l cylindroconical tanks. The fertilized eggs were incubated with constant exchange of filtered seawater and slightly aeration.

The newly hatched larvae (24-48 hours after collected) were stocked in indoor rearing tanks of different volume (10 m^3 square tanks, 32 circular tanks and 40 m^3 circular tanks) at densities ranging from 3 to 18 larvae l^{-1}.

The tanks with black bottom were previously filled with filtered (5µm) natural seawater and the rate of seawater renewal was increased progressively during the rearing.

Aeration was also provided in the tanks by means of pipes distributed in the perimeter and the center of the tank in order to maintain the larvae in gentle rolling suspension.

The internal initial filter screen of 363 µm mesh size changed to 500 µm mesh size at 25 culture day. A surface skimmer was used during the rotifers feeding period to keep the surface free from lipids to improve the swim bladder inflation.

All larval rearing trials were developed under natural conditions of temperature, salinity and photoperiod.

Feeding

The duration and type of feeding item that was included in the rearing protocols during the experimental procedure was based in the previous information obtained.

The live microalgae *Chlorella sp*, cultured in IEO facilities, was added daily from start until 22-25 dph at 100-200x10^3 cell ml^{-1}. Feeding was based on daily administration of rotifers (*Brachionus plicatilis*) harvested from the stock culture and enriched with DHA Protein Selco (INVE S.A., Belgium) distributed two times a day (8.00 and 16.00 h) from 3 to 25 dph. The initial density of rotifers in the rearing tank was 3 rot ml^{-1} and increased progressively to 5-10 rot ml^{-1} at 8-10 dph and then decreased. The remaining rotifer concentration in the larval rearing tank was estimated daily before adding the new enriched rotifers. During the rotifers feeding, copepods were introduced to the rearing tanks due to the natural productivity in the rotifers culture, which potentially contributed to larvae feeding.

At 12 dph, and during 5-7 days, *Artemia* AF nauplii were added to rearing tanks and *Artemia* EG 1-day enriched with A1 DHA Selco (INVE S.A., Belgium) were offered from 14 dph. The *Artemia* was supplied two times a day and density in the rearing tanks ranged between 0.05 and 0.5 *Artemia* ml^{-1}.

The time to start feeding with artificial feeds depends on the larva size and development, but in all trials it began at 18-20 dph. Size, frequency and quantity of artificial feeds increased progressively according to fish size (NRD 2/4 size of 200–300 µm, and NRD 3/5 size of 300–500 µm, INVE S.A., Belgium)

Sampling

During rearing trials, larvae were sampled periodically to evaluate growth parameters. Total length and eye diameter were measured with a Nikon Digital Sight DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) and the percentage of larvae with inflated swim bladder was also determined. Prey intake was determined by analysis of stomach contents.

Larvae samples were flushed with N_2 and kept frozen at -80 °C until analysis to determine nutritional condition, stress oxidative and immune system.

Survival of larvae was calculated based on the number of surviving fish that were individually counted at the end of the experiment.

Results

The natural seawater conditions of salinity were 35 psu and the temperature ranged between 20.3-25.1 ºC during the different trials, and the photoperiod and light intensity were natural.

Daily renewal rate increased progressively according with the previous information ranging from 15-40% day^{-1}at day 1, 30-40% at day 10, 100-120% at 20 days, and 200-240% at 30 days (**Figure 15.4.1.1**). Dissolved oxygen varied from 6.1 to 6.7 mg l^{-1} during the larval rearing and oxygen saturation tended to decrease presenting the lower levels at 20-25 days in 32 m^3 tanks.

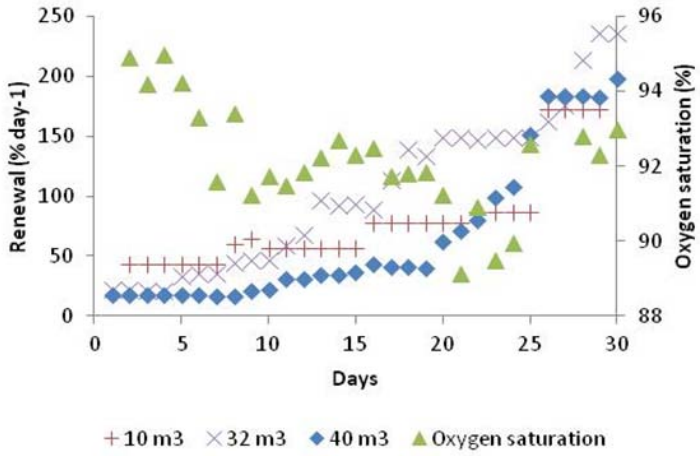


Figure 15.4.1.1. Daily renewal rate (% day^{-1}) in 10, 32 and 40 m^3 tanks and oxygen saturation (%) in 32 m^3 tank.

The temperature as well as light intensity varied depending on the tank volume (depth). In 40 m^3 tanks the light intensity (~1000-2500 lux) decreased to 50% at 20-40 cm of depth and about 80% at 140 cm of depth. The temperature also decreased 0.4 ºC at the first 40 cm of depth and no changed after. However, in 10 m^3 square tanks with a depth of 1 m, the light intensity (~500-700 lux) decreased to 50% in the first 20-30 cm and remained unchanged about 30% after 60 cm of depth.

Survival obtained differed widely between the different trials, ranging from < 1% to ~ 10%, although in some cases no larvae were obtained. Logically, survival depended on the age at which the larvae were removed from the rearing tank. The mortality by cannibalism was significantly high after 25 dph, but also pathologies might be responsible for important mortalities. The transfer of the larvae from initial rearing tank to new tanks (preferably of smaller volume) increased the larval survival.

The total length increased exponentially during the larval rearing (**Figure 15.4.1.2**), and the growth rate and the percentage of larvae with inflated swim bladder (**Figure 15.4.1.3**) were higher in tanks of 32 m^3 than in tanks of 40 m^3. In 32 m^3 tanks, all larvae showed the swim bladder inflation at 7 dph with a total length of 3.84±0.30 mm, and in 40 m^3 tanks, at 14 dph with 4.59±0.29 mm.

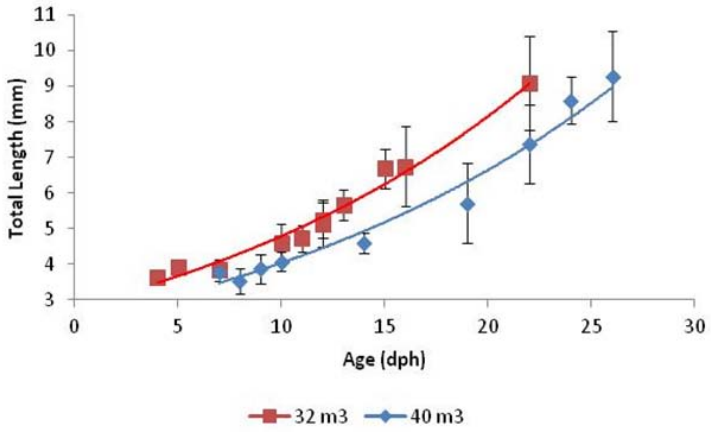


Figure 15.4.1.2. Total length (mm) of greater amberjack larvae reared in 32 and 40 m^3 tanks. Values are mean ± SD (n=3).

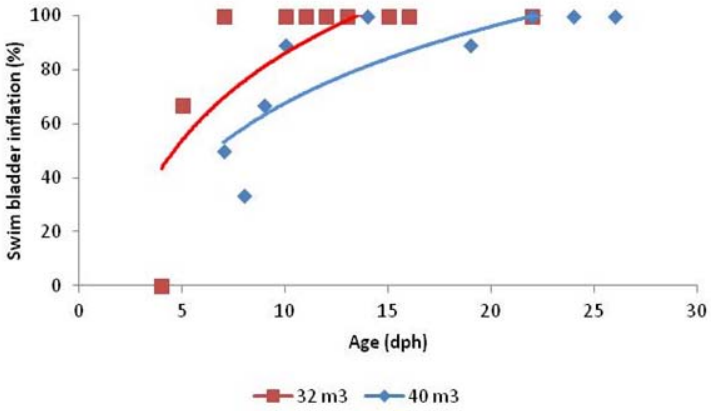
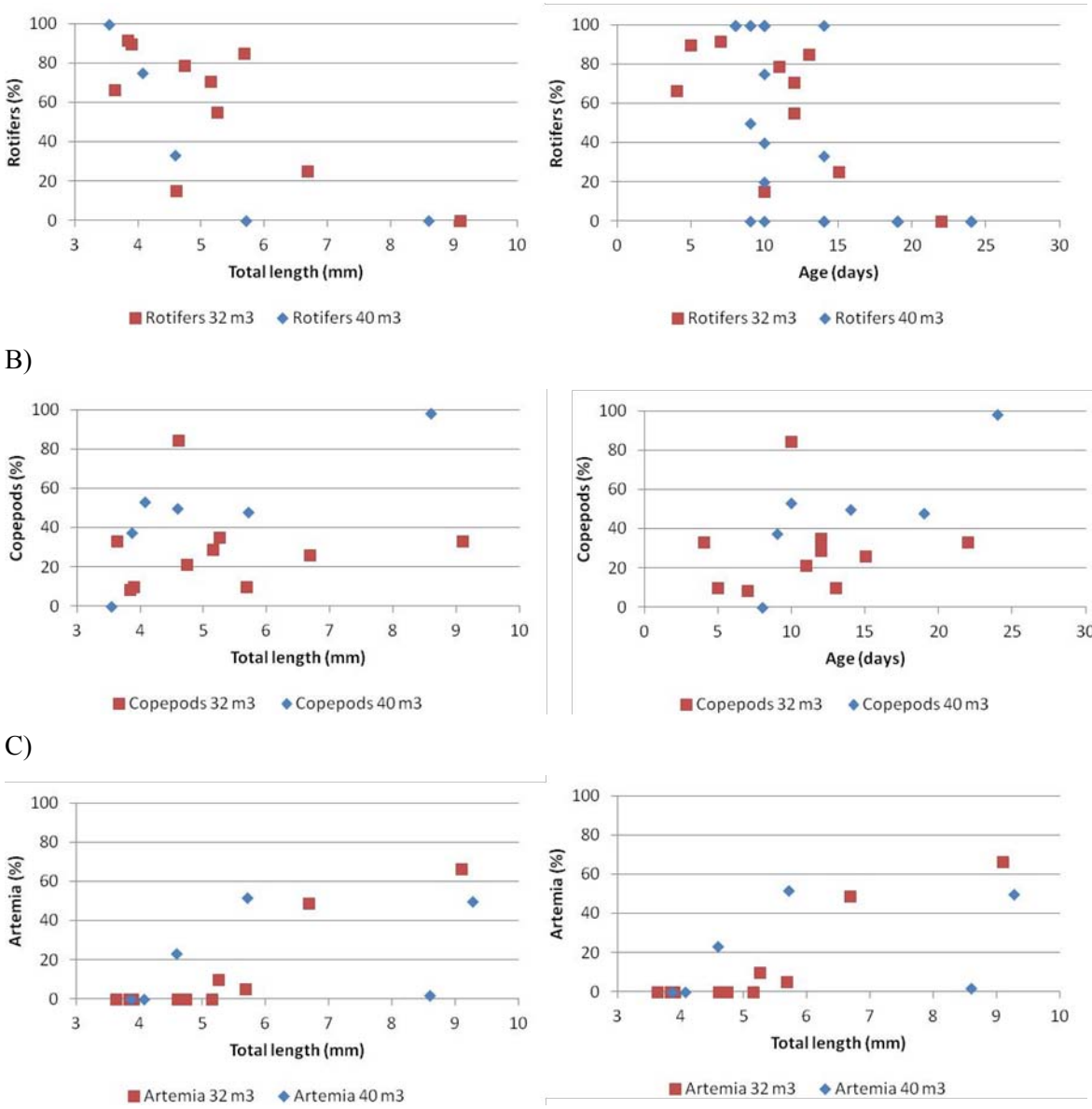


Figure 15.4.1.3. Swim bladder inflation (%) of greater amberjack larvae reared in 32 and 40 m^3 tanks.

The prey items in the stomach was related to total length of larvae showing a decrease of the rotifers intake coinciding with an increase of *Artemia* intake irrespective of the rearing larvae tank volume (**Figure 15.4.1.4**). Larvae ingested a higher number of rotifers at 8-10 dph and they did not show rotifers in their

stomach after 19 dph. Copepods were present in the larvae stomach from first feeding to 25 dph. Also, *Artemia* nauplii were added to rearing tanks at 12 dph and found in the larvae stomach up to 26 dph.

A)



B)

C)

Figure 15.4.1.4. Relationship between prey intake and total length (mm) or age (days) in greater amberjack larvae reared in 32 and 40 m^3 tanks A) rotifers, B) copepods and C) *Artemia*.

Sub-task 15.4.2 Ossification pattern and incidence of skeletal deformities for amberjack larvae (by FCPCT, Carmen Maria Hernández Cruz)

To achieve this objective, samples of larvae from hatching to end of metamorphosis from Subtask 15.2.2 were collected to evaluate ossification pattern, staining protocols were tested to evaluate the samples.

The cranial and body skeleton development ontogeny are described on this experience

Material and methods

Osteological analysis

Total length was recorded during larval development from samples of 30 larvae per tank, every 5 days, from hatching to 30 dph.

Samples preparing

Amberjack larvae were collected from each tank to kill them on ice. Then, the larvae were washed with distilled water and kept at 4 ºC until its use. It is necessary to ensure that all larvae have died for a good fixation. To study the bone ossification, all specimens were fixed in 10% buffered formalin.

To carry out these analyses, Boglione (2014) manual was followed. In this experiment, single staining was performed to dye cartilage. The first step was to measure the larvae in the profile projector (Mitutoyo PJ-R3000, San Antolin, Elgoibar). After, melanophores bleaching were transferred to fishes, where a solution of potassium hydroxide (KOH) and 0.5% hydrogen peroxide (H2O2) 3% was used until eyes colour turn from black to brown (in this case 1h, Boglione, 2014). Then, the larvae were washed with 96% ethanol quickly. To dehydrate, the larvae were kept 24 h in 50% ethanol and other 24 h to 95% ethanol. These times are set by the size of the larvae study. Furthermore, absolute alcohol was necessary to prevent demineralization small bone, thus to reduce water in the samples improvement cartilage staining and extending the life of this solution (Boglione, 2014).

The next step was alcian blue staining, which was performed with a composite solution of ethanol absolute (70 ml) alcian blue (20 mg) and acetic acid (30 ml), (Boglione, 2014). Larvae were exposed for 2 h until the cartilage structures were stained. Due to the solution acidity was necessary to neutralize the staining solution. For this, the fishes were subject to 12 h in 0.5% KOH. The solution was removed and the specimens washed with distilled water to carry out the fixation. The stained samples were treated with several dilutions. First, a solution of 30% glycerol and 70% KOH (1%) for 12 h, and finally, the larvae were transferred to solution with 60% glycerol and 40% KOH (1%) other 12 h.

Larvae were individually examined using stereomicroscopy, **Figure 15.4.2.1.**

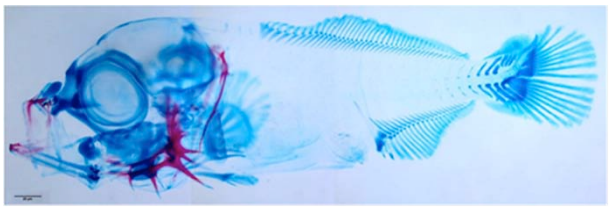


Figure 15.4.2.1. Stained larvae greater amberjack *Seriola dumerili* (5.64± 0.11 mm)

The drawings of the different developmental stages were made using Adobe Photoshop CS3-10.0 (1990-2007 Adobe System Incorporated, United States) directly from digital photographs. Bone description, followed the terminology suggested by different authors (Manod, 1968; Matsuoka, 1985; Collette & Gillis, 1992; Suda, 1996; Cubbage & Mabee, 1996; Faustino & Power, 2001).

Results

The cranial structures began to ossify by 3.38 ± 0.15 mm **Figure 15.4.2.2**, with the calcification of upper jaw (premaxilla) and cleithrum. The jaw structures differentiated in two regions, the upper maxilla and premaxilla and the lower jaw.

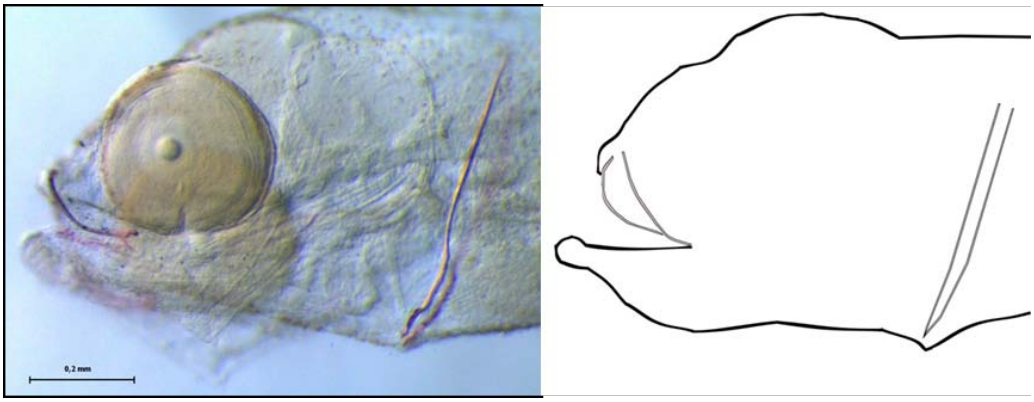


Figure 15.4.2.2. Cranial development to a size of TL (mm): 3.38 ± 0.15

The first visible structure in greater amberjack larvae was the maxilla, followed by the dentary and the premaxilla, and the last structures were the Angulo-articular and the retro-articular by 4.49 ± 0.14 mm TL, **Figure 15.4.2.3**. At this time, small premaxillary teeth were first seen, whereas the dentary teeth developed.

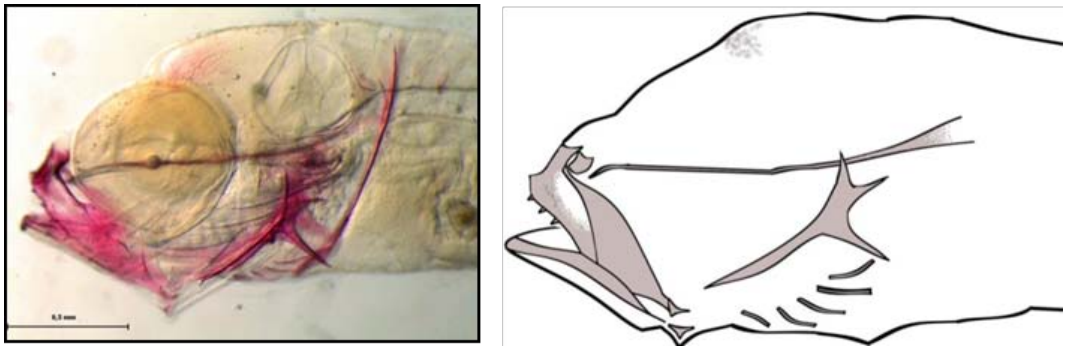


Figure 15.4.2.3. Cranial development to a size of TL (mm): 4.49 ± 0.14

The last bones to calcify were registered on the otic region at 10.15 ± 1.86 mm **Figure 15.4.2.4.**

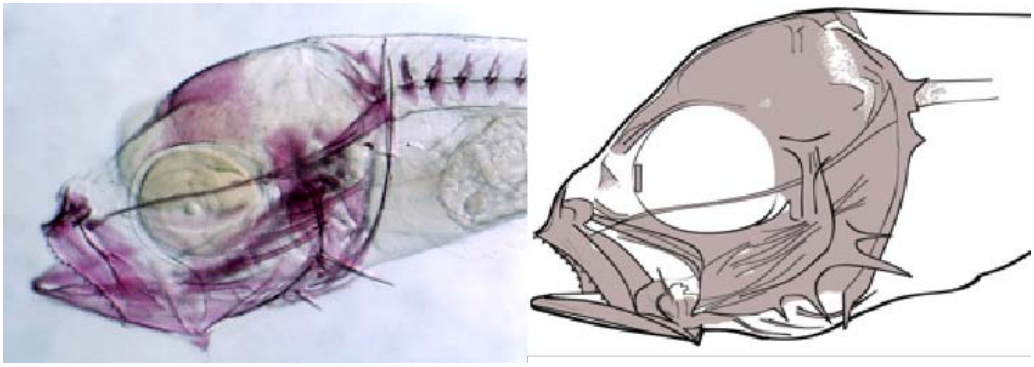


Figure 15.4.2.4. Cranial development to a size of TL (mm): 10.15 ± 1.86

In the vertebral column and fin ontogeny of greater amberjack, the first structures that began to ossify were the neural arches (4.49 mm) **Figure 15.4.2.5**. and continued with the haemal arches and the centrum.

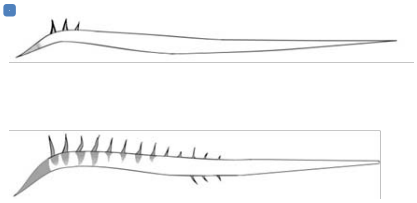


Figure 15.4.2.5. The vertebral column ontogeny of greater amberjack to a size of TL (mm): 4.49 mm± 0.14

The caudal fin began to ossify with the calcification of the caudal rays (Lepidotrichia) by 5.29 mm, **Figure 15.4.2.6** and continued with flexion of the notochord by 5.36 mm.

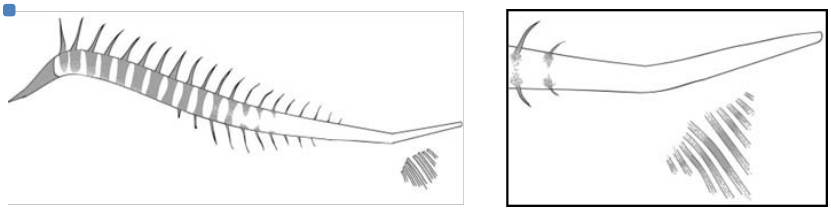


Figure 15.4.2.6. The caudal fin and the flexion of the notochord of greater amberjack to a size of TL (mm): 5.36 mm± 0.2

The last bones to calcify were the haemal arches (up to 12.87 mm) and the caudal fin was totally ossified by 13.03 mm, **Figure 15.4.2.7** and, **Figure 15.4.2.8**.

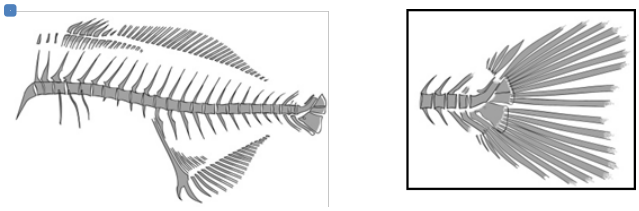


Figure 15.4.2.7. The caudal fin ontogeny of greater amberjack to a size of TL (mm): 12.87 mm± 0.12

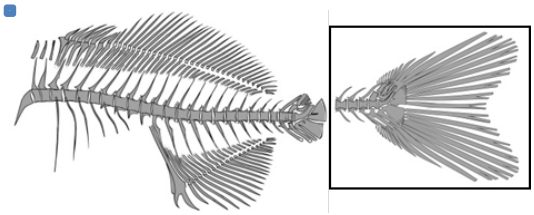


Figure 15.4.2.8. The caudal fin ontogeny of greater amberjack to a size of TL (mm): 13.03 mm± 0.09

The skeletal deformities was determined of this task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.3. Optimun hydrodynamics and light conditions during greater amberjack larval rearing.***

Sub-task 15.4.3 Validation of the developed protocol initially at FCPTC and over two successive years in an SME hatchery (by HCMR, Nikos Papandroulakis and GMF, Giorgos Iakovopoulos and Popi Tsakoniti)

Validation of the developed protocol initially at FCPTC and over two successive years in an SME hatchery

For the implementation of Task 15.4.3 Industrial application of greater amberjack larval rearing, a meeting was organized in August 2016 between HCMR and FORKYS. During the meeting the parameters of the trial were discussed and it was decided that for an efficient implementation, FORKYS ought to implement some modifications in the hatchery facility. Namely, (a) the water quality in the larval rearing tank ought to be improved by including a sea-water inlet and (b) install a small unit for phytoplankton culture. The representatives of FORKYS explained that they have to consider some administrative issues before proceeding with the implementation. Unfortunately, on February 2017 the company decided that it was not possible for them to proceed with the required adaptations in the hatchery facility.

A contingency plan for this includes the transfer of this partner's activities to another partner that has the required infrastructure and has shown the will to participate in the DIVERSIFY project. Task 15.4.3 will be allocated to a partner SME (Galaxidi Marine Farms, GMF), which is member of the consortium and has the required facilities.

The above was part of the last amendment of the DOW. Furthermore, the evaluation of the developed protocols was also tested in the hatchery of another Greek company that expressed the will to participate without any financial support. NIREUS Aquaculture hatchery at Nafpaktos performed also some larval rearing trials during 2016 and 2017 in collaboration with HCMR.

GMF Larval rearing trials

The trials in GMF were performed in the hatchery of the farm at Galaxidi. The hatchery has already performed larval rearing of greater amberjack during 2015 and 2016 unsuccessfully. Therefore, the personnel were experienced with this species.

The purpose of the specific trial was to test the environmental parameters considered as important for the performance of the larvae as were resulted from the work under task 15.3. In particular, the light conditions were the principal factor tested. For this, the larval rearing tanks were equipped with submerged lights (**Figure 15.4.3.1**) that provided the required conditions.

The eggs from induced spawning of breeders kept in GMF and Argosaronikos SA farm were used for the rearing. After collection, eggs were transported to the hatchery facilities in polystyrene boxes in ~ 4 hours, and then were incubated.

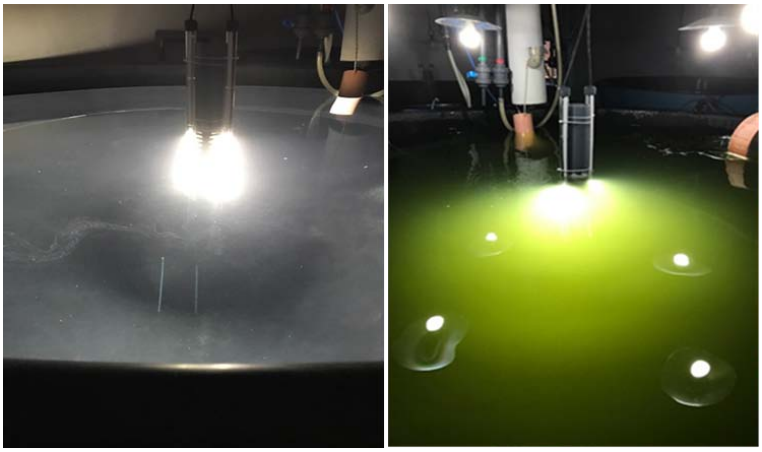


Figure 15.4.3.1 Larval rearing tanks equipped with submerged lights

Incubation was directly in the larval tanks at a density of apx 120 eggs l-1. Following hatching the density of the larvae was at about 75 ind l^{-1}, indicating a survival rate of apx 62%. Phytoplankton was added in the larvae tanks since day 2 and until day 15 post hatching. Light intensity was 800 lux on 3 dph, increased to 1200 lux on 6 dph until 12 dph when it was decreased to 1000 lux and gradually to 500 lux until 20 dph. The photophase was continues (24L:00D) from mouth opening to 20 dph when it was decreased to 18L:06D until 30 dph when it was set to natural.

Feeding was based on enriched rotifers and subsequently with *Artemia* and dry feeds. Frozen copepods were added from 10-15 dph while frozen eggs were also added in the tanks after 20dph.

An indicative growth curve of the larvae is presented in **Figure 15.4.3.2**.

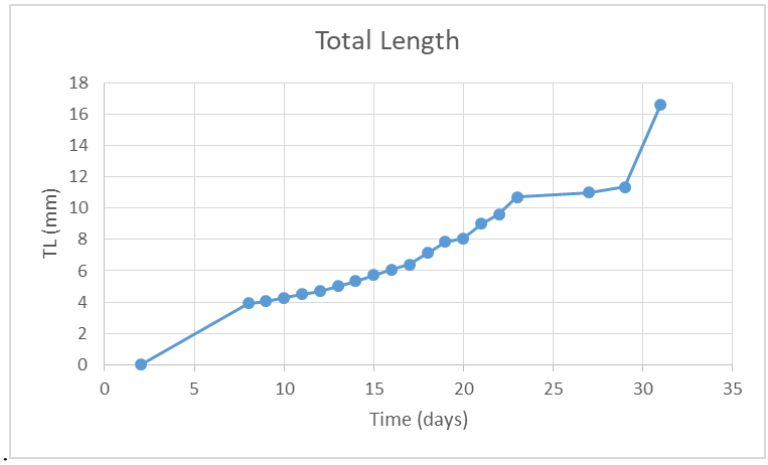


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Figure 15.4.3.2. Total length of the larvae during the trial

Following the hatchery phase, individuals were transferred for weaning and selected in size. The final number of juveniles transferred for pre-growing was approximately 15.000 that were classified in 4 size-classes between 0.3 and 2.5 g.

NIREUS Larval rearing trials

The trials were implemented in the hatchery of the company at Nafpaktos, where the previous years some first trials for the larval rearing of greater amberjack were performed. In fact, during 2016 the company received almost 2.5 million eggs from P23. ARGO farm that following transport were incubated following the protocol of the hatchery and were transferred after mouth opening to larval rearing tanks. This procedure proved to be lethal for the larvae and resulted to no actual larval rearing.

During the 2017 period, the hatchery received 4 batches of eggs again from P23. ARGO on the June 9, 10, 11 and 25. The received quantities were 1.0, 1.2, 0.65 and 0.5 million eggs respectively. The eggs were directly incubated in the rearing tanks and the rearing temperature was set at 24.5 to 25.0 ºC. Larval rearing was performed following the standard protocol of the hatchery and the feeding was based on enriched rotifers, instar I and enriched instar II *Artemia* nauplii followed by artificial diets.

The light conditions in the tanks were modified where possible in order to increase the light intensity on the surface of the tanks at >1000 lux. This resulted in significant higher survival in the tanks, which modified light conditions applied.

Furthermore, following 20 dph fish were selected in size and grouped accordingly. The result of the adaptations was a significant improvement in the performance of the larvae and in particular their survival rate.

The hatchery finally transferred in cages 48.300 juveniles of 25-50 g. Part of these juveniles are now used for the implementation of Task 21.1.1.

Deviations from Annex I and their impact:

Delay in the submission of deliverable 15.5: Due to the fact that the number of eggs obtained in 2016 greater amberjack spawning was scarce, most of the work had to be done in 2017, from the beginning of spawning in July. The biological samples needed to analyse the required parameters (physiological and nutritional condition) have been obtained over the current spawning period and they are been analysed now. For this reason we have requested an extension of 6 months in the submission of deliverable 15.5.

WP 16 Larval husbandry – pikeperch

WP No:	16	**WP Lead beneficiary:**		P9. UL
WP Title (from DOW):	Larval husbandry – pikeperch			
Other beneficiaries (from DOW):	P3. IRTA	P21. DTU	P29. F2B	
Lead Scientist preparing the Report (WP leader):	Pascal Fontaine			
Other Scientists participating:	Enric Gisbert (P3), Ivar Lund (P21), Jiri Bossuyt (P39)			

Objectives

1. Improvement of pikeperch larval rearing protocols by using a multifactorial approach,
2. Reduction of cannibalism rate to increase survival,
3. Development of industrial protocol to improve larval performance during rearing.

Summary of work reported in the previous Reporting Period (1-12 Mo):

An experiment was planned in April 2014 to study the effects of four environmental parameters on the efficiency of rearing larvae using a factorial design experiment (4 factors tested in 8 experimental units). Four factors were selected (two modalities per factor): light intensity (50 lx *vs* 200lx), water renewal rate (50 % *vs* 100% per hour), direction of the water flow (ascending *vs* descending) and tank cleaning time (early morning *vs* late afternoon). The remaining variables (*e.g.* water temperature and photoperiod) remained constant throughout the experiment. However, due to an unstable RAS, all larvae died and this first experiment was postponed to January-March 2015.

Summary of work reported in the previous Reporting Period (13-30 Mo):

1 – The first experiment (exp. 1), initially planned in 2014 was repeated in January-March 2015. In this experiment, the effects of four environmental factors (light intensity, water renewal rate, water flow direction, tank cleaning time) on the efficiency of rearing of pikeperch larvae were determined. Results have been analysed and included in the Deliverable 16.1. The deliverable D16.1 was ended on month 31.

2 – A second experiment (exp. 2), aiming at the determination of the effects of four feeding-related factors on the efficiency of pikeperch larviculture was performed between February and March 2016. In this study, four factors (feeding frequency, co-feeding or not, weaning timing, weaning duration) were tested.

Summary of progress towards objectives (31-48 Mo):

In the **Task 16.1** optimal combinations of factors to improve pikeperch larval rearing were tested. The results related to the experiments 2 (effects of feeding-related factors) and 3 (effects of population parameters) are presented. In experiment 2 it was demonstrated that weaned juveniles of 1.0-1.5 g mean body weight can be

produced in 53 days, with relatively good survival (3.6-13.1%). A longer weaning duration increased mean swim bladder inflation (18% vs 67%) and final biomass increase. In addition, discontinuous feeding increased the final biomass produced in tanks while co-feeding (6 days) and the onset of the weaning period (10 or 16 days dph) had no significant effect on the final biomass and the percent of inflated swim bladders, while the method of food distribution only affected the rate of swim bladder inflation. During the course of the experiment, the mean specific growth rate (SGR) was 15.6% day^{-1}. There was a strong effect of the interaction between the onset of weaning (10 vs 16 dph) and its duration (3 vs 9 days) on the mean larval size and weight measured at 25 and 53 dph, which were higher when fish were weaned later with a longer weaning duration. Pikeperch larvae growth was also influenced by the interaction between the method of food distribution and whether or not co-feeding was implemented. In fact, when co-feeding was applied, no effect of the method of food distribution was observed, whereas in the absence of co-feeding, the larvae were heavier and larger with continuous feeding. On the other hand, this effect was no longer observed after 25 dph suggesting that this interaction is effective only during the weaning period. In conclusion, our results suggest that a later onset and longer duration of weaning followed by discontinuous feeding improved larval survival, growth and reduced deformities in pikeperch populations.

In experiment 3 (effect of population parameters), it was demonstrated that the production of juveniles of 1.8-1.9 g mean body weight can occur in 52 days as well as high levels of swim bladder inflation and tank biomass which is a marked improvement of pikeperch juvenile production in RAS conditions. Final biomass correlated with a higher initial larval density (100 larvae l^{-1}) and the use of larvae supplied by bigger females. In the comparison of the predatory behaviour of cannibals vs non-cannibals**,** predation tests revealed that cannibals show less predatory behaviour than non-cannibals, but they were significantly more efficient in prey capture. In the comparison of the digestive enzymatic activity of predators vs non-predators, results showed that trypsin and amylase activity values were higher in non-predator larvae than in predator larvae. Furthermore, pepsin activity values were lower in non-predators than in predator larvae. These results indicate that predatory larvae have a more developed digestive system development (higher levels of acid proteases in comparison to alkaline proteases) at the same age of non-predator larvae.

Details for each Task

Task 16.1 Optimal combinations of factors to improve larval rearing (led by UL, Pascal Fontaine).

This task was based on four successive experiments using multifactorial designs in order to integrate the effects of each simple factor tested and interactions between them, and to identify *in fine* an optimal combination of factors that significantly increase both larval survival and growth. The four experiments focused on the effects of environmental factors (deliverable D16.1), feeding-related factors (deliverable D16.2) and population parameters (deliverable D16.3), and finally the identification and validation of the optimal combinations of factors (deliverable D16.4). The results related to the first experiment have been completed during the previous reporting periods and the full description of the work and results have been provided in the ***Deliverable 16.1 Determine effect of environmental factors on pikeperch larval rearing.***

In this periodic report, the results related to the experiments 2 (effects of feeding-related factors) and 3 (effects of population parameters) are presented.

Experiment 2: effects of feeding related factors

The effects of four feeding-related factors (frequency of food distribution, co-feeding, onset of weaning, duration of weaning; **Table 16.1.1**) have been studied in 2016. For the experiment, 240 000 newly hatched larvae (<1 dph) were obtained from Asialor (Pierrevillers, France) and transferred to the UL experimental platform (UR AFPA, Vandœuvre-lès-Nancy, France). The study was carried out in 8 tanks (700-l. each) which were stocked with 30,000 larvae each (ca. 43 larvae l^{-1}). Water temperature was set at 15-16°C

throughout the experiment. For more details concerning the rearing system and the standardized rearing conditions, see *the* ***deliverable D16.2 Determine effect of nutritional factors on pikeperch larval rearing***.

Table 16.1.1. Modalities applied in the experiment 2, where four feeding-related factors were tested.

Factors	Modality 1	Modality 2
Frequency of food distribution	Continuous	Discontinuous
Co-feeding	Yes	No
Beginning of weaning	10th day	16th day
Time of weaning	3 days	9 days

The experiment lasted 49 days (from 5th February until 24th March 2016). Larvae were sampled every 7 days and designated as days after first feeding (daff), which began at 4 dph: T0, T7, T14, T21, T28, T35, T42 and T49. The following variables were studied: (a) Morphometric measures (total length (TL), body weight (W), coefficient of variation of total length (CV TL) and coefficient of variation of weight (CV W) done on 30 larvae tank^{-1} sampling date^{-1}; (b) Observations during 5 min day^{-1} of each tank in order to detect cannibals. Cannibals were identified and counted as individuals responsible for a direct attack or with a big abdomen. At the end of the experiment, the total fish biomass was weighed for each tank and the percentage of swim bladder inflation was calculated.

Statistical analyses were carried out using the statistical software Analys (Kobilinsky, 2000; Gardeur et al., 2007). The detection of the potentially active effects of tested factors on the output variables was given by Daniel's graphics (Half Normal probability plot of basal estimation function, Daniel, 1959) using an oversaturated model of variance analysis. The interactions between three or more factors were considered insignificant. When an interaction between two factors was found significant ($P < 0.05$), the potential single effects of these factors were also considered insubstantial.

Final production of pikeperch juveniles (53 dph)

In this experiment, compared to the results obtained in the first trial (see ***D16.1 Determination of the effect of environmental factors on pikeperch larval rearing***), it was demonstrated that weaned juveniles of 1.0-1.5 g mean body weight can be produced in 53 days, with relatively good survival (3.6-13.1%). By implementing several combinations of factors (tanks 3, 4 and 9), the overall percent survival was above 10% (see **Table 16.1.2**). To the best of our knowledge, this is the first time that pikeperch larvae have been reared successfully in large tanks (700 l) and over an extended period of time (53 dph), including the initial phase of feeding on live prey and the weaning period (see ***D16.2 Determination of the effect of nutritional factors on pikeperch larval rearing***) . The higher survival and swim bladder rates obtained in the tanks 3 and 9 are very promising. The multifactorial analysis showed that a longer weaning duration increased mean swim bladder inflation (18% *vs* 67%) and final biomass increase (+ 62%). In addition, discontinuous feeding increased best the final biomass produced in tanks (+ 66%). The use of co-feeding (6 days) and the onset of the weaning period (at 10 or 16 days dph) had no significant effect on the final biomass and the percent of inflated swim bladders, while the method of food distribution only affected the rate of swim bladder inflation.

Table 16.1.2. Final swim bladder inflation rate, final fish biomass, mean body weight, and survival rate in the 8 tanks.

Tanks number	Swim bladder einflation (%)	Final biomass (g)	Mean weight (mg)	Survival (%)
2	22.51	1026	919.27	5.5
3	98.11	1962	1502.31	10.5
4	15.97	2110	623.57	11.3
5	10.43	1361	677.11	7.3
6	86.29	766	861.50	4.1
7	24.63	678	770.95	3.6
8	15.17	1489	913.10	8.0
9	70.81	2443	1022.20	13.1

Growth and weight heterogeneity

During the course of the experiment, the mean specific growth rate (SGR) was 15.6% day^{-1}, ranging from 14.9 to 16.7% day^{-1}. There was a strong effect of the interaction between the onset of weaning (10 *vs* 16 dph) and its duration (3 *vs* 9 days) on the mean larval size and weight measured at 25 and 53 dph, which were higher when fish were weaned later with a longer weaning duration. Pikeperch larvae growth was also influenced by the interaction between the method of food distribution and whether or not co-feeding was implemented. In fact, when co-feeding was applied, no effect of the method of food distribution was observed, whereas in the absence of co-feeding, the larvae were heavier and larger with continuous feeding. On the other hand, this effect was no longer observed after 25 dph suggesting that this interaction is effective only during the weaning period.

The regulation of the size or weight heterogeneity is of major importance to limit the impact of cannibal individuals in the rearing of predatory fish (Kestemont et al., 2003). Numerous biotic and abiotic factors influence the heterogeneity of a population. The present study suggested that a late onset of weaning increased weight heterogeneity (simple effect observed at 25 dph). However, as significant lower coefficients for weight were noted at 53 dph when the larvae were weaned later, the effect of the timing of the weaning on fish heterogeneity is confirmed. In contrast, the effects of the method of food distribution remain unclear as a higher homogeneity in weight was recorded with continuous feeding (interaction with the weaning duration) at 32 dph, whereas pikeperch batches were more homogenous with a discontinuous feeding at 39 and 53 dph.

We observed no cannibalism before 14 dph, followed by two periods of intense cannibalism (first between 28 and 34 dph and the second between 49 and 53 dph).

In conclusion, our results suggest that a later onset and longer duration of weaning followed by discontinuous feeding improved larval survival, growth and reduced deformities in pikeperch populations.

Experiment 3: effects of population parameters

The effects of four feeding-related factors (initial larval density, sorting out fish' jumpers, stocking sibling or not sibling larval groups, female weight; **Table 16.1.3**) were studied in 2017. For the experiment, 420 000 newly hatched larvae (<1 dph) were obtained from Asialor (Pierrevillers, France) and transferred to the UL experimental platform (UR AFPA, Vandœuvre-lès-Nancy, France). The study was carried out in 8 tanks (700-l. each). Water temperature was set at 15-16°C throughout the entire experiment. For more details concerning the rearing system and the standardized rearing conditions (see ***the deliverable D16.3 Determine effect of population factors on pikeperch larval rearing)***.

Table 16.1.3. Modalities applied in the experiment 3, where four population parameters were tested.

Factor	**Modality 1**	**Modality 2**
Density	50 larvae.L^{-1}	100 larvae.L^{-1}
Sorting of fish jumper	Yes *	no
Sibling or not sibling	Sibling	Not sibling **
Female weight	Small (< 2.8 kg)	Large (> 3.3 kg)

*: Every day, after tank cleaning, a sorting out of "big" fish called jumpers was done using a net.

**: Mixing (50/50) of larvae from spawning of two females.

Larvae hatched 19-20th February 2017. The experiment lasted 53 days (from 19th February until 13th April 2017). Larvae were sampled every 7 days and designated as days after first feeding (daff), which began at 4 dph: T0, T7, T14, T21, T28, T35, T42 and T49. The variables studied and the behavioural observations were similar to those in the experiment 2. Due to the unexplained high mortality in tank 7, this experimental unit was not considered in the analysis. All statistical analyses were performed using the free software R version 3.2.4 (R Core Team 2016) (see ***the deliverable D16.3 Determine effect of population factors on pikeperch larval rearing)***.

It was demonstrated that the production of juveniles of 1.8-1.9 g mean body weight can occur in 52 days (**Fig 16.1.1**). However, the more significant results were (1) the very high levels of swim bladder inflation (mean 92.8%) and (2) the high final biomass harvested in some tanks (tanks 3 and 9). In these tanks, the final densities were respectively (7.9 and 8.3 kg per m^3). These results constitute a marked improvement of pikeperch juvenile production in RAS conditions. Two combinations (tanks 3 and 9, see **table 16.1.4**) appear particularly effective. High final biomass seemed correlated to a higher initial larval density (100 larvae.L-1) and the use of larvae supplied by bigger females, but independent of jumper sorting and the use of sibling population.

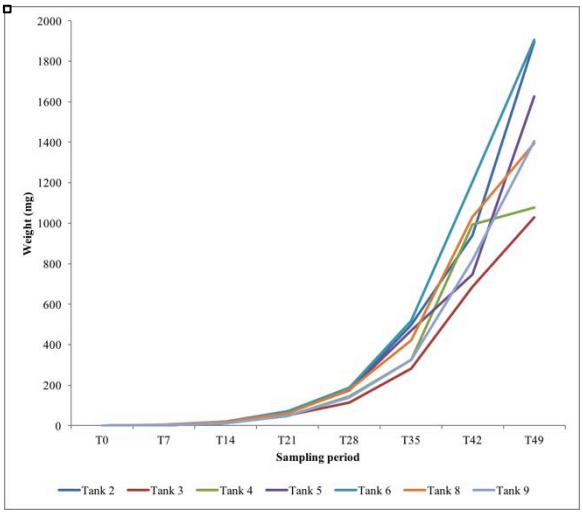


Figure 16.1.1. Growth curve of pikeperch larvae submitted to the seven combinations of factors.

Table 16.1.4. Final swim bladder inflation rate, fish biomass, mean body weight, and survival in the 7 tanks.

Tanks number	Swim bladder inflation (%)	Final biomass (g)	Mean weight (mg)	Survival (%)
2	96.66	2073	1896.37	3.1
3	90.00	5596	1029.58	7.7
4	93.33	3606	1076,00	9.5
5	100.00	3527	1626.94	3.1
6	93.33	3046	1905.66	4.5
8	86.66	1345	1395.8	2.7
9	90.00	5837	1406.90	5.9

After three experiments (see ***Deliverables 16.1*** *Determine effect of environmental factors on pikeperch larval rearing,* ***D16.2*** *Determine effect of nutritional factors on pikeperch larval rearing* and ***D16.3*** *Determine effect of population factors on pikeperch larval rearing)***,** we have identified an optimal combinations of environmental, nutritional and population factors which allow the production of pikeperch juveniles of 1.8-2.0 g mean body weight with a global survival rate around 10% and a rate of swim bladder inflation above 90%. The corresponding combinations must be validated in a final experiment planned in 2018.

Complementary experiments

Comparison of the predatory behaviour of cannibals vs non cannibals

For this additional experiment, fertilized eggs were obtained from one female and one male (supplied by the P29. ASIALOR, Pierrevillers, France). Larvae hatched on February, 1st, 2017 and were then transferred to and reared at the P9. UL facilities (Unit of Animal Research and Functionality of Animal Products). When a cannibalistic larva was found, it was isolated in a small rack and its behaviour monitored the next day. Non-cannibalistic larvae were obtained and groups of four larvae were stocked in racks. Indeed, if all larvae were still there the following day, this implied that 4 non-cannibals could be tested at a given time.

The following behaviours of cannibals and non-cannibals were analysed:

1. Fish orientation where tested larva turned its body head-first towards prey (zebrafish larvae) or congener and visually tracked it (Bell and Sih, 2007).
2. Fish approach, was defined as the movement of the tested larva towards the other larva with slow swimming.
3. Fish attack, characterized by a rapid movement of the pikeperch towards the zebrafish larvae or congeners, with the mouth open. This behaviour was easily identifiable: just before the attack, the larva stopped and adopted an “S” position (Houde, 2001, Turesson et al., 2002), or just modified the orientation of its caudal fin.
4. Fish capture, which corresponded to the bite of the prey by the pikeperch.

All details concerning the experimental design, material and methods and statistical analysis used are explained in ***the deliverable D16.3 Determine effect of population factors on pikeperch larval rearing.***

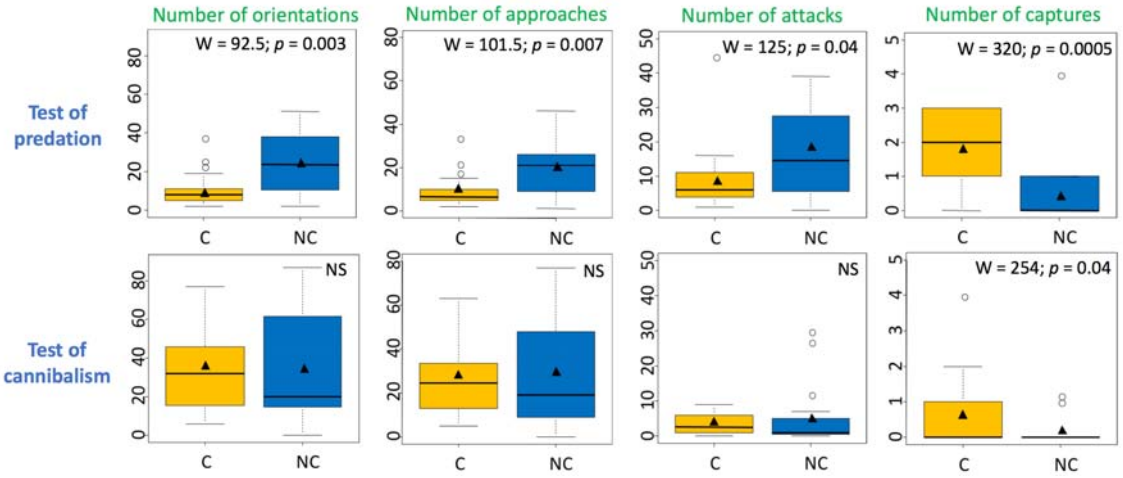


Figure 16.1.2. Comparison of the behavioural profile of cannibals (C, yellow) and non-cannibals (NC, blue) over the period 17-33 dph. The black line is the median, the black triangle is the mean, white dots are outliers and top and bottom lines are first and third quartiles.

Our behavioural studies allowed improving the emergence of cannibalistic behaviour in pikeperch larval rearing. Predation tests revealed that cannibals show less predatory behaviours than non-cannibals (less orientation changes, approaches and attacks on prey), but they were significantly more efficient in prey capture; this last point was confirmed during the test of cannibalism (**Fig 16.1.2**). There was no effect of the tested factors on the rate of cannibalism.

Comparison of the digestive enzymatic activity of predators vs non predators

For this experiment, pikeperch larvae hatched in the French farm Asialor (Pierrevillers- 57120 France) on February, 1st 2016 were brought back to the laboratory (PEA- URAFPA- University of Lorraine, Nancy-France). Larvae were obtained from the stripping of one female fertilized by one male. Eggs were put in 500 l tank. The water was maintained between 16°C and 17°C until 23 dph and then increased by one degree per day until reaching 21°C. The water parameters (mean ± SD) were: dissolved oxygen rate = 8.2 ± 0.6 $mg.l^{-1}$, pH = 7.8 ± 0.2, and salinity = 0.2 ± 0.05 $g.l^{-1}$. Four days' post-hatching (dph) larvae were fed *Artemia* nauplii, until weaning (21 dph), and then they were fed artificial food (Larviva and Inicio Plus, BioMar).

The predatory behaviour of pikeperch larvae was tested at two different ages: 30 dph (10.8 ± 1.4 mm) and 52 dph (28.8 ± 4.9 mm). In a behavioural test, larvae were confronted with three prey (zebrafish larvae: 4.08 ± 0.81 mm). A behavioural analysis allowed us to categorize pikeperch larvae as a predator or not. Then larvae were frozen and dried (INRA 54-Champenoux France) and sent to IRTA (Sant Carles de la Rapita, Spain) for enzyme analyses. These analyses were conducted after larvae dissection (tail and head were removed). A fluorimetry technique was used to quantify the activity of digestive enzymes. A *EnzChek® Protease Assay Kit* (Thermo Fisher Scientific) was used to quantify proteases (pepsin and trypsin), and a *EnzChek® Ultra Amylase Assay Kit* (Thermo Fisher Scientific) was used to quantify amylase. Enzyme concentrations were compared between predator and non-predator larvae with a t-test of Student.

Data analysis showed that trypsin and amylase activity values were higher in non-predators' larvae than in predators (**Fig. 16.1.3**). Furthermore, pepsin activity values were lower in non-predators than in predators' larvae (**Fig. 16.1.3**).

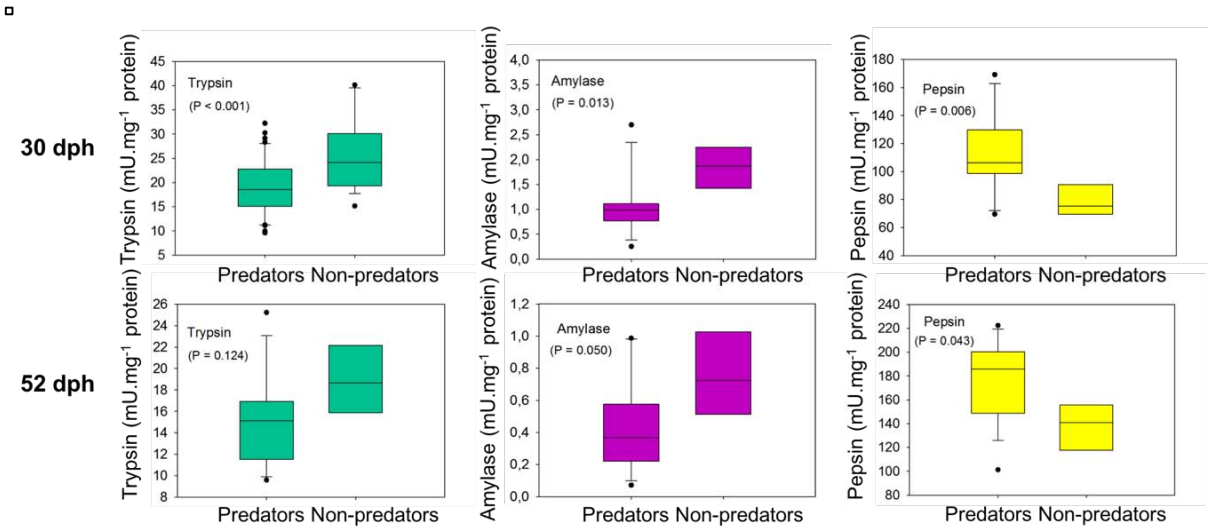


Figure 16.1.3. Results of the t-test of Student for enzymatic activity values at 30 and 52 days post-hatching (dph). Pikeperch larvae, which attack prey (predators) were compared to those, which did not attack prey (non-predators). The black line is the median, black dots are outsiders and top and bottom lines are first and third quartiles.

These results indicate that predatory larvae have a more developed digestive system (higher levels of acid proteases in comparison to alkaline proteases) at the same age of non-predator larvae.

Task 16.2 Development of an industrial protocol (led by F2B, Jiri Bossuyt).

The development of an industrial protocol will be undertaken in the year 2018 as foreseen initially in the DOW.

Deviations from Annex I and their impact:

The deliverable D16.2 initially planned on month 24 was delivered on month 36 because the experiment was delayed in early 2016, as a consequence of the delay of the D16.1.

The deliverable D16.3 initially planned on month 36 was delivered on month 47 because the experiment was delayed in spring 2017, as a consequence of the delay of the D16.1 and D16.2.

The deliverable D16.4 initially planned on month 48 will be delivered on month 54 because the experiment will be done in February-March 2018, as a consequence of the delay of the D16.1, D16.2 and D16.3.

WP 17 Larval husbandry – Atlantic halibut

WP No:	17	**WP Lead beneficiary:**		P7. IMR
WP Title (from DOW):	Larval husbandry – Atlantic halibut			
Other beneficiaries (from DOW):	P17. NIFES	P22. SWH		
Lead Scientist preparing the Report (WP leader):	Birgitta Norberg			
Other Scientists participating:	Øivind Bergh (P7), Torstein Harboe (P7), Audun Nerland (P7), Sonal Patel (P7), Nina Sandlund (P7), Kristin Hamre (P17), Borre Erstad (P22)			

Objectives

1. Improve larval survival and quality during early development of Atlantic halibut.

Summary of work reported in the previous Reporting Period (1-12 Mo):

A recirculating aquaculture system (RAS) for Atlantic halibut yolk sac incubators was constructed. Samples were taken for analysis of bacterial activity in the water, and for identification of candidates for a probiotic treatment protocol. Larval mortality was higher in the RAS system the first week after hatching. Thereafter there were no differences in mortality. No differences in larval size at the end of yolk sac stage were found. There was however, a higher proportion of jaw-deformed larvae in the RAS system. Previous work with halibut yolk sac larvae in silos has strongly indicated that jaw deformities are more frequent when the larvae are exposed to water movement the first days after hatching. Most likely, there was a difference between the siloes in such water movement, which may have been responsible for the difference.

Thirty-five different *Vibrio* spp. strains were tested for virulence towards Atlantic halibut larvae in a major challenge experiment. Some of the strains are known to be associated with bacteriophages, making phage therapy possible. Other possible strains were to be used as model strains in challenge experiments with probiotics following the model by D`Alvise et al., 2012.

Summary of work reported in the previous Reporting Period (13-30 Mo):

RAS first feeding:

Halibut larvae, at an age of 265 day-degrees, were transferred from a yolk sac incubator to 6 first feeding tanks. Numbers of larvae were approximately 5000 in each tank. Three of the tanks were connected to a RAS system (Tropical Marine Center). The three other tanks had a standard flow through water system with water coming from 160m depth. The tanks had a volume of 1400l and water flow of 5 $l \cdot min^{-1}$. Water temperature was 12 ± 0.3°C during the whole period. Highest growth was obtained for the larvae reared in the FT system. However, survival was not different between the FT and RAS groups. The lower growth in the RAS group was probably due to high ammonia concentrations in the RAS system.

Artemia on-growing protocol:

A production protocol, based on Olsen et al., 1999, for on-grown *Artemia* was further developed, where water renewal and quality were crucial parameters. The protocol includes feeding, washing and disinfection

of the *Artemia*, and has been tested both at an experimental (P7. IMR) and commercial scale (P22. SWH). The experiments that led to this protocol were followed by analyses of biochemical profiles of macro- and micronutrients of the on-grown *Artemia* that are presented in ***Deliverable D11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia***

First feeding on-grown Artemia:

A strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality is to use on-grown *Artemia*. Ongrown *Artemia* are larger, contain more protein and phospholipids and have a different micronutrient status from *Artemia* nauplii (Hamre and Harboe, unpublished; Task 11.2). They also have a lower shell to nutrient content. Olsen et al., (1999) showed that halibut larvae fed on-grown *Artemia* develop into juveniles with better pigmentation and eye migration than Atlantic halibut fed *Artemia* nauplii. There was no difference in larval performance. Survival, measured as number of halibut fry 70 days after first feeding, was between 42 and 48% of incubated larvae. Growth data, except for the end point, has so far not been measured. However, at the end point there were no differences between the two groups. Both groups showed 100% normal pigmentation and good eye migration (score: more than 2.5/3).

Summary of progress towards objectives (31-48 Mo):

A brief summary of progress towards objectives, highlighting clearly significant results (max 0.5 page)

During this period halibut fry were produced in RAS systems applied both during yolk sac incubation and first feeding stages. The RAS unit used for first feeding was started 6 weeks prior to larvae incubation and the ammonia concentration was low during the entire period, even when clay was added to create turbidity. The larvae in the RAS had better growth and survival compared to the larvae in the FT system.

Details for each Task

Task 17.1 Recirculation (RAS) vs Flow through (FT) systems during yolk sac and first feeding stages and the effects on larval survival, quality and growth (led by IMR, Torstein Harboe).

Introduction

The commercial production of halibut fry is currently carried out in flow through systems (FT), while there is a growing consensus that a recirculation system, RAS, would offer more stable environmental and chemical water parameters, and lead to establishment of probiotic bacterial communities, that would lead to improved larval performance. The yolk sac and first feeding stages in halibut are performed in different rearing tanks. RAS systems for both these stages are presented here. The first test of a RAS system for the yolk sac stage was done in 2015 and a second, modified, was conducted in 2017. Both experiments were done without replicate silos. For the first feeding stage, experiments were conducted in 2016 and 2017. These experiments were done using triplicate tanks both for the RAS system and control tanks. The protocol is based on the second trial for both yolk sac and first feeding stages.

Yolk sac stage:

The yolk sac stage lasts for 43 days at 6 C° in halibut. Eggs are transferred to the silos approximately 3 days prior to hatch. At this time, a salinity gradient has been established in the upper part of the silo by use of freshwater. Hatching is synchronized by use of light, which arrests hatching, and thereafter darkness to induce hatching. The salinity gradient is present during hatching and for one or two more days, depending of the buoyancy of the larvae. Recirculation is not used in this period. The silos used for water treatment and for larval rearing, are 5000 litres in volume. Approximately 1 to 2 litres of eggs (40 000-80 000 eggs) are

normally incubated in one silo, depending on the size of the egg batch. There is no feeding or any addition of organic material during this period.

Materials and methods:

Two trials were conducted before the protocol was finalized. In the first trial, water temperature was adjusted between the RAS silo (without larvae) and the silo inoculated with larvae. In the second trial this temperature adjustment was done within the RAS silo, resulting in a more even temperature profile (**Fig. 17.1.1**).

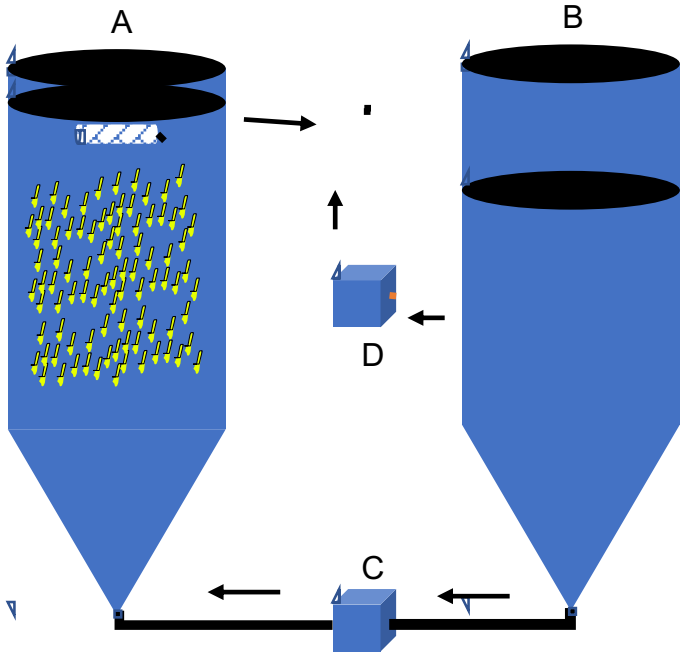


Figure 17.1.1. Illustration of the RAS used for yolk sac larvae. A= silo with larvae, B=water treatment, C= water pump including flowmeter. D= water cooler.

Results:

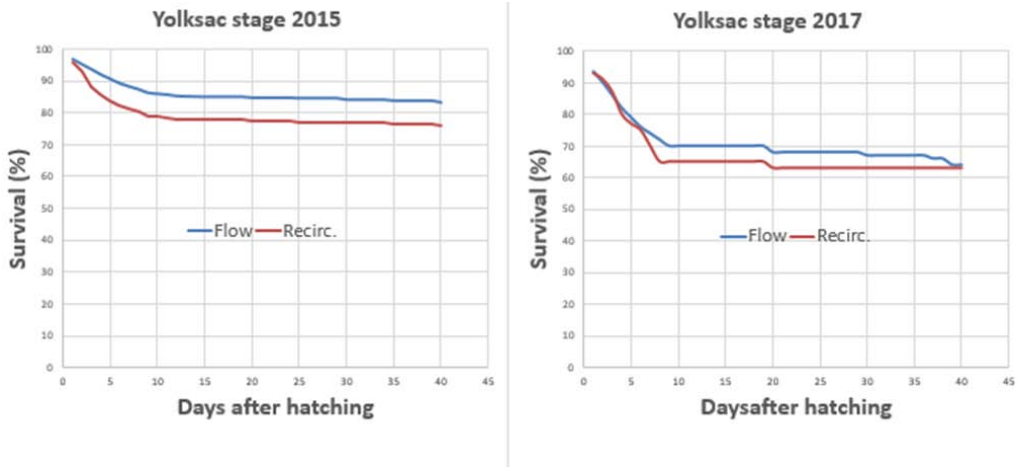


Figure 17.1.2. Larval survival (%) in 2015 and 2017.

Only small differences in survival were observed during yolk sac incubation between FT and RAS both in 2015 and 2017 (Fig. 17.1.2). Proportion of jaw deformed larvae was 14% in 2015 and 11% in 2017 for the RAS larvae and 9% in 2015 and 17% in 2017 for the FT larvae (**Fig 17.1.3**).

Figure 17.1.3. Atlantic halibut larvae with deformed (left) and normal (right) jaw.

First feeding stage:

At the IMR, it is standard practice to treat the larvae with antibiotics the first three days of the first feeding period, to prevent a drop in appetite during this period. To avoid use of antibiotics and to decrease mortalities use of a RAS was tested, in order to establish a stable microbial environment that would have a probiotic effect. It is not clear whether the intestinal microflora of halibut larvae is determined by the feed or by water quality parameters (see Bergh et al., 1994; Attramadal, 2011). Short time enriched *Artemia* is most widely used for first feeding of halibut larvae. The feeding period is normally 45 to 50 days before they are weaned to a dry diet.

Materials and methods:

A RAS system from Tropical Marine Centre (TMC) was used (**Fig. 17.1.4**). This system has been tested previously by the IMR for use in research on several cold-water and warm-water marine species. In this setup three first-feeding tanks were connected to the system. The system consists of a reservoir (650 l), filter bags, sand filter, re-gassing / trickling biofilter and a protein skimmer.

Figure 17.1.4. RAS system P5000P MARINE from Tropical Marine Centre.

Figure 17.1.5. First feeding tanks.

The first-feeding tanks had a flat bottom, with a volume of 1100 l and a water flow of 5 $l \cdot min^{-1}$ (**Fig. 17.1.5**). Water temperature was held at 12 ± 0.3°C during the whole period. The tanks had shadow frames to avoid illumination of the walls and a fluorescent daylight source placed 70 cm above the water surface, giving a light intensity of approximately 400 lux at the surface. The tanks had central aeration near the bottom. The water outlet sieves were placed in the centre of the tanks, reaching from the bottom to the surface. Water inlets were placed near the tank wall, approximately 10 cm below the surface. Automatic cleaning devices (windshield wipers) were mounted in each tank and were run once a day. After one rotation, dead material was removed by a siphon. The water volume that was removed daily by siphoning represented the water exchange in the RAS system. The recirculating volume was calculated to 97%.

Water turbidity was created by use of dissolved clay (Sibelco, Vingerling K148, white) to an initial turbidity of 2NTU. Approximately 10g of clay was dissolved in one l of freshwater and added to each tank twice a day.

Before the water returned to the RAS unit it was filtered to remove *Artemia* and part of the clay (**Fig. 17.1.6**). The reminding clay was left in the RAS unit, mostly in the reservoir.

Figure 17.1.6. Bag filter and sedimentation tank.

Results

First feeding larvae in the RAS had higher growth than larvae in the FT system, and had attained nearly twice the weight of larvae reared in the FT system at the end of the experiment (**Fig. 17.1.7**). Larval survival

was 35% or more for the larvae in the RAS (**Fig 17.1.8**). One of the FT tanks had high mortalities during the second half of the first feeding period. The concentration of un-ionized ammonia was low in all tanks.

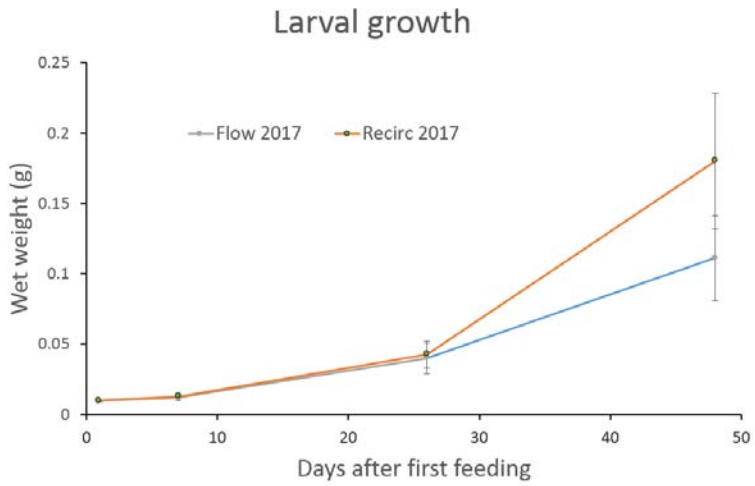


Figure 17.1.7. Larval growth during the first feeding period.

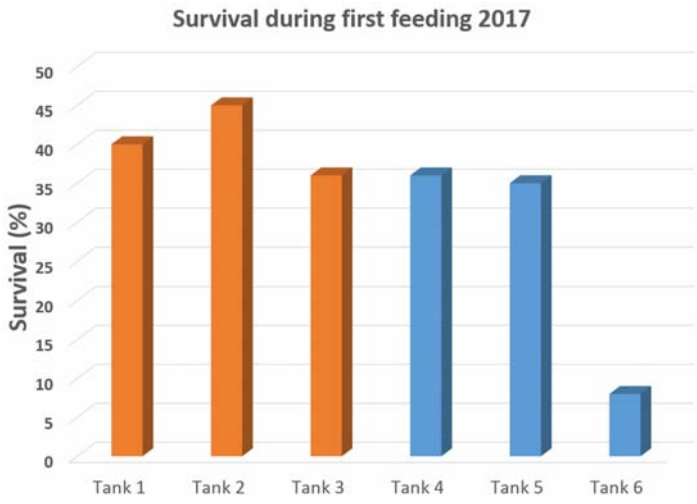


Figure 17.1.8. Larval survival (triplicate tanks) during 48 days of first feeding.

Metagenomic studies of the larvae and the environment.

Samples were taken from individual larvae (126 samples) and the environmental water (36 samples), both during yolk sac incubation and first feeding. The individual larvae were rinsed in distilled water, homogenized in 500 ul distilled water followed by centrifugation at 13000 g for 15 minutes at 4°C. Microbes in the water were sampled by centrifuging 45 ml water at 3000 g for 10 minutes at 4°C. DNA from the resulting pellets was isolated by using the CTAB-method (Reid et al 2017, Mitchell and Takacs-Vesbach 2008). The samples are currently being analyzed by deep sequencing of the V3-V4 region of the 16S-gene

according to the Illumina protocolhttps://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Results from the metagenomic studies will be reported in the next periodic report.

References

Mitchell KR, Takacs-Vesbach CD. A comparison of methods for total community DNA preservation and extraction from various thermal environments. J Ind Microbiol Biotechnol. 2008; 35:1139–1147.

Reid KM, Patel S, Robinson AJ, Bu L, Jarungsriapisit J, Moore LJ, Salinas I. Salmonid alphavirus infection causes skin dysbiosis in Atlantic salmon (Salmo salar L.) post-smolts. PLoS One. 2017 Mar 6;12(3).

Task 17.2 The effect of probiotics on larval microbiota and survival and development of an industrial protocol (led by IMR, Øivind Bergh).

Protocols for larval rearing under stable bacteriological conditions are described below, for both yolk-sac and first-feeding stages. Metagenomic studies aiming to identify probiotic candidates in the systems are underway (see task 17.1).

Yolk sac stage:

1. Fill RAS and larvae unit (silo) approximately 5 days prior to incubation.
2. Do not connect the units until salinity gradient is removed.
3. Establish salinity gradient two days before incubation.
4. Use light and darkness to synchronize hatching.
5. Adjust waterflow according to larvae placement.
6. Connect the silos when the salinity gradient is no longer present.
7. Adjust waterflow to maximum level (5litre/min).
8. Observe larvae placement and temperature daily.
9. Remove the freshwater layer above the outlet sieve and transfer the larvae to first feeding units.

First feeding:

1. Fill the RAS unit with seawater 45 days prior to larvae incubation. During this period, the recirculation unit without tanks, a total of 650 litres, must be conditioned by addition of a daily amount of 1.5 g NH_4Cl.

Preparation:

1. Measure NH_4 concentration and pH value once a week to see if the biofilter in the unit removes NH_4.
2. Keep water temperature stable at 12°C.
3. Fill the first feeding tanks with seawater the day before larvae incubation. Adjust aeration, waterflow and turbidity. Connect the tanks to the RAS unit.
4. Incubate approximately 5000 larvae per tank. Feed short-time enriched *Artemia* according to feeding protocol.

Daily routines: Check and if necessary adjust water flow in protein skimmer (venturi pump), sand filter and biofilter in the RAS unit.

1. Exchange and clean bag filters prior to the RAS unit (excess *Artemia* and clay) and the bag filters in the RAS unit.

2. Refill seawater after the larvae tanks have been tended. Use a water level mark in the reservoir.

Weekly routines:

1. Measure NH_4 concentration and pH value.
2. Siphon clay from the bottom of the reservoir.

Other:

1. Feed and remove dead larvae according to larvae rearing protocol.

Task 17.3 Production of on-grown *Artemia* (led by IMR, Torstein Harboe).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia***.

Task 17.4 Comparison of feeding on-grown *Artemia* versus *Artemia* nauplii on larval performance (led by IMR, Torstein Harboe).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance.***

Deviations from Annex I and their impact:

Sequencing of the bacterial strains established in the RAS and FT systems is not finished, and results from will not be available until early 2018. This is our means to detect the probiotic bacteria in the different larvae and aquaculture systems, which is required in order to complete Deliverables ***D17.3 The effect of probiotics on Atlantic halibut larval microbiota and survival***, and ***D17.5 Development of an industrial protocolfor probiotic treatment of halibut larvae***, which therefore will not be completed until June 2018.

WP 18 Larval husbandry – wreckfish

WP No:	18	**WP Lead beneficiary:**		P8. IEO
WP Title (from DOW):	Larval husbandry – wreckfish			
Other beneficiaries (from DOW):	P1. HCMR	P19. CMRM	P32. MC2	
Lead Scientist preparing the Report (WP leader):	Blanca Álvarez-Blázquez			
Other Scientists participating:	Nikos Papandroulakis (P1), Fatima Linares, Jose Luis Rodriguez (P19), Antonio Vilar Peron (P32), Evaristo Perez, Montserrat Perez, Pedro Domíngues			

Objectives

1. Development of larval rearing protocol based on the most effective prey density, succession of prey type, temperature and culture system.
2. Description of ontogeny of digestive system, vision, taste and smell organs in response to larval rearing methods.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The main objectives of WP18 during the first 12 months of the Project were to develop a culture protocol and influence of different temperatures, as well as the description of the ontogeny of the digestive system according to the culture protocol. Only from stock of P32. MC2 was spawning obtained, possibly due to the young age of the fish in the different stocks, and also unexpected oceanographic variations in temperature and extreme weather phenomena.

As a result, spawns were few and of poor quality. A 1,000 ml spawn was obtained at the P32. MC2 with 70% fertilization, and 270,000 eggs were incubated at 14.6ºC. The hatching rate was 14% and egg diameter was 2.405±32. After the start of the culture period (n=11,340 larvae), the protocol normally used was applied until 20 dph, when mortality was 100%. Larvae had functional, but empty stomachs. Embryonic development at 14.6±0.5ºC lasted for 168 h and yolk sac was consumed after 120 h.

Summary of work reported in the previous Reporting Period (13-30 Mo):

Task 18.1 Development of feeding methodology. The objective of this Task is to test different feeding regimes (prey densities and succession of prey type) in order to develop a feeding protocol and avoid periods of food deprivation. Testing includes rearing in semi-intensive culture system (Mesocosm with 40,000 l) tanks, from the end of endogenous feeding to the change to inert feeding (weaning phase). The culture system will be evaluated in terms of ontogeny of larval digestive and visual system (influenced by feeding) through histological and image analysis procedures. I

During the reported period some preliminary trials were performed due to the low availability of eggs, that did not allow the implementation of a full-scale trial.

The problem was identified as similar to a syndrome related to swollen yolk sac (SYSS). Although it seems that for the wreckfish this syndrome seems to be the case, further studies are required.

Summary of progress towards objectives (31-48 Mo):

Important advances have been made in the understanding of ontogeny and larval development (**D18.**1) as well as the initial stages to develop an adequate larval feeding protocol. Knowledge of the optimal incubation temperature and larval culture (**Subtask 18.2.1**) has also increased. Improving the technical conditions of culture that include aeration, water flow rate and tank circulation as well as continuing to investigate the high percentage of larval malformation (**Subtask 18.2.2**), will be objectives during the last period of the project and comply with the delivery of the following deliverables that have been delayed: **D18.2**, **D18.3** and **D18.4**.

Task 18.2 Defining optimum conditions for larval rearing

Sub-task 18.2.1 Testing (IEO, MC2) the effect of two temperature ranges (14-17 and 19-22°C) in triplicate trials in 2000 l tanks in flow-through systems and using the same photoperiod regime from the end of endogenous feeding to the change to inert feeding (weaning phase). These studies will be evaluated in terms of growth, survival, larval quality and size.

Poor results from 2015 possibly due to unsuitable feeding protocol and early age of the stocks (sexual maturity is achieved > 10 Kg) as well as the variable and unsuitable environmental conditions, resulted in few spawns, with poor quality, compared to previous years at MC2 (P32). Spawn quality at MC2, is improving considerably, and the stock at the IEO (P8) has started to deliver good quality spawns, which assures experimental work for 2016 in WP18. Nevertheless, it is likely that we will have to postpone the deadline of deliverables D18.1, D18.2 y D18.3, which depend on larval availability, in order to obtain more information to develop more accurate feeding protocols.

Details for each Task

Task 18.1 Development of feeding methodology (lead by HCMR, Nikos Papandroulakis)

During this period the deliverable ***D18.1 Development of the digestive system of wreckfish***, has been delivered. A summary of this it is provided below.

INTRODUCTION

Larval rearing of wreckfish is considered as the major bottleneck for the successful culture of this species, due to the low survival rates observed during this period. One of the main scientific goals for wreckfish larval rearing is the development of protocols according to the specific requirements of the larvae during the early developmental stages. The study of the development of the organs related with larval feeding behavior offers part of the necessary information for the optimization of the larval rearing protocols.

During the first developmental stages until the transformation into a juvenile, numerous changes appear in the digestive system of fish larvae, in terms of morphology and functionality (Przybył et al., 2006). Therefore, the knowledge of the digestive system ontogeny is essential, in order to be able to understand the digestive physiology of larvae. Most teleost larvae are mainly visual predators. Under rearing conditions, the signals received by the visual system are defined by the lighting conditions and these signals are coming from the type and concentration of food items. Therefore, if the visual ability and the light requirements of the species under commercial rearing conditions are known, the farmers could modify the light conditions in the tank according to larval requirements.

The aim of this study was the description of the eye, and the digestive system ontogeny. The information will be used for the improvement of the rearing protocols for the successful wreckfish larval production.

MATERIALS AND METHODS

Larval rearing

The rearing trials were performed at the facilities of the Aquarium Finisterrae (MC2) of La Coruña City Council. Larvae were released into a 10-m ^{3} tank where two species of copepods were previously cultured: *Tisbe battagliai Harpaticoide* and *Acartia tonsa Calanoide*. The histological procedure was performed at the HCMR. The study of histological procedure was focused on the digestive ontogeny the area covered with lipid vacuoles (ACLV) in the liver, the eye ontogeny and the estimation of histological visual ability (visual acuity).

RESULTS

Digestive system ontogeny

As concerning the developmental status of the digestive system, most of the organs (except for the maxillary teeth at the upper jaw that became visible at 19 dph) appeared by 8 dph (**Fig. 18.1.1**). From the ontogenetical point of view, the digestive system ontogeny up to 23 dph can be categorized into two distinct periods.

Lipid deposition in the liver (ACLV) and stomach content.

No lipid deposition was detected during the analysis in the liver (ACLV) of wreckfish larvae. Furthermore, no food items were detected in any part of the digestive canal.

Evolution of intestinal villi.

The formation of intestinal villi started between 7-8 dph. Then their size had an increasing trend until 16 dph. After that day their size was reduced and at 23 dph no more microvilli were found in the intestinal area.

Retina development.

During the first day of rearing there were no differences regarding the ontogenesis of the eye. At the day of hatching (0 dph) the retina appeared as a simple hemispherical sheet of undifferentiated neural epithelium (UNE) enclosing the lens, which comprises of a spiral of unspecialized cells. The first differentiation in the different layers was visible at 3 dph. The pigment epithelium (PE) was not formed by this day.

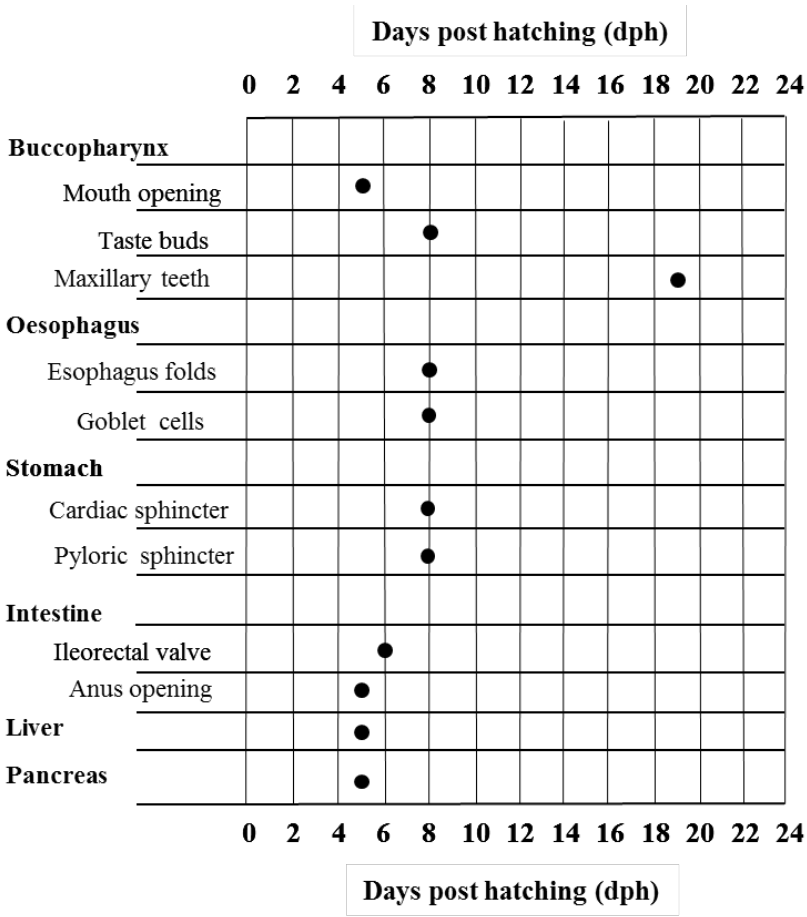


Figure 18.1.1. Schematic representation of the main structures of the digestive system that were studied. The time of appearance of each structure is presented with a black solid circle as a function of days post hatching (dph, horizontal axis).

From 6 dph onwards, the PE appeared on the external area of the retina. The nucleus of the cone cells appeared at 6 dph in the outer nuclear layer, along with all the other neutral cells in the inner nuclear layer (amacrine, bipolar and horizontal cells), which were now completely distinct. Rod cells were not detected until 23 dph.

Visual acuity and visual distance

The visual acuity (expressed as minimum separable angle, MSA) decreased until 6 dph and then remained constant with values fluctuating between 2 and 1.5 degrees (Fig. 6a). As the MSA decreased the distance that the rotifers and the Artemia nauplii could be perceived by the wreckfish larvae, increased (Fig. 6b).

DISCUSSION AND CONCLUSIONS

In wreckfish, the ontogenesis of the digestive system is considered as a slow procedure in comparison with other species. The ontogenesis of the organs related to the digestive and the vision system was not completed until 23 dph. Major structures like the gastric glands or the pyloric caeca, the appearance of which characterizes the time when the development of the digestive system is completed, were not identified in this study. The ontogeny of the retina of the wreckfish was found to be similar to the general pattern shown in most fish species. At hatching, the retina was an undifferentiated and non-functional tissue, as occurs in most marine fishes with pelagic early life stages. Wreckfish visual acuity - the distance the eye can differentiate between two points - improved over time, as shown by the histological assessment. Therefore, the density of the rotifers, which are considered the smallest food particle provided in the rearing tank, could be theoretically calculated according the visual abilities of the larvae of wreckfish. Although both the digestive and the visual system had developed to such an extent that would allow the larvae to feed, we did not detect any food item in the digestive channel, which means that their diet during that period was based exclusively on the reserves of the lecithotrophic sack. The inability of larvae to feed on exogenous food sources resulted in limited growth performance, as the endotrophic reserves were not enough to allow further growth. The study of ontogenesis and the formation of the basic systems of the rearing organism, as has been also proved in other cases, seem to be the basis on which the optimization of the protocol of wreckfish larval rearing should be carried out. As the main organs like the gastric glands did not appear until the length of 5.5 mm, a combination of easily captured and more digestible preys as rotifers or different types in different developmental stages of copepods, have to be included in the larval rearing feeding protocol of wreckfish. The above, in combination with the optimization of the rearing conditions, such as the tank hydrodynamics, the temperature protocol during the rearing procedure and the photic conditions in the rearing water, is considered necessary for the development of the wreckfish larval rearing protocol.

Task 18.2 Defining optimum conditions for larval rearing (lead by IEO, Blanca Álvarez)

Sub-task 18.2.1 Testing (IEO, MC2) the effect of two temperature ranges (14-17 and 19-22ºC).

2016

During May of 2016 two trials with testing different incubation temperatures (14±0,5 ºC and 17±0,5 ºC) with eggs from two different spawns (Ch5/16 and Ch6/16) from broodstock of Instituto Español de Oceanografía (IEO) facilities (**Fig. 18.2.1.1**) were made, taking advantage of the improved spawns and the availability of eggs, in order to advance with the deliverable D18.2 and the optimization of the environmental parameters. The optimal incubation temperature in this trials was shown to be 16±0.8°C. At this temperature range the best results, in terms of normal embryonic development and the hatching rate, were achieved.

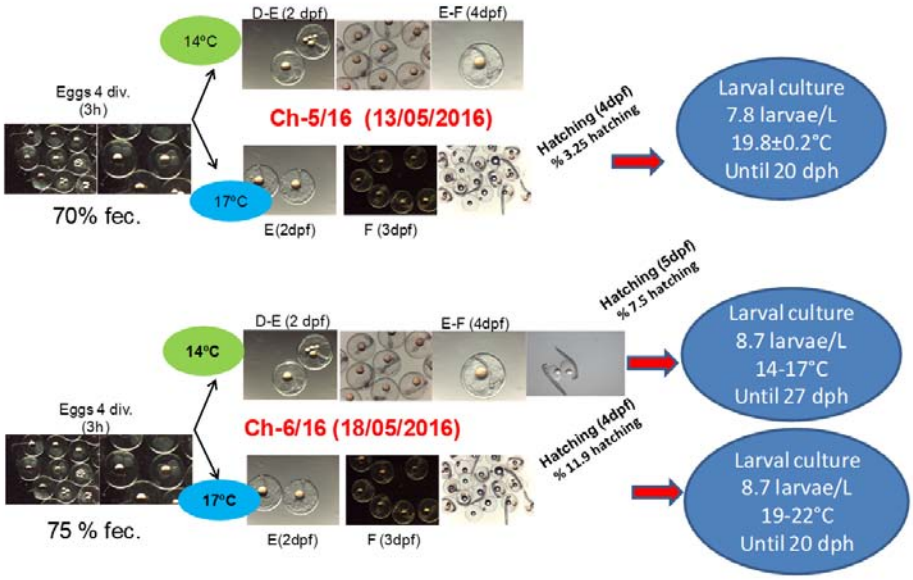


Figure 18.2.1.1. First incubation and larval wreckfish culture trials with two spontaneous spawns of IEO

These preliminary results are very interesting for setting an optimal range of incubation temperatures in future experiments, but not conclusive because the assays are insufficient. Regarding larval rearing, the maximum period that the larvae survived never exceeded 27 days post hatching (dph).

During larvae rearing some malformed individuals were observed. This problem could be related to inadequate nutrition, environmental conditions, oxidative stress, husbandry conditions or some of this factors together.

Larval data recorded:

- Larvae total length was 4.70±0.27 mm at 1 dph.
- Yolk sac was consumed by 11 dph at 14-17°C and by 8 dph at 17-20°C sea water temperature.
- Mouth opening occurred at 7 and 4 dph at 14-17°C and 17-20°C, respectively. Following mouth opening, larvae were fed with enriched rotifers and Artemia nauplii, which maintained the larvae until 27 dph.

There was no evidence that the food ingested by the larvae in these trials (**Fig 18.2.1.2**), was digested, because the larval growth was negative, i.e. both length and dry weight decreased (**Fig. 18.2.1.3**) indicating that the surviving larvae were not properly fed while some of them exhibited deformities as well. On the other hand, the fact that the larvae ingested prey was considered a step forward in these preliminary trials.

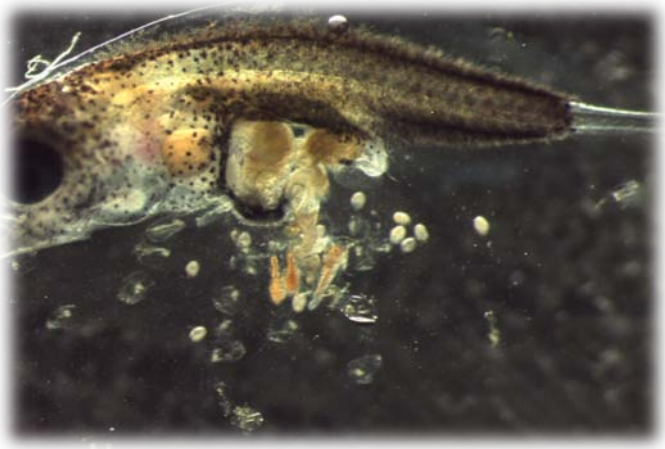


Figure 18.2.1.2. Larvae at 18 dph demonstrating gut content including *Artemia* nauplii, rotifer eggs and their squelets.

Therefore the rearing conditions were obviously not appropriate and further work is required. It is also not possible to conclude whether the deformities appeared at an earlier or later stage of the rearing, although it seems that they increased during larval rearing.

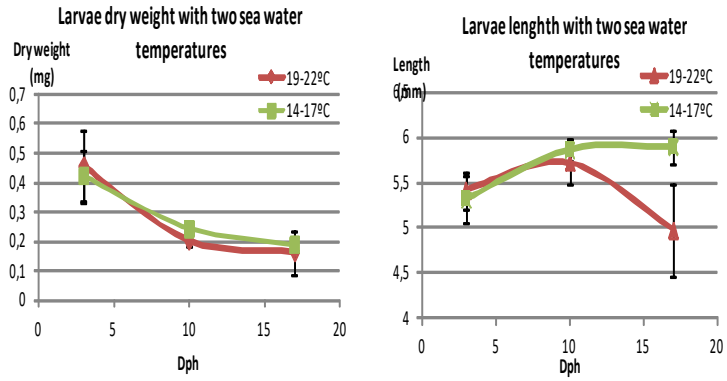


Figure 18.2.1.3. Larvae dry weight (mg) and length (mm) cultured in two different water temperature ranges (19-22ºC and 14-17ºC).

2017

During April 2017 an incubation experiment with larvae was carried out with three temperatures to extend and validate the results obtained during 2016. The temperature ranges were 13-14ºC, 16-17ºC and 19-20ºC. Floating egg samples were taken from three spawns (one of which was finally rejected as being of poor quality) between April 3rd and 17th, 2017. Two eggs concentrations,(350 and 450 eggs/l) were placed in 2.5 l cylindrical vessels with 500 µm mesh base (3 containers/concentration) **(Fig. 18.2.1.4)**. The water used in the experiment was filtered (1 µm), while the flow rate was continuous for 2-3 hour renewals with soft aeration to facilitate egg mixing. The temperature was regulated with a heat pump and was recorded every three hours between 9:00 am and 9:00 pm each day to verify that it was within the desired limits.

Figure 18.2.1.4. Tanks with three different water temperature, where the different cylindrical vessels with a mess base were introduced to incubate the eggs.

Each morning, a sample of 10 floating eggs was taken from each container to identify the stage of embryonic development and all the dead eggs from the bottom were siphoned and counted. All hatched larvae were counted, differentiating between those that were of good quality and those that had some type of deformity. With this data, the hatching rate and deformity for each temperature was calculated as follows:

$$\% \text{ Eclosion} = N_L/N_H \times 100$$

% Deformities = N_D/N_L x 100

Where N_L is the total number of hatched larvae, N_H the number of eggs hatched and N_D the number of deformed larvae.

The average temperatures obtained during the whole experiment were 13.7 ± 0.2ºC, 16.6 ± 0.4ºC and 19.5 ± 0.4ºC for each of the temperature ranges. Embryonic development was different for each temperature, and was of 4, 5 and 7 days. at 19.5ºC, 16.6ºC and at 13.7ºC, respectively. The quality and number of individuals hatched is shown in the **Fig. 18.2.1.5.**

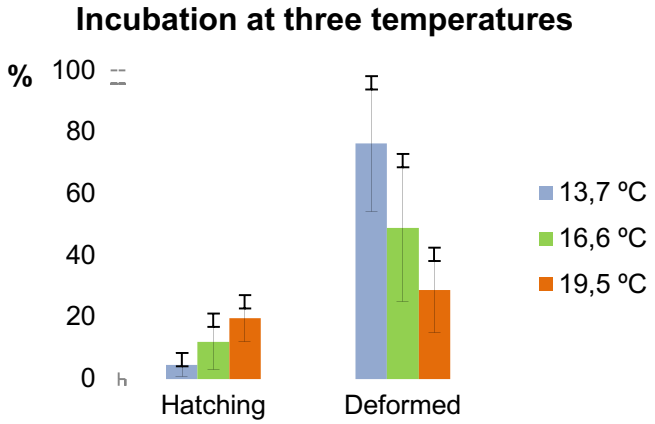


Figure 18.2.1.5. Hatching rate (%) and larvae quality (% deformed) during the trial with three water temperatures.

The best hatching % were at 19.5 ºC (between 13% and 31.2%) and decrease with lower temperatures, with a minimum % at 13.7 ºC (between 1% and 9.8%). More larvae of poor quality and exhibiting deformities hatched at 13.7 ° C (between 47% and 100%), at 16.6 ° C (between 18% and 81%) and at 19.5 ° C (between 12% and 43%). The average values of both parameters are shown in the **Table 18.2.1.1.**

Table 18.2.1.1. Average values of hatching (%) and deformities (%) of larvae during the trial with three water temperatures.

13,7 ºC		16,6 ºC		19,5 ºC	
% Hatching	% Deformed	% Hatching	% Deformed	% Hatching	% Deformed
4,61 ± 3,9	76,25 ± 21,9	12,11 ± 9,1	49,0 ± 23,9	19,7 ± 7,5	28,8 ± 13,8

Significant differences were observed ($p < 0.05$) in both hatching rate and deformed larvae between the temperatures of 13.7ºC and 19.5ºC, while the differences were not significant with respect to 16.6ºC. However, the trend suggests that low temperature incubations promote very low hatching % and high deformity.

The critical days regarding the viability of embryogenesis at the three temperature ranges were also calculated. The Table 18.2.1.2 shows the percentage of eggs collected from the bottom of each glass during the first three days of incubation with respect to the total of eggs that were incubated at first.

Table 18.2.1.2. Eggs collected from the bottom (%) the first three days of incubation at three water temperatures.

Tª	Days of incubation		
	1	2	3
13,7	24% ± 9,7%	30,2% ± 9,5%	29,5% ± 10,9%
16,6	35,4% ± 23,8%	40,8% ± 20,9%	4,7% ± 3,4%
19,5	56,8% ± 8%	18,2% ± 7,5%	-

At 13.7 ° C eggs mortality was around 30% during the first three days of incubation. At 16.6ºC, the highest levels, around 75%, are concentrated in the first two days. At 19.5ºC the majority of the mortality, over 60%, was during the first day of incubation. This fact indicates that during the first stages of egg development, vulnerability to external conditions is higher; therefore, the incubation parameters must be adjusted and the facilities and systems optimized in order to increase the quality of the embryogenesis, and larvae in the best conditions, and increase survival.

Sub-task 18.2.2 Test of two culture systems RAS(CMRM) and flow-through (IEO).

These experiments could not be carried out due to the lack of sufficient larvae stock to carry out experiments with guarantees, in terms of evaluating the results obtained.

With respect to the biochemical profiles, larvae batches were analyzed at different days of life, which were useful for designing enrichments for food of larvae (Task 12.1). We hope to be able to test next year 2018.

Deviations from Annex I and their impact:

During this period, important advances have been made in the understanding of ontogeny and larval development. The deliverables **D18.2**, **D18.3** and **D18.4** have not been delivered so far. Some factors have been previously mentioned. It will be necessary to increase the quality of the hatched larvae in order to allow us to follow the initiated trials. To this end, changes are being made in husbandry and larval culture that can be decisive to avoid the problem of larval deformities and achieve greater survival.

WP 19 Larval husbandry – grey mullet

WP No:	19	**WP Lead beneficiary:**		P4. IOLR
WP Title (from DOW):	Larval husbandry – grey mullet			
Other beneficiaries (from DOW):	P2. IRTA	P25. DOR		
Lead Scientist preparing the Report (WP leader):	Bill Koven			
Other Scientists participating:	Alicia Estevez, Enric Gisbert (P3), Hagay Sarusi (P25)			

Objectives

1. Investigating environmental and nutritional factors that affect larval rearing.
2. Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production.
3. Determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The experiment addressing Task 19.1 Effect of algal type and concentration on larval performance and the Sub-task 19.1.1 (IOLR) Determine the effect of algal type and concentration in rearing tanks on larval performance had begun on Oct 31st 2014 and was being carried out at the writing of the previous report. Consequently, only the experimental design was reported then but will now be presented in the present report.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In sub-tasks 19.1.1 and 19.1.2, two tank turbidity levels (0.76 and 1.20 NTU) from two algal species (*Nannochloropsis oculata* and *Isochrysis galbana*) and the no-algae control (0.26 NTU) were tested on 2-25 dph grey mullet larvae. This study concluded that rotifer consumption and survival of grey mullet larvae and juveniles were dependent ($P<0.05$) on algal turbidity but independent of algal type. (2) Rotifer consumption in early development markedly influences later juvenile survival. (3) Higher survival resulted in large numbers of smaller fish, which contributed to a skewed size distribution in the population.

Summary of progress towards objectives (31-48 Mo):

In **Sub-task 19.1.1**, which determined the effect of algal type and concentration in rearing tanks on larval performance demonstrated that the higher turbidity (1.2 NTU) increased rotifer consumption independently of algal type although common biochemical factors between *Nannochloropsis oculata* and *Isochrysis galbana* may still influence larval performance. The significant ($P<0.05$) effect of turbidity level on rotifer consumption in 5 dph larvae was markedly similar to the treatment effect on survival in 51 dph fish despite that more than three weeks had elapsed since the fish were exposed to the algal treatments suggesting the long-term effect and importance of rotifer feeding. The results of this study were described in the submitted

deliverable ***D19.1 Determine most effective type and concentration of algae used in grey mullet larval rearing***.

Sub-task 19.1.2 determined if the benefit of algal addition to rearing tanks was due to background lighting or other factors that contribute to larval performance. The results verified that high *N. oculata* turbidity (1.2 NTU) improved larval performance over the low *N. oculata* turbidity treatment (0.8 NTU). Moreover, larvae in the high *N. oculata* turbidity treatment significantly consumed more rotifers, as well as displaying better growth and survival than larvae exposed to the same turbidity derived from clay. This suggests a further advantage that live algae provides over its ability to produce turbidity in the larval rearing of grey mullet.

Task 19.2 Compared the selected microalgae type and protocol (**Task 19.1**) with a lyophilized substitute demonstrated that larvae exposed to lyophilized and live *Nannochloropsis oculata* , which gave a turbidity of ca 1.2 NTU, in the rearing tanks resulted in very similar larval performance in terms of rotifer ingestion rate, swim bladder inflation, growth and survival. These results suggest that lyophilized algae, which is an available commercial product, can be used to replace live algae. This would lead to a significant saving in time, labour and infrastructure. Worthy of note is that the advantage of algae over clay is not lost during the lyophilisation process. Consequently, the results of the present study recommends the use of lyophilized *Nannochloropsis oculata* in the larval rearing of grey mullet.

Task 19.4 Determined when to wean larvae and which weaning diet to use according to DT maturation and the shift from carnivorous to omnivorous feeding. The results showed that mullet juveniles grew significantly less, in terms of length and final weight, when fed only an ulva based herbivorous diet compared to a commercial carnivorous feed, while fish fed the 1:1 omnivorous mix of ulva and the commercial feed exhibited markedly ($P<0.05$) superior growth than all the treatments. Fish fed the herbivorous diet demonstrated significantly higher numbers of smaller fish (<100 mg), than the carnivorous and omnivorous diet fish and, in general, exhibited a population skewed to slower growing individuals. Conversely, 200-300 mg carnivorous and omnivorous treatment fish represented a significantly ($P<0.05$) higher percentage of the population than the herbivorous diet fed fish. Mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch rich microalgae and macroalgae, as well as benthic protein rich organisms characterizing the lower salinity estuarine waters they move into at this developmental stage. Furthermore, the high amylase and maltase activity in the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing resulting in improved growth. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds.

Details for each Task

Task 19.1 Effect of algal type and concentration on larval performance (led by IOLR, Bill Koven).

Sub-task 19.1.1 (IOLR) Determine the effect of algal type and concentration in rearing tanks on larval performance

Introduction

In the commercial rearing of marine fish larvae, the tanks are frequently "greened" with algae such as *Nannochloropsis oculata* or *Isochrysis galbana*. It is widely believed and demonstrated that the provision of these algae to the tanks significantly improves larval performance and has become an inseparable part of commercial rearing protocols in fish farms around the Mediterranean basin. On the other hand, how algal supplementation contributes to larval growth and survival or if different algal species are equally effective remains unclear. The biochemical composition of algal species (e.g. fatty acids) varies and it is entirely possible that species-specific compounds secreted from the algal cell (e.g. polysaccharides) released during digestion might stimulate the immune system or enhance the digestive process. Having said that, water turbidity from specific algal concentrations may provide optimal backlighting for larvae to facilitate prey identification (e.g. rotifers) and thereby enhance hunting success. The question whether the benefits of algal addition to rearing tanks is due to back lighting and/or compounds secreted by the algae that promote larval

growth and survival remains speculative and has not been addressed at all in the larval rearing of the grey mullet.

Consequently, the aims of the present study were to (1) compare the effect of microalgae type (*Nannochloropsis oculata* vs *Isochrysis galbana*) and concentration (cells ml^{-1}) on larval rotifer ingestion rate, biochemical composition, growth, survival and digestive tract enzyme ontogeny. (2) Determine if the benefit of live algal addition to the rearing tank is due to the effect of tank turbidity on efficient prey capture or to other factors.

Materials and methods

Grey mullet eggs (gastrula stage) were stocked in fifteen 1500 l tanks (100 eggs l^{-1}) in an open system where filtered (10 µm), UV-treated, 40 ‰ sea water (25 ^{0} C) entered from the tank bottom at a rate of two tank exchanges day^{-1}. Two turbidity levels (0.76 NTU, 1.20 NTU) and the no-algae control (0.26 NTU) were tested using two algal species (*Nannochloropsis oculata* and *Isochrysis galbana*) on 2-23 dph mullet larvae. Each of the 5 treatments was investigated with three replicate tanks.

All turbidity values were determined on triplicate water samples (including the no-algae control) that were first filtered (40 µm) before being read with a Turbidometer (Lovibond Turbi-check, Amesbury, England). The turbidity treatments were added twice daily, during the morning and afternoon. The afternoon turbidity reading was measured to calculate the amount of algae necessary to add to the tanks to compensate for algae washed out as a result of the water exchange rate in the rearing protocol. This algal amount was added in order to reach the designated control, A and B turbidity levels of 0.26, 0.76 and 1.19, respectively (Table 19.1.1) defined in the experiment.

Table 19.1.1. Turbidity levels of the no algal control treatment as well as the *Isochrysis galbana* and *Nannochloropsis oculata* concentrations added to the experimental tanks in order to give two higher turbidity levels (A, B). Turbidity values having different letters were significantly ($P<0.05$) different.

Treatments	Turbidity (NTU)
Control (no algae)	0.26^{a}
Isochrysis galbana A ($0.0144x10^{6}$cells ml^{-1})	0.77^{b}
Nannochloropsis oculata A ($0.2x10^{6}$ cells ml^{-1})	0.75^{b}
Isochrysis galbana B ($0.0228x10^{6}$cells ml^{-1})	1.18^{c}
Nannochloropsis oculata B ($0.4x10^{6}$cells ml^{-1})	1.20^{c}

To determine rotifer consumption, five larvae were sampled from each tank in the experimental system 90 min after feeding and were then fixed in 10% buffered formalin and stored at 4 ^{0} C until counting of the gut mastaxes.

Larval samples for fatty acid body composition were taken at hatching, 15, 18 and 25 dph while fish for digestive tract enzyme specific activity were sampled at hatching, 18, 25, 40, 61 and 79 dph. Lyophilized larvae were shipped to P3.IRTA for determining the activity of digestive enzymes.

Results

Turbidity values of the control and the two treatment levels were significantly ($P<0.05$) different from each other each day after the morning algal addition (**Figure 19.1.2**) and shortly before the morning rotifer feeding. The significant ($P<0.05$) differences between the A and B turbidity levels were maintained independently of the different *Nannochloropsis oculata* and *Isochrysis galbana* concentrations added to the tanks in each treatment. On the other hand, there were no turbidity differences among the treatments in the

afternoon as a result of algal loss from the water exchange protocol. Nevertheless, the treatment turbidity levels returned to designated values, after calculation, with the afternoon algal addition which was shortly before the afternoon rotifer feeding.

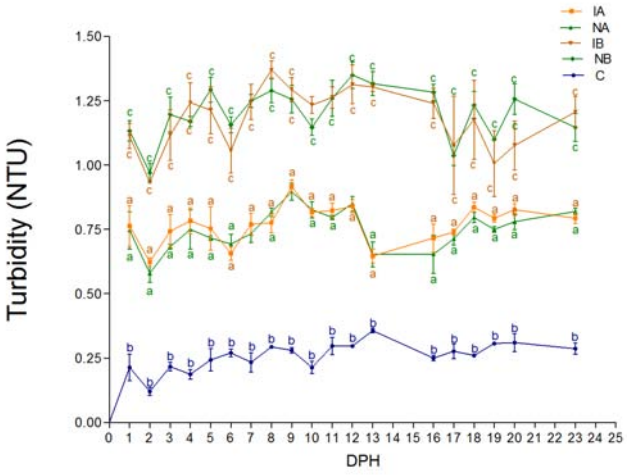


Figure 19.1.2 Turbidity levels (NTU) measured after morning algal addition to the tanks. The algal treatments no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) were applied from first feeding at 1 to 23 dph. NTU values having different letters within each day were significantly ($P<0.05$) different.

Figure 19.1.3a shows a significant ($P<0.05$) turbidity effect on rotifer consumption in 5 dph larvae that is independent of algal type. The larvae in the IB and NB treatments consumed significantly ($P<0.05$) more rotifers than the NA and C fish, although this was not significant ($P>0.05$) in the IA larvae. Interestingly, this pattern of rotifer consumption was similar to that of survival (**Figure 19.1.3b**), where fish exposed to high algal turbidity levels from 2-23 dph survived significantly ($P<0.05$) better at 51 dph than fish feeding at the lower turbidity values or in clear water. However, despite the turbidity effect on prey consumption and survival, there was no significant ($P>0.05$) treatment effect on their growth (not shown).

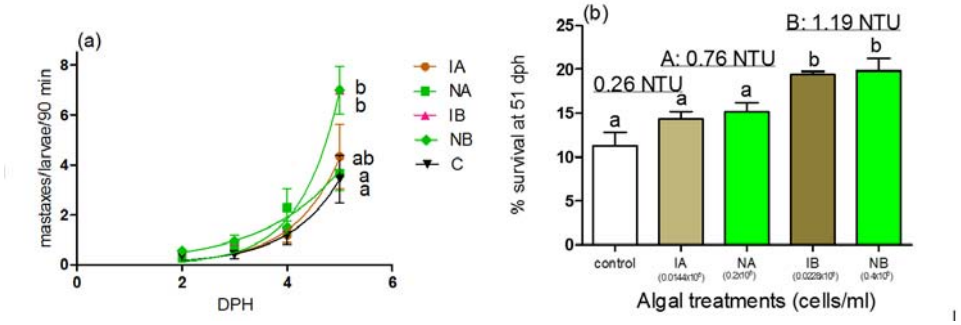


Figure 19.1.3 The effect of turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB) on (a) average rotifer (mastax count) consumption larva^{-1} found 90 min after feeding from 2-5 dph and (b) average percent (%) larval survival at 51 dph. Mastax number at 5 dph and percent survival values of the algal treatments on 51 dph having different letters were significantly ($P<0.05$) different.

In 15 dph larvae, there were significant (P<0.05) treatment effects on larval essential fatty acid profiles and fatty acid class content (Figure 19.1.4). On the other hand, these results cannot be correlated to the high DHA and low EPA found in *Isochrysis galbana* or the low DHA and high EPA found in *Nannochloropsis oculata*. Moreover, there were no significant (P>0.05) treatment effects on these essential fatty acids or fatty acid classes in all other larval ages sampled (not shown).

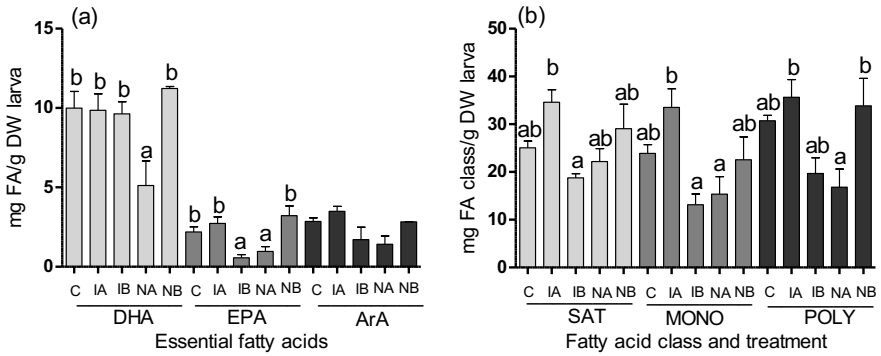


Figure 19.1.4. Essential fatty acids and fatty acid classes in 15 dph mullet larvae reared in the different turbidity treatments; no algae (C), *Isochrysis galbana* low turbidity (IA), *Isochrysis galbana* high turbidity (IB), *Nannochloropsis oculata* low turbidity (NA), *Nannochloropsis oculata* high turbidity (NB). Values within an essential fatty acid or fatty acid class having different letters were significantly (P<0.05) different**.**

In general, it appears that the turbidity treatments tested on 3 to 23 dph larvae had no lasting effect on the specific activity of digestive tract enzymes when measured between 18 and 79 dph (**Figures 19.1.6-19.1.15**).

On the other hand, diet composition during the period of enzyme sampling did affect specific activity in some enzymes. Lipase decreased (**Figure 19.1.6**) from 18 dph, when larvae were feeding on high lipid containing rotifers and *Artemia* (**Table 19.1.5**), to the lower lipid level characterizing the co-feeding of Caviar: *Ulva lactaca* (1:1 w/w) from 25-57 dph (**Table 19.1.6**). However, after switching at 57 dph to feeding only on the inert diet RDF, the increased lipid of this inert diet (14%) resulted in an increase of the lipase specific activity (P<0.05). Similarly, dietary protein decreased from the high levels found in rotifers and *Artemia* (2 to 25 dph) (**Table 19.1.5**), which corresponded to high protease activity, to the reduced protein levels of the Caviar: *Ulva lactaca* (1:1) diet fed between 25 and 57 dph with the subsequent decrease in alkaline protease activity (**Figure 19.1.7**). Nevertheless, the activity of this enzyme tended to increase when the fish were ingesting the higher protein of RDF from 58 to 79 dph (**Figure 19.1.7**). On the other hand, trypsin specific activity was not markedly (P>0.05) affected by the diet throughout the entire sampling period (**Figure 19.1.8**). The cytosolic enzyme leucine-alanine peptidase significantly (P<0.05) decreased in all algal treatments from 18 to 61 dph, but then increased (P<0.05) in 79 dph fish when the fish were fed the RDF diet (**Figure 19.1.9**). Importantly, the digestive tract marker for brush border membrane (BBM) development, alkaline phosphatase (AP; **Figure 19.1.10**), demonstrated significantly (P<0.05) increasing activity that was on average 9.6 times higher in fish from 61 to 79 dph (**Figure 19.1.10**). Consequently, the AP/leu-ala ratio, an indicator of gut maturation, peaked at 61 dph, but then declined in 79 dph fish from all algal treatments (**Figure 19.1.11**). During the period between 61 to 79 dph, amylase specific activity (**Figure 19.1.12**) increased 2.3 times signaling a more herbivorous/detritivorous mode of feeding. The alkaline protease/lipase ratio values showed higher protease activity over lipase activity at 18 dph when the larvae were feeding on highly digestible rotifer and *Artemia* protein (**Figure 19.1.13**). However, protease activity dropped compared to lipase activity at 25 dph, when the fish began to feed on the Caviar: *Ulva lactaca* diet. On the other hand, the ratio increased significantly (P<0.05) from 25 to 61 dph, possibly signaling the fish's increasing ability to breakdown dietary carbohydrates (**Figure 19.1.13**) and exposing more *Ulva lactaca*

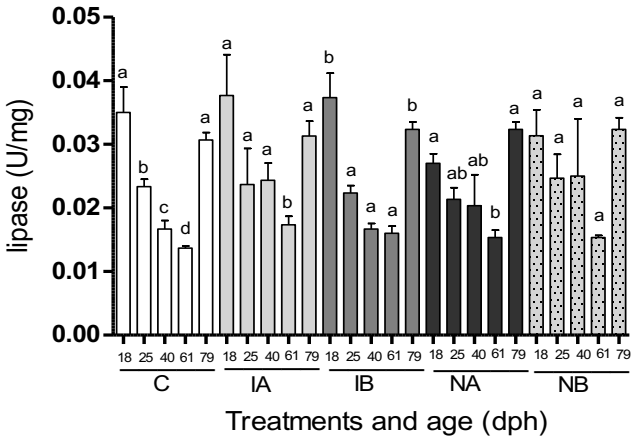
protein for protease digestion (**Figure 19.1.13**). Moreover, the amylase/trypsin and amylase/protease ratios supported the increasing capability of amylase production with age (**Figures 19.1.14** and **19.1.15**).

Table 19.1.5 Composition (%) of food used to feed the grey mullet at different stages of development.

Feed	Enriched Rotifers*	Unenriched *Artemia***	Enriched *Artemia****	Caviar (Bernaqua, Belgium) †	*Ulva lactaca* (IOLR, Israel)*****	Ranaan Dry feed (RDF, Israel)†
Days fed (dph)	1-23	15	16-24	25-50	25-50	50-79
Protein	48.2	56.2	53.1	55	34	56
Lipid	14.0	17.0	28.7	15	7.4	14
Carbohydrate	18.5	3.6	3.5	8	56	1
Ash	9.3	7.6	2	12	2.6	14.8

*Demir and Diken 2011, **Garcia-Ortega et al. 1998, ***Koca et al 2015, ****analysis at IOLR, †according to manufacturer

Figure 19.1.6. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* that were given to 3-23 dph grey mullet larvae on lipase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.



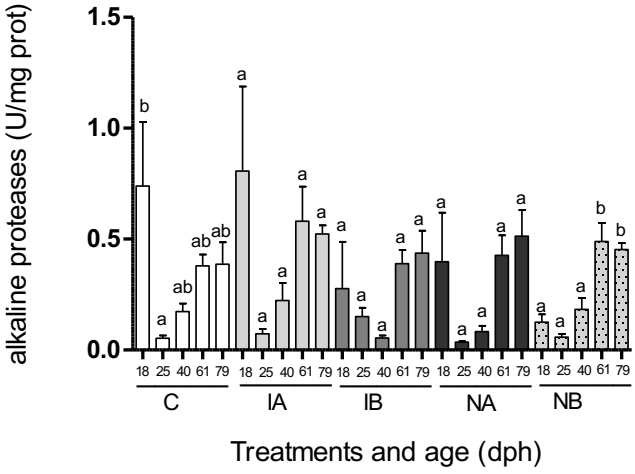


Figure 19.1.7 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on alkaline proteases activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.

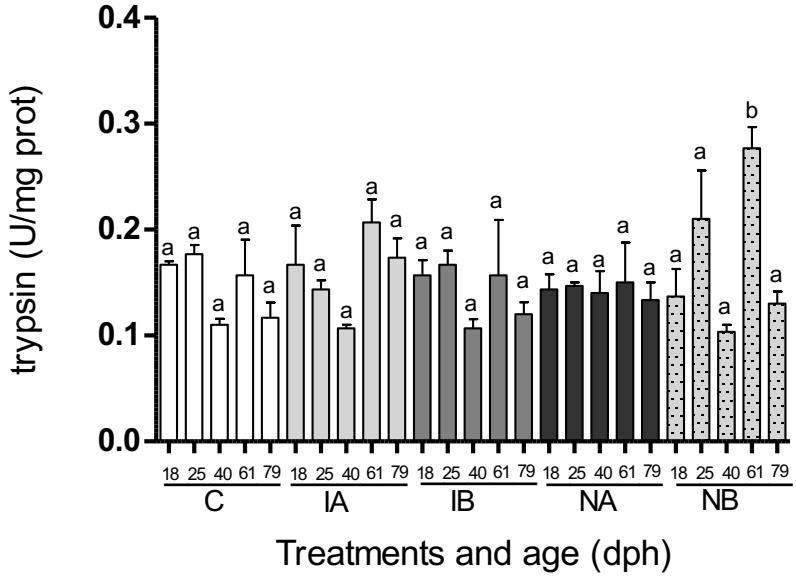


Figure 19.1.8 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on trypsin activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.

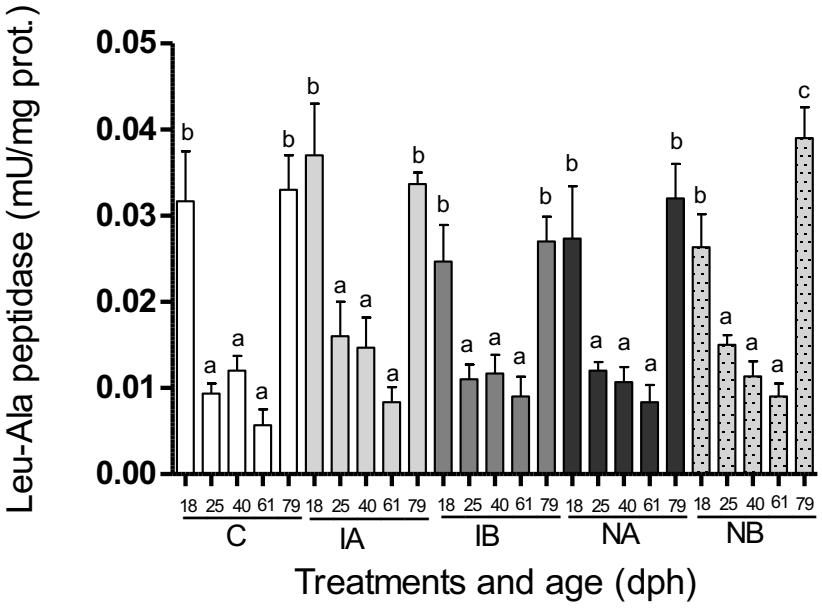


Figure 19.1.9. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on leu-ala peptidase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.

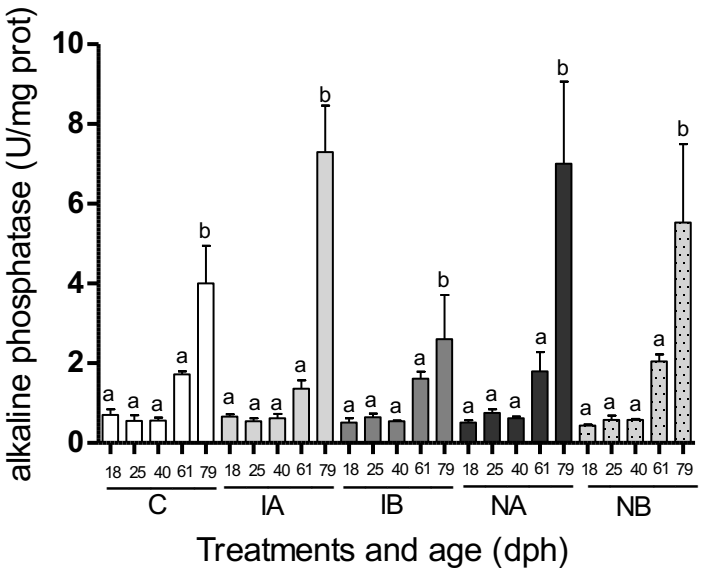


Figure 19.1.10 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on alkaline phosphatase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

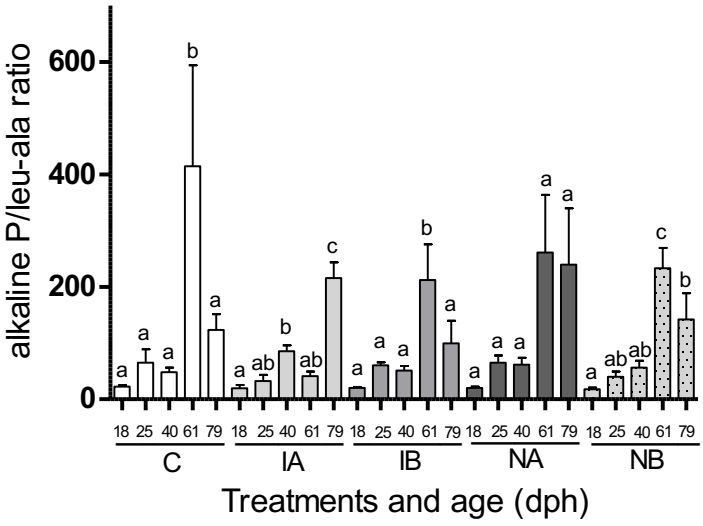


Figure 19.1.11 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on the alkaline phosphatase/leu-ala ratio sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly (P<0.05) different.

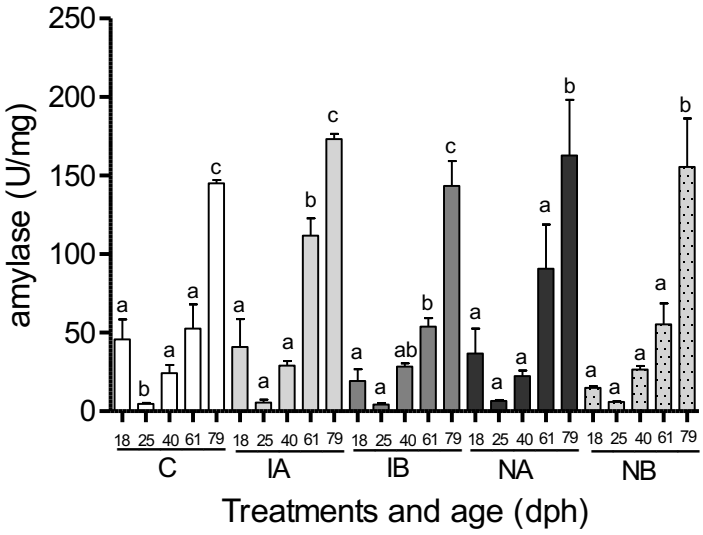


Figure 19.1.12 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-23 dph grey mullet larvae on amylase activity sampled at different fish ages (18, 25, 40, 61, 79 dph). Specific activity values within a treatment having different letters were significantly ($P<0.05$) different.

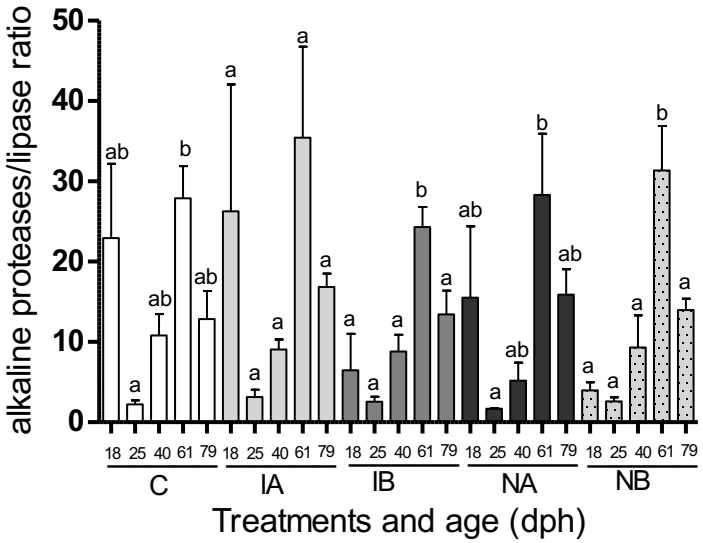


Figure 19.1.13. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on alkaline protease/lipase ratios at different fish ages (18, 25, 40, 61, 79 dph). Ratios within the same treatment having different letters were significantly ($P<0.05$) different.

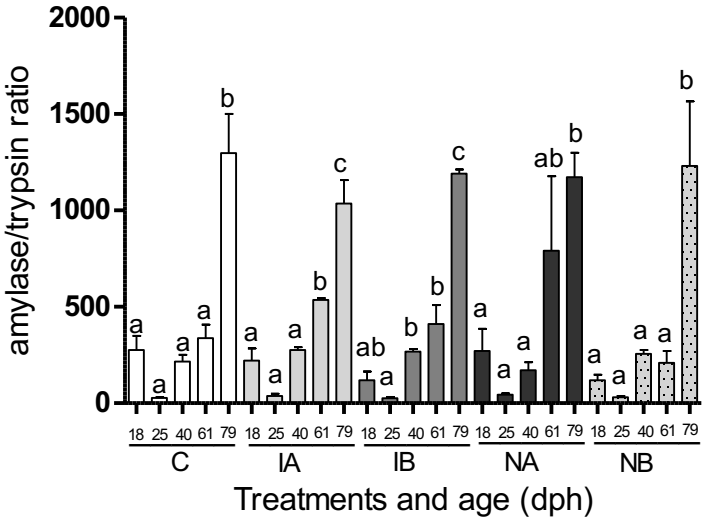


Figure 19.1.14 The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on amylase/trypsin ratios at different fish ages (18, 25, 40, 61, 79 dph). Amylase/trypsin ratios within the same treatment having different letters were significantly ($P<0.05$) different.

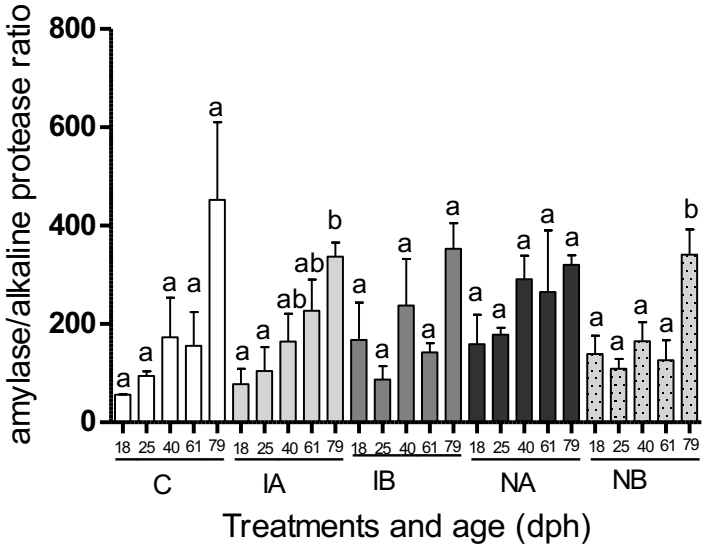


Figure 19.1.15. The effect of the turbidity treatments; no algae (C), low turbidity *Isochrysis galbana* (IA), high turbidity *Isochrysis galbana* (IB), low *Nannochloropsis oculata* (NA) and high *Nannochloropsis oculata* (NB) given to 3-25 dph grey mullet larvae on amylase/proteases ratio at different fish ages (18, 25, 40, 61, 79 dph).

Discussion

The beneficial effects of the presence of microalgae in the rearing tanks for the larvae of many farmed species have long been recognized. Various hypotheses have been put forth explaining how the algae might

benefit the larvae that include (1) providing a direct supply of micronutrients that trigger key physiological processes, (2) releasing of appetite stimulating components and (3) influencing the bacterial composition of the rearing water and consequently the larval gut microbial flora. Apart from these potential advantages, microalgae might be indirectly contributing to larval welfare by affecting the diffusion of light in the water column and thereby, creating a backlighting effect. A number of authors have suggested that this would contrast the zooplankton prey against its background facilitating detection by the larvae, which would contribute to hunting success. In the present study, the increased rotifer consumption at the higher turbidity, independent of algal type, suggests that the developing fish could detect and hunt the prey more effectively under these conditions. Moreover, turbidity appears to be a more dominant factor affecting prey consumption than the algal biochemical content. Although the biochemical composition considerably differs between *Nannochloropsis galbana* and *Isochrysis oculata* microalgae, particularly in essential fatty acid content, these phytoplankton species similarly affected prey consumption at the same turbidity level.

Nevertheless, green water may be still contributing to larval welfare in other ways. The significant ($P<0.05$) effect of turbidity level on rotifer consumption in 5 dph larvae was markedly similar to the treatment effect on survival in 51 dph fish. This means that 2-23 dph larvae, which were exposed to the high turbidity treatments, survived significantly better at 51 dph than the lower turbidity and control treatments. This occurred despite the fact that more than three weeks had elapsed since the fish were exposed to the algal treatments and that during this period the fish were all fed an identical diet.

The composition of the diets the grey mullet were consuming during development appears to have influenced the specific activities of lipase and total alkaline proteases, while there did not appear to be an age or treatment effect on trypsin. The level of lipase and total alkaline protease activities increased when ingesting the relatively lipid and protein rich rotifers and *Artemia* (18 dph), while decreased when co-fed the lower lipid and protein levels in the Caviar: *U. lactaca* diet (25-57 dph) and increased again when ingesting the moderate lipid and protein levels of RDF (> 57 dph). Protease activity dropped compared to lipase activity at 25 dph, when the fish commenced to feed on the Caviar: *U. lactaca* diet. The considerable increase in total alkaline protease activity may also be due to the improved ability to breakdown carbohydrates. It should be noted that no acid protease activity was found (data not shown); thus, protein digestion in grey mullet larvae is mainly accomplished by alkaline proteases and not acid proteases as in other marine fish larvae and early juveniles. *Ulva* spp. are a relatively rich source of starch where amylase can hydrolyze the α-1, 4 glycoside bonds in glycogen and starch. This suggests that the increased amylase activity from 25-61 dph, which was likely genetically based, resulted in an increasing ability to digest *Ulva lactaca* carbohydrate and potentially exposing more *Ulva* protein for protease digestion.

In fact, the ontogeny of digestive tract enzyme activity in the grey mullet larvae and juveniles appears to be more a function of age and genetic programming than dietary modulation. Nevertheless, the suggested late age of the carnivorous-herbivorous shift in grey mullet is supported by the steadily increasing activity of amylase from 25-79 dph (particularly between 61 to 79 dph). Amylase activity is much higher in herbivorous and omnivorous fish compared to carnivores. Overall, the results suggest that 61-79 dph grey mullet juveniles, which approximate the size of juvenile mullet moving to estuaries, have the capacity to digest protein and starch allowing for the exploitation of the relatively starch rich microalgae and macroalgae, as well as benthic organisms characterizing these lower salinity estuarine waters. Moreover, the results broadly suggest that aquaculture feeds at this developmental stage should include high levels of starch or other low cost amylolytic energetic compounds.

Sub-task 19.1.2 (IOLR) Determine if the benefit of algal addition to rearing tanks due to background lighting or other factors that contribute to larval performance

Introduction

In **Sub-task 19.1.1** and deliverable **D19.1**, it was suggested that algae derived turbidity, whether from *Nannochloropsis oculata* or *Isochrysis galbana*, in the larval rearing tanks was the main factor determining the benefit of algae and its effect on rotifer ingestion and survival independent of algal type and biochemical

composition. This was supported by the commercial rearing of halibut, which uses clay and not algae to produce turbidity (Attramadal et al. 2012 and is much more economical. However, the question remained if live algae nevertheless provides a benefit and if the replacing of algae with clay is applicable to the larval rearing of grey mullet

Methods and Materials

Grey mullet eggs were spawned at P4.IOLR where the hatching percent was 65% and survival to the end of dph 0 was 92.5%. The experimental system consisted of twelve 1500 l V-tanks in a flow through system where 40 ‰ ambient, temperature controlled (24-25 ^{o}C), filtered (10 µm) and UV treated sea water enters at the bottom of the tank and exits through a 150 µm filter. Larvae were exposed, after depletion of the yolk sac and the onset of exogenous feeding, to three turbidity treatments from 2-30 dph which were tested in replicates of 4 tanks/treatment under a photoperiod of 14D/10L with a light intensity of 500 lux. Two of the treatments were the control and high turbidity treatment (0.8 and 1.2 NTU, respectively) produced by the addition of two concentrations of *Nannochloropsis oculata*; 0.25 and 0.5 x 10^{6} cells/ml, respectively. The third treatment mimicked the high algae turbidity (1.2 NTU) but was produced by potter's clay (Red DAS Terracotta. F.I.L.O., Pero, Italy). Turbidity was measured (Turbicheck, Lovibond, Amesbury, England) after, filtering (40 µm) during the morning and afternoon addition of algae. The larvae were fed rotifers enriched on taurine and the commercial enrichment "Red Pepper" (Bernaqua Ltd., Belgium) and then *Artemia* from 15 to 21 dph, which were enriched with the same protocol. From 22 to 32 dph, the fish were fed a combination of ulva and weaning diet caviar (1:1 DW). Fatty acid samples of eggs and freshly hatched and 28 dph larvae were taken and frozen at -70 ^{o}C.

Statistics

One way ANOVA, two way ANOVA and regression (linear and non-linear) analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. ANOVA analyses and Barlett's test for equal variances were carried out simultaneously. If significance ($P<0.05$) was found for ANOVA while Barlett's test was not significant ($P>0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P<0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P<0.05$) differences among treatments. All data are presented as mean ± SEM.

Results and discussion

Figure 19.1.2.1a shows that turbidity levels and treatment conditions were maintained from 3 to 30 dph. The rotifer ingestion rate of larvae exposed to Nanno B was significantly higher ($P=0.0004$) higher than the Nanno A and Clay B rates while these latter treatments were not significantly different from each other. The marked difference between Nanno A and Nanno B results verify the findings of **D19.1** that the higher N. oculata derived turbidity was more effective than the lower algae derived turbidity. However, larvae exposed to Nanno B outperformed cohorts exposed to Clay B, in terms of rotifer consumption (**Figure 19.1.2.1b**), growth (DW and length) as well as survival at the juvenile stage (**Figure 19.1.2.2a,b,c**). This is true despite the fact that these two treatments had identical turbidity levels and strongly suggests that live algae provides a further advantage over its ability to produce turbidity in the larval rearing of grey mullet. Moreover, the turbidity effect still influenced survival 20 days after the turbidity treatments had ceased. It should be noted that this study is currently being repeated with white clay (the present experiment used red clay) and the preliminary results appear to be very similar and reinforces the result that clay was less effective as a contributor to turbidity than algae. Taken together the present results recommend the use of algae (N. oculata or I. galbana) to produce a turbidity of 1.2 NTU in the rearing tanks and not to use clay (red or white).

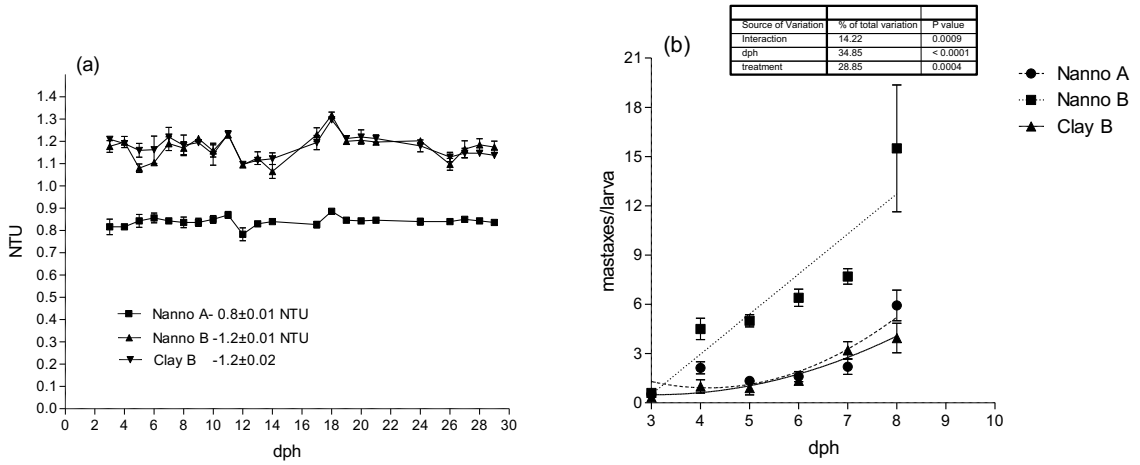


Figure 19.1.2.1 (a) NTU levels after the morning low and high *N. oculata* and clay addition to the tanks (0.8, 1.2, 1.2, respectively); n=4. (b) Average number of mastaxes consumed per larvae 90 minutes after feeding; n=4. Regression and AIC analysis showed that the probability that Nanno A, Nanno B and Clay B were generated by a 2nd order polynomial, linear and 2nd order polynomial models, respectively, were 86.4, 57.5 and 57.9%, respectively. The Nanno B curve was significantly (P=0.0004) different than the Nanno A and Clay B curves while the latter two curves were not significantly (P>0.05) different from each other. The source of variation in the values was significantly affected by both age and treatment as well as their interaction.

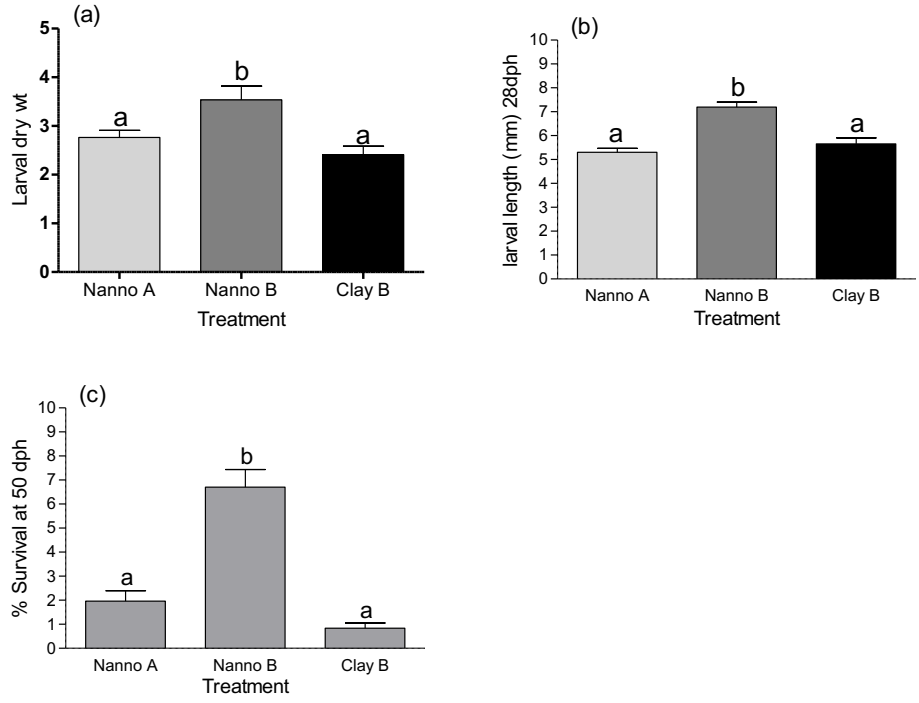


Figure 19.1.2.2 The effect of the Nanno A, Nanno B and Clay B turbidity treatments (0.8, 1.2, 1.2 NTU, respectively) on 30 dph larval (a) length and (b) dry weight as well as (c) survival in 51 dph fish; n=4. ANOVA was significant (P<0.05) in all three parameters and found that Nanno B fish grew (DW and length) and survived markedly (P<0.05) better than the control (Nanno A) and Clay B.

References

Attramadal, K.J.K., Tøndel, B., Salvesen, I., Øie, G., Vadstein, O., Olsen, Y., 2012. Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. Aquacult. Eng. 49, 23-34.

Task 19.2 Comparing the selected microalgae type and protocol (Task 19.1) with lyophilized substitute (led by IRTA, Alicia Estevez/Enric Gisbert)

Methods and Materials

Grey mullet eggs were spawned from F1 broodstock at ARDAG Fish Farms Ltd., Eilat, Israel and stocked in a 6 m^3 V-tank (170 eggs/l). After 3 days, first feeding larvae were transferred and stocked (25 larvae/l) in eight 400 l V-tanks in a flow through system where 40 ‰ ambient, temperature controlled (24-25 ^oC), filtered (10 µm) and UV treated sea water enters at the bottom of the tank and exits through a 150 µm filter. Sea water salinity was incrementally reduced over a few days to 25 ‰ (P4.IOLR grey mullet rearing protocol). This allowed the testing of live or lyophilized *Nannochloropsis oculata* derived turbidity (*ca* 1.22 NTU or concentration of 0.5 x 10^6 cells/ml) on 2-30 dph larval performance in replicates of 4 tanks per treatment. The study was carried out under a photoperiod of 14D/10L with a light intensity of 500 lux. Turbidity was measured (Turbicheck, Lovibond, Amesbury, England) after, filtering (40 µm) during the morning and afternoon addition of algae. The larvae were fed rotifers enriched on taurine and the commercial enrichment "Red Pepper" (Bernaqua Ltd., Belgium) and then *Artemia* from 15 to 21 dph, which were enriched with the same protocol. From 22 to 30 dph, the fish were fed a combination of ulva and weaning diet caviar (1:1 DW). at 30 dph, larval samples were collected for fatty acid and digestive tract enzyme analyses as well as RNA extraction.

Statistics

One way ANOVA, two way ANOVA and regression (linear and non-linear) analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. ANOVA analyses and Barlett's test for equal variances were carried out simultaneously. If significance ($P<0.05$) was found for ANOVA while Barlett's test was not significant ($P>0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P<0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P<0.05$) differences among treatments. All data are presented as mean ± SEM.

Results and Discussion

Figure 19.2.1a demonstrated that the lyophilized (Nanno-D) and live (Nanno-L) algae treatments provided almost identical daily turbidities during the 3-30 dph experiment. This resulted in very similar larval performances in the two treatments in terms of rotifer ingestion rate (**Figure 19.2.1b)**, swim bladder inflation (**Figure 19.2.2**), growth (TL and DW) (**Figure 19.2.3a,b**) and survival (**Figure 19.2.4**). These results suggest that lyophilized algae, which is an available commercial product, can be used to replace live algae which would be a significant saving in time, labour and infrastructure. Worthy of note is that the advantage of algae over clay is not lost during the lyophilisation process. Consequently, the results of the present study recommends the use of lyophilized *Nannochloropsis oculata* in the larval rearing of grey mullet. Nevertheless, the isolation of the active compound that provides the algal advantage remains elusive and requires further study.

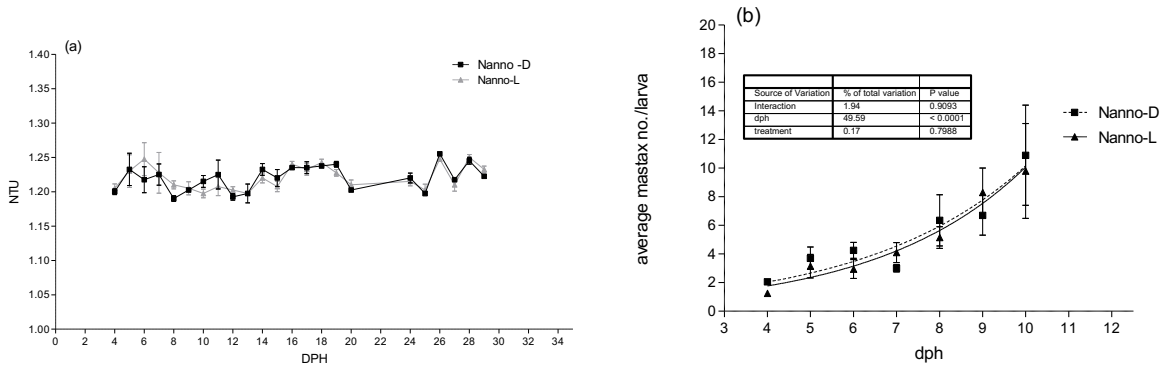


Figure 19.2.1 (a) Average NTU levels measured in the experimental tanks demonstrating that the lyophilized and live algae (Nanno-D, Nanno-L, respectively) provided very similar turbidity values from 4-30 dph. (b) The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) turbidity on rotifer ingestion (mastax no.) in 4-10 dph mullet larvae. Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by an exponential growth equation were 75.5 and 67.1%, respectively. The curves were not significantly (P>0.05) different from each other. The source of variation was a result of age (dph) and not algal treatment.

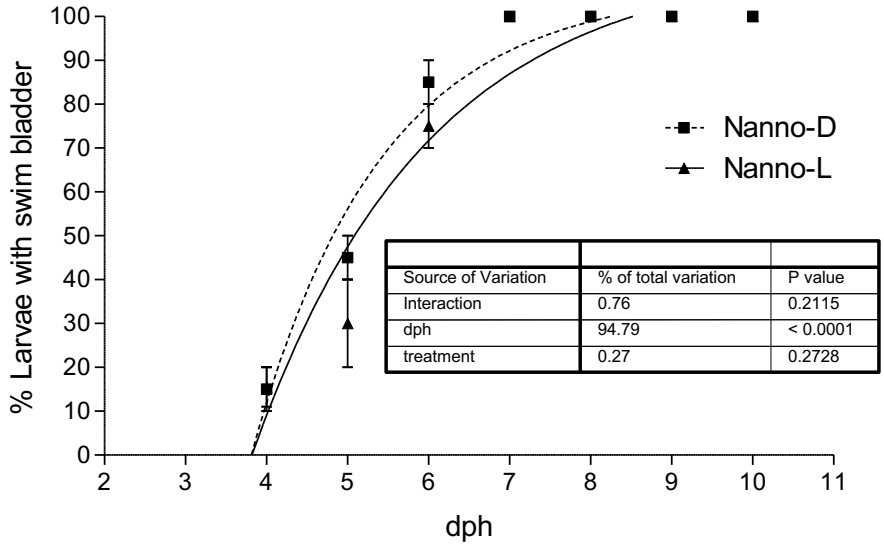


Figure 19.2.2 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) turbidity on percent (%) larvae with swim bladder inflation; n=4. Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by a one phase association equation was 99.9% for both curves. The curves were not significantly (P>0.05) different from each other. The source of variation was a result of age (dph) and not algal treatment.

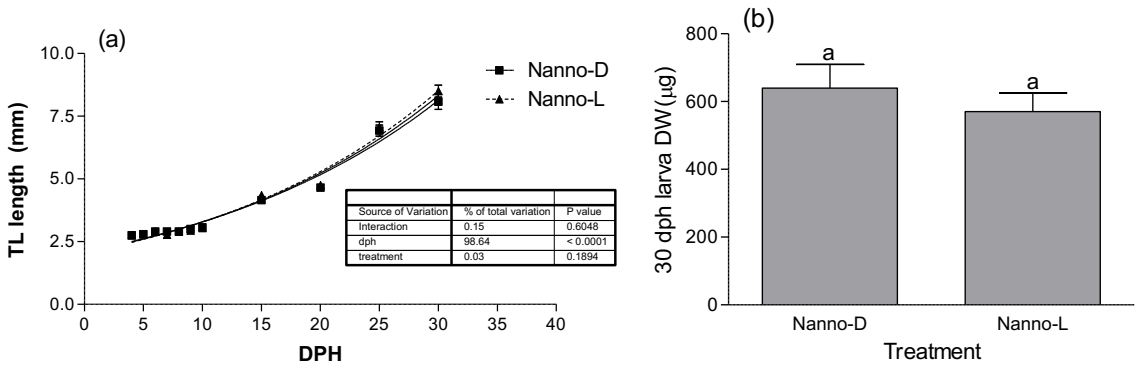


Figure 19.2.3 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) turbidity on (a) 3-30 dph larval length; n=4. Regression and AIC analysis showed that the probability that the lyophilized (Nanno-D) and live (Nanno-L) *N. oculata* values were generated by an exponential growth equation was 99.9% for both curves. The curves were not significantly (P>0.05) different from each other. The source of variation was a result of age (dph) and not algal treatment. (b) Dry weight (DW) in 30 dph larvae. One way ANOVA of DW values was not significant (P>0.05).

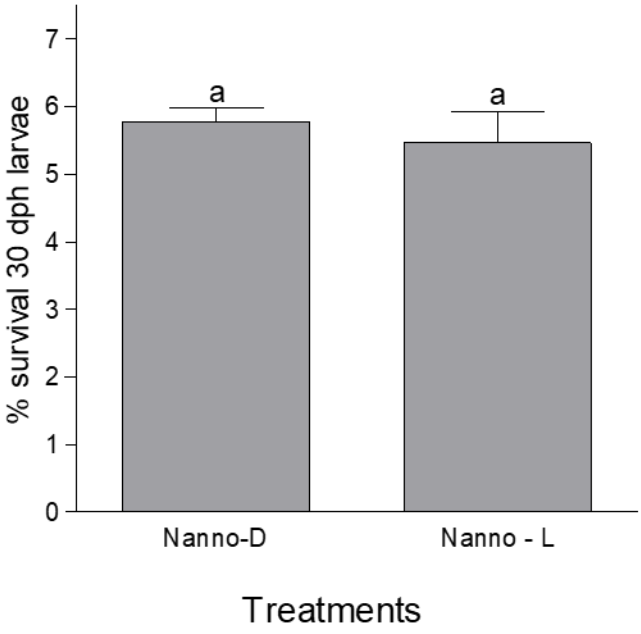


Figure 19.2.4 The effect of using lyophilized (D) or live (L) *Nannochloropsis oculata* derived turbidity in the grey mullet larval rearing tanks on percent survival (%) in 30 dph fish; N=4. One way ANOVA of survival values (after arsine transformation) was not significant (P>0.05).

Task 19.3 Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production (led by IOLR, Bill Koven).

As the company that produced the ciliates has been closed, P4.IOLR received permission to replace ciliates with copepods. Problems producing sufficient numbers of copepods to run the experiment this year. It is hoped that the experiment will be carried out during the final season next fall.

Task 19.4 Determine when to wean larvae and to feed weaning diet type according to DT maturation and the shift from carnivorous to omnivorous feeding (led by IOLR, Bill Koven).

This was a deliverable (D19.3) that was accepted during 2017 and is presented here in brief.

Introduction

Grey mullet larvae, as in all marine early developing fish, are strict carnivores feeding on zooplankton such as rotifers (*Brachionus spp.*) and *Artemia spp*. in commercial hatcheries. However, after the mullet larvae have metamorphosed into juveniles they begin to change their mode of feeding from a carnivorous to an herbivorous/omnivorous diet as they are programmed, in nature, to search out less saline estuaries with higher primary productivity of micro and macroalgae. In captivity, D19.1 demonstrated that the digestive tract reaches full maturation around 61 days and considerable pancreatic amylase production at 79 dph (an increase of 5.3 fold from 40 dph), while maintaining alkaline protease activity as the mullet adapt to a high carbohydrate, low protein diet. From 24-38 dph, mullet early juveniles can be weaned off live *Artemia* and onto a dry, more energy dense starter diet. As the weaning period appears to overlap the transition period where the mullet juveniles change their mode of feeding, the question remains if weaning diets should be carnivorous, herbivorous or omnivorous in nature in order to maximize growth and survival.

Consequently, the aim of the present study was to evaluate the efficacy, in terms of growth, survival, weight distribution and digestive tract (DT) enzyme activity, of weaning on to and feeding a carnivorous, herbivorous or omnivorous diet to mullet juvenile fish. These fish have been weaned according to a protocol developed at the IOLR (P.4) in a previous DIVERSIFY study (Deliverable D19.1) and have begun their trophic shift to a more herbivorous/omnivorous mode of feeding.

Materials and methods

Experimental design

Fifteen 17 l aquaria in a flow through system with 40 ‰, UV treated, temperature (24.5°C) controlled seawater were stocked with eighty-five 23 dph larvae aquarium^{-1}. (**Fig. 19.4.1**) This allowed the testing of three weaning dietary treatments, differing in their protein and carbohydrate content, in replicates of 5 aquaria treatment^{-1}. Diet 1 was comprised of only dried *Ulva lactuca* produced at the IOLR in Eilat, Israel (34% plant protein, 56% carbohydrate). Diet 2 was the commercial starter diet "caviar" (Bernaqua, Belgium; 55% animal protein, 8% carbohydrate) where the protein fraction is comprised of krill, fish and squid and represented a strictly carnivorous ration. Diet 3, which was a 1:1 w/w mixture of the plant diet 1 and the animal diet 2 and represented an omnivorous feed. All fish were weaned from the zooplankton diet of rotifer (*Brachionus rotundiformis*) and *Artemia* spp. to the experimental diets over a period from 24-38 dph. The fish from 38 to 53 dph were hand fed to satiation 1-5 times daily only their respective experimental treatments. At the end of the experimental period all fish were counted and individually weighed and samples taken for DT enzyme analysis carried out at P3. IRTA, Spain.

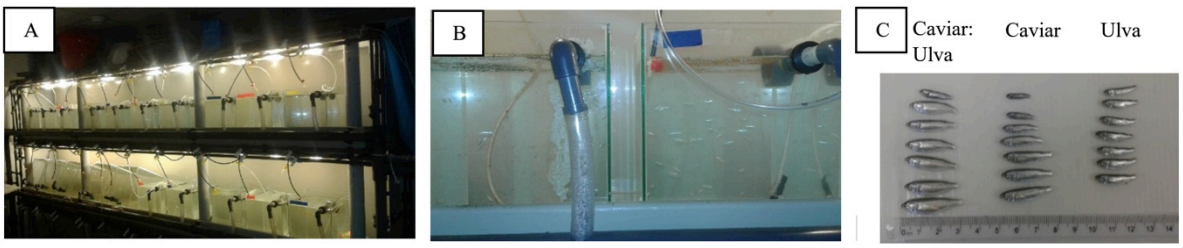


Figure 19.4.1 (A) The experimental aquaria system and (B) fish used in the weaning study. (C) Size variability at the end of the study among the experimental treatments; Ulva, Caviar and Caviar:Ulva.

Results

In **Figure 19.4.2** the average length of fish fed the carnivorous and omnivorous diets at the end of the study were 2.50 and 2.66 cm, respectively, which was significantly (P<0.05) longer than mullet consuming the herbivorous diet (2.22 cm). However, final weight is generally a more sensitive growth indicator than length. The average final weight of the omnivorous feeding fish (**Figure 19.4.2b**) was significantly (P<0.05) higher (203.94 mg) than their carnivorous feeding (163.32 mg) and herbivorous (111.76) cohorts. In addition, the carnivorous feeding fish were markedly (P<0.05) heavier than the herbivorous feeding mullet. Although there was a large size range in each of the treatments (**Figure 19.4.4**), there was no cannibalism and no significant (P>0.05) dietary effect on percent survival. Nevertheless, there was a significant (P<0.05) dietary effect on the pattern of weight distribution at the end of the experiment. In **Figure 19.4.4**, the population of fish fed the herbivorous diet was skewed to smaller fish so that there were significantly (P<0.05) more smaller individuals weighing less than 100 mg , representing an average 47.4% of the population, than the carnivorous and omnivorous feeding fish, representing 23.9 and 14.0%, respectively. In contrast, there were significantly (P>0.05) more 200-300 mg fish fed the carnivorous and omnivorous diets (21.72 and 31.08% of the population, respectively) than those consuming the herbivorous feed (6.02% of the population). In fact, the pattern of increase of larger fish in the population, as a function of diet type, can be described as omnivorous > carnivorous > herbivorous feeding (**Figure 19.4.4**).

In **Figure 19.4.5** the activities of pancreatic enzymes showed a diet modulated response where amylase increased significantly (P<0.05) when dietary carbohydrate (Ulva) was introduced into the omnivorous and herbivorous treatments. Surprisingly, the proteolytic enzymes; alkaline protease and trypsin also increased significantly (P<0.05) as dietary carbohydrate increased. Although lipase showed a non-significant (P>0.05) increase with increased inclusion of dietary carbohydrate, chymotrypsin activity was independent of diet type and composition. On the other hand, the brush border membrane (BBM) enzymes alkaline phosphatase (AP), which is a marker for nutrient absorption and leucine aminopeptidase (AN), an indicator of gut maturation, both increased with more animal protein and less carbohydrate in the diet. In fact, the decreasing (P<0.05) AP/LAP (leucine alanine peptidase-cytosolic enzyme) ratio with increasing Ulva carbohydrate suggests gut maturation decreases with more ulva and carbohydrate in diet. The level of maltase activity, which would provide glucose from maltose (originating from starch breakdown) for transport into the enterocyte, was independent of diet type.

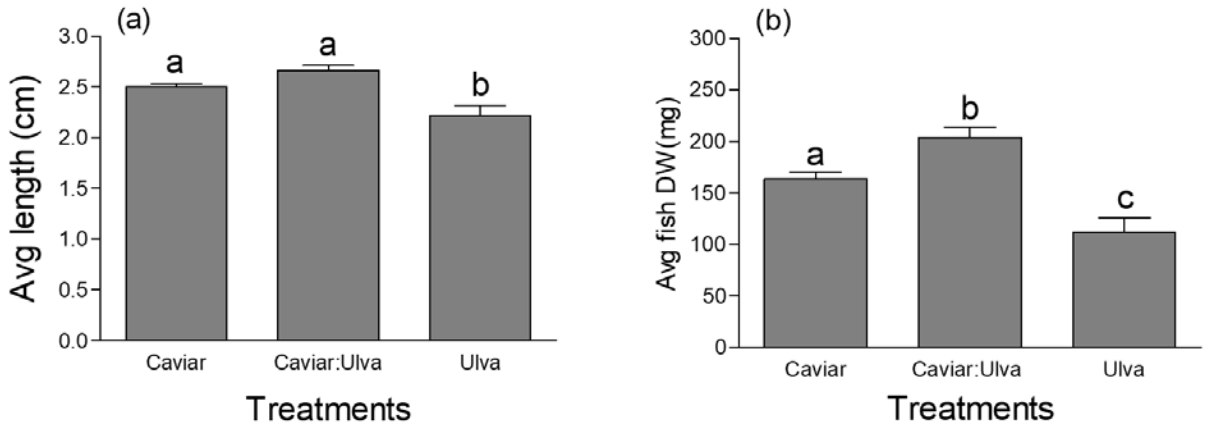


Figure 19.4.2. The effect of the commercial starter diet Caviar, macroalgae Ulva and the 1:1 mix Caviar: Ulva on (a) fish length and (b) dry weight (DW) at the end of the experiment. Length and DW values having different letters were significantly (P<0.05) different.

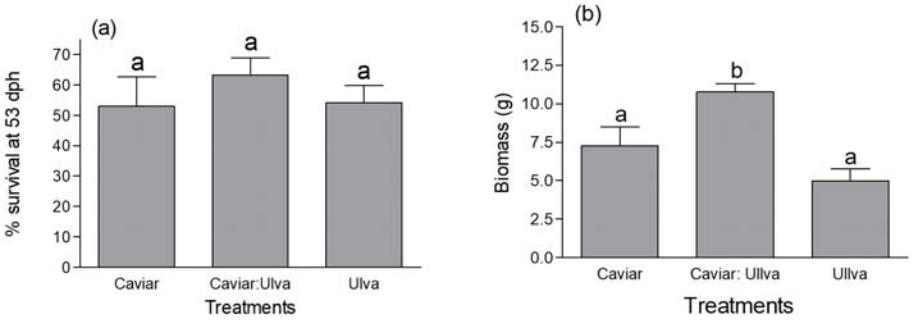


Figure 19.4.3. The effect of the commercial starter diet Caviar, macroalgae Ulva and the 1:1 mix Caviar: Ulva on (a) Percent (%) survival and (b) aquarium biomass at the end of the experiment. Percent and biomass values having different letters were significantly (P<0.05) different.

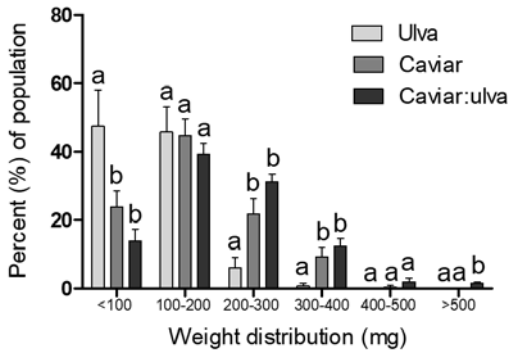


Figure 19.4.4. The effect of the commercial starter diet Caviar, macroalgae Ulva and the 1:1 mix Caviar: Ulva on weight distribution (mg). Percent values having different letters within a weight class were significantly (P<0.05) different. All Percent values were arcsine transformed before analysis.

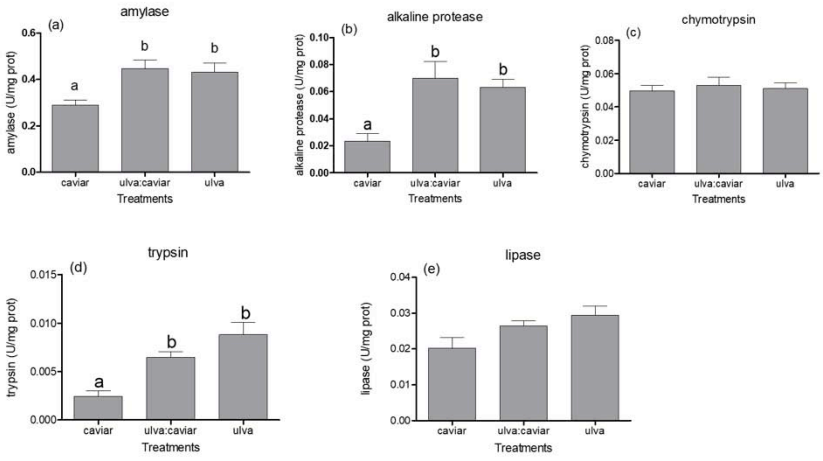


Figure 19.4.5. The effect of carnivorous, omnivorous and herbivorous weaning diet on the pancreatic enzymes (a) amylase, (b) alkaline protease, (c) chymotrypsin, (d) trypsin and (e) lipase. Enzyme values (u/mg protein) having different letters were significantly (P<0.05) different.

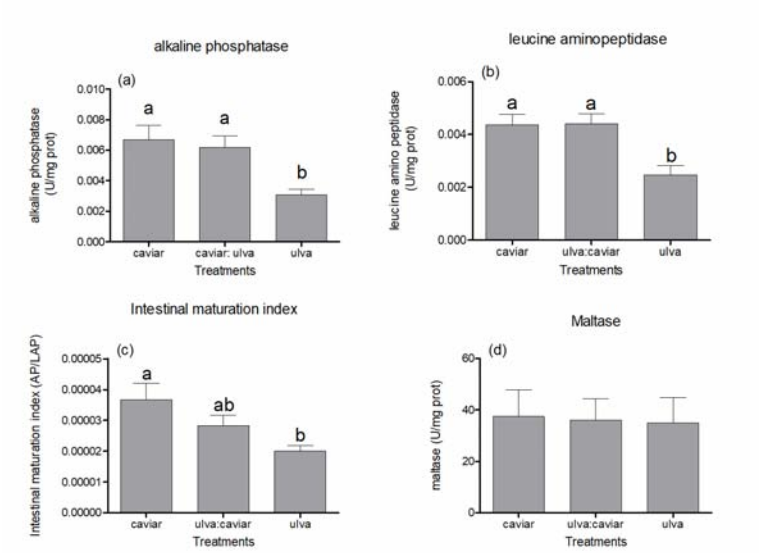


Figure 19.4.6. The effect of carnivorous, omnivorous and herbivorous weaning diet on the brush border and cytosolic enzymes (a) alkaline phosphatase (AP) and (b) leucine aminopeptidase (LAP), respectivel, as well as (c) intestinal maturation index (AP/LAP) and (d) maltase. Enzyme and index values having different letters were significantly (P<0.05) different.

Discussion

The results showed that the fish grew significantly (P<0.05) less, in terms of length and final weight, when fed only an ulva based herbivorous diet (ulva) compared to the carnivorous feed (Caviar), while fish fed the 1:1 omnivorous mix of Ulva and Caviar exhibited markedly (P<0.05) superior growth than all the treatments. A similar result was shown in aquarium biomass, where the omnivorous diet produced the largest fish. Animal protein has generally a more balanced amino acid profile including free taurine compared to plant based proteins and the macroalgae *Ulva lactuca* which can be deficient in methionine and lysine amino acids as well as containing anti-nutritional factors.

A wide weight distribution was demonstrated in fish fed the different treatments (**Figure 19.4.4**). Nevertheless, fish fed the herbivorous diet demonstrated significantly (P<0.05) higher numbers of smaller fish (<100 mg), than the carnivorous and omnivorous diet fish and, in general, exhibited a population skewed to slower growing individuals. Conversely, 200-300 mg carnivorous and omnivorous treatment fish represented a significantly (P<0.05) higher percentage of the population than the herbivorous diet fed fish. Moreover, the weight percentages of the omnivorous fish population appeared to be representative of a normal population distribution. In aquaculture, populations that are skewed to smaller fish would mean a poor food conversion ratio (FCR), a serious limitation as feed represents the largest single expense in commercial operations. However, the present results showed that population weight distribution can be modulated by diet type. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds.

The previous deliverable (19.1) demonstrated that juvenile mullet were genetically programmed to incrementally increase amylase activity from 25 to at least 79 dph. However, the present study showed that increasing dietary carbohydrate significantly augmented this predisposition further. Amylase activity is much higher in herbivorous and omnivorous fish compared to carnivores. Interestingly, the proteolytic capacity to breakdown protein also markedly (P<0.05) increased with increasing dietary carbohydrate content. Enhanced protease capability might be necessary to digest less available plant proteins. On the other hand, increased

amylase activity and starch breakdown would potentially expose more macroalgae protein for protease digestion. Nevertheless, mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch rich microalgae and macroalgae, as well as benthic protein rich organisms characterizing the lower salinity estuarine waters. Furthermore, the high amylase and maltase activity in the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing resulting in improved growth.

The results of the BBM and cytosolic enzyme activity argues that the ulva high carbohydrate diet delayed gut maturation and mucosal absorption, which would also contribute to the sub-optimal growth performance of fish feeding on this diet and the prevalence in the population of smaller fish compared to their omnivorous feeding cohorts. Taken together, the present IOLR (P.4) weaning protocol is most effective when juvenile mullet are weaned onto an omnivorous diet and not an herbivorous or carnivorous diet.

Task 19.5 Testing the improved grey mullet larval rearing protocol in a commercial hatchery (led by DOR, Hagay Sarusi).

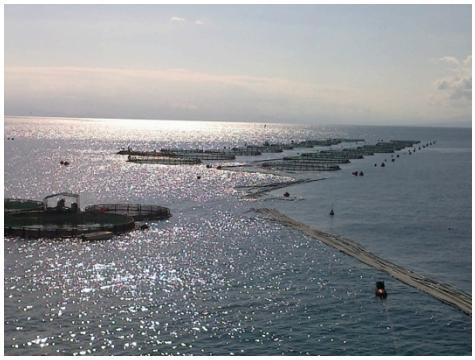
This task has been postponed to 2018.

Deviations from Annex I and their impact:

The objective “Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production” has not yet been addressed due firstly to the closing of the company that produces ciliates. After getting permission from the EU to alternatively test the effect of co-feeding copepods and rotifers, there was a problem producing enough copepods to satisfy the demands of the experiment. It is now planned to run the experiment during the next spawning season in 2018.

Group Work Packages

Grow out husbandry



The work during the reported period was targeted to define optimal grow out conditions and develop appropriate feeding method for meagre. Task 20.1 has been completed and deliverable 20.1 presented. Task 20.2 Effect of rearing environment continues. Sub-task 20.2.1 was completed and reported during reporting period 2. Sub-task 20.2.2 examined the effect of shading and light intensity on grow out in cages. The work was completed on schedule and the data is being processed. A substantial amount of work was completed on Task 20.3 feeding methodology during the reporting period. Sub-task 20.3.1 on feeding stimuli has been completed. Both visual (light) and mechanical (aeration) ques stimulated feeding in small (50-100 gr) and large fish (700-900 gr). Natural sunlight was also observed to affect feeding behaviour. The structure of the visual system was closely connected to the development of different behavioural patterns of meagre. A high proportion of rod cells indicated that meagre are a nocturnal species that prefers low light intensity environments. Sub-task 20.3.2 on different feeding methods was completed. All three feeding systems, self-feeding, hand feeding and automatic feeding gave similar and satisfactory growth in small (50-100 gr) and large fish (700-900 gr). The self-feeding indicated an increased feeding period at lower light intensities. A total of 50% of the stomach content had been transferred to the rest of the digestive channel after 8 hours. Sub-task 20.3.3 on two feed distribution methods in cages was initiated. Similar growth was obtained in cages fed during the day and night. Feeding methods that delivered feed at the surface and at the bottom of the cage were compared and the data is being analyzed. The experimental work for sub-task 20.3.4 to compare automatic and demand feeding in tanks has been completed. During an entire year the growth was similar between fish feed with automatic and demand feeders. Demand feeding fish feed during the entire 24 hour period throughout the year. Together these results indicate that cage feeding can be automated with feed delivery early and late in the day when light intensity if lowest.

In WP21 (greater amberjack), the first trial implemented in Greece resulted in significant mortalities due to parasite infection (*Zeuxapta seriolae*) that forced a change in the objectives of the trial. Hence instead of testing the different rearing volumes (and the cage depth) information on the husbandry practices in cage aquaculture of g. amberjack was gathered. Methods to treat parasites were developed with oxygen peroxide that resulted in survival of more than 65% of the originally introduced individuals. Regarding the growth performance, during the first period of the rearing growth was high (apx 5g d^{-1}). Significant differences in growth were presented between the individuals resulting in size variability of almost 100% a problem that requires further investigation. A second trial in the Canary island has been implemented but results are still under analysis.

Environmental temperature significantly affects the performance of g. amberjack and the study implemented during the reported period was with individuals with 350g mean body weight. Fish held at 21º C showed significantly higher body weight compared with fish held at 26ºC while fish held at 16ºC showed the lowest final body weight. The survival rate was higher at 16ºC but there was no significant difference in the FCR for the whole experimental period (3 months). Plasma Cortisol levels were analogous to temperature and showed a high inter-individual variability, illustrated by high standard deviation values and consequently high coefficients of variation, which ranged from 97.2 ± 41.3% for 21 ºC to 157.3 ± 41.3% for 16 ºC and 119.7 ± 46.1 for 26 ºC. Nutrient digestibility values of amberjack were in line with the observations made in earlier studies. Overall, the digestibility coefficients were high indicating the good quality of the diets. Although temperature is one of many parameters affecting gut transit time it did not affect energy fat, protein and dry matter digestibility of amberjack.

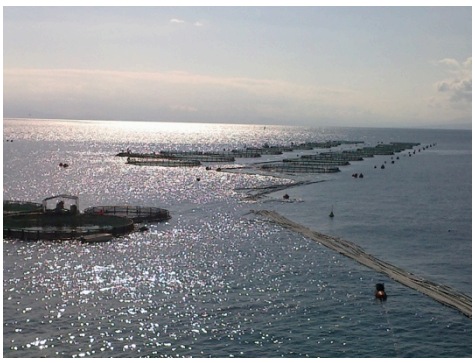
For the digestive characteristics of the species during on growing, results showed, as a conclusion from these preliminary assays, that the optimal range for the digestion of the amberjack is between 22ºC and 26ºC and

the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the enzymatic activity in that range is maximum, while in the intestine the maximum activity range is between 12 and 18h.

Regarding the stocking density, for greater amberjack juveniles, the conditions tested was for values 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium-Low (MLD), Medium-High (MHD) and High (HD) densities, respectively. The results showed that stocking density affects growth rates and feed intake. Fish maintained at High density presented better specific growth rate although not significantly different, while the condition index presented no difference between the groups. Further to this, results showed that stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four month during which the feed intake decreased with the increase of density, although no significantly.

During the 3rd PR in WP 22 (pikeperch), the objectives were to characterize growth, immune and physiological status of pikeperch in order to validate the effects of the best identified rearing variables in farm conditions. The ongrowing experiment from juveniles to marketable size started around April 2017 and will finish around January 2018. Since light characteristics may be an important factor in pikeperch culture (see Task 22.1), it has been decided to maintain fish under the two, defined as, optimal experimental modalities but testing only red vs. white light spectrum, since other factors modalities induced less variability. Several samplings of organs are done along this *in vivo* experiment. Then, the analyses for stress and immune markers will be performed from February to April 2018. A complementary *in vivo* experiment was performed in the laboratory FUNDP facilities in order to further validate and deepen the effects of the light intensity and light spectra on stress status, humoral innate immune response and expression profiles of immune-relevant genes in pikeperch. While light spectrum had little influence on tested variables, the use of a high light intensity was followed by long-term stress and an immune suppression.

The *in vivo* experiment for WP22.3 task which aimed to assess the effects of pikeperch domestication level and geographical origin on growth and stress sensitivity has just started in October 2017 in the URAFPA facilities (Nancy, France) and will last 3 months. Fish will be examined for physiological stress responses and immune competence. This task will allow the further basis knowledge for selection studies of pikeperch strains according to the rearing conditions of commercial fish farms.



WP 20 Grow out husbandry – meagre

WP No:	20	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Grow out husbandry – meagre			
Other beneficiaries (from DOW):	P1. HCMR	P23. ARGO		
Lead Scientist preparing the Report (WP leader):	Neil Duncan			
Other Scientists participating:	Alicia Estévez (P3), Ignasi Gairín (P3), Nikos Papandroulakis (P1), Yannis Papadakis (P1), Tasos Raftopoulos (P23)			

Objectives

1. Adaptations in the existing methodology for grow out in cages related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed,
2. Development of an appropriate feeding method that respects the species' specificities.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The task related to meagre was targeted to adaptations of the existing methodology for grow out in cages. In particular the conditions related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed will be studied. Also the development of an appropriate feeding method that respects the species specificities will be addressed.

Size variability of meagre juveniles: the first trial showed that (a) high cannibalism at the early stages may result in significant size variability of juveniles and (b) different size classes performed similarly following grading. For the definition of the optimum rearing environment during on growing, there is an on-going trial and another one in preparation. The results obtained until now show that depth of the cage net during on-growing affect the behaviour of the fish, but it is not yet clear whether it has any effect on growth. There is also a significant difference of fish behaviour during day and night.

Summary of work reported in the previous Reporting Period (13-30 Mo):

As before, the tasks related to meagre were targeted to the development of appropriate feeding to provide improvements related to the size dispersion that is observed frequently. Regarding size variability of meagre juveniles (Task 20.1) a second trial in agreement with the first trial in the first reporting period showed that (a) high cannibalism at the early stages may result in significant size variability of juveniles and (b) different size classes performed similarly following grading. However, no compensatory growth was observed in smaller grades and the slightly poorer growth indicated that these grades represented a commercial disadvantage. An economic analysis found an extra six months of on-growing with associated costs was required to grow small grades of juveniles to 500 g.

For the definition of the optimum rearing environment during on growing (Task 20.2), trials have been completed to examine the benefits of depth of cages and shading cages. No differences in growth were observed between deep (8 m) and shallow (6 m) cages, or shaded vs unshaded cages. However, mortality and feed conversion ratio (FCR) were lower in deep cages. A range of blood parameters was followed in the deep and shallow cages throughout the year and glucose, lactate and lysozyme were significantly elevated in fish in shallow cages. No differences in mortality were observed between shaded and unshaded cages.

Behaviour profiles of the fish described a significant difference of fish behaviour during day and night with fish being close to the bottom during the day and dispersed throughout the water column at night. There was also evidence of feeding during the night and this will be explored in the feeding methodology (Task 20.3) experiments programmed for the third reporting period.

Finally, a feeding behavior study demonstrated that (a) meagre is able to learn, to be trained and to remember specific stimuli that are associated with feeding time, (b) light is an acute stimulus to which the fish respond very quickly (from the second day of its application) and (c) environmental conditions, particularly light intensity, affect meagre feeding behavior.

Summary of progress towards objectives (31-48 Mo):

The work continued to define optimal grow out conditions and develop appropriate feeding method for meagre. Task 20.1 has been completed and deliverable 20.1 presented. Task 20.2 Effect of rearing environment continues. Sub-task 20.2.1 was completed and reported during reporting period 2. Sub-task 20.2.2 examined the effect of shading and light intensity on grow out in cages. The work was completed on schedule and the data is being processed. A substantial amount of work was completed on Task 20.3 feeding methodology during the reporting period. Sub-task 20.3.1 on feeding stimuli has been completed. Both visual (light) and mechanical (aeration) ques stimulated feeding in small (50-100 gr) and large fish (700-900 gr). Natural sun light was also observed to affect feeding behaviour. The structure of the visual system was closely connected to the development of different behavioural patterns of meagre. A high proportion of rod cells indicated that meagre are a nocturnal species that prefers low light intensity environments. Sub-task 20.3.2 on different feeding methods was completed. All three feeding systems, self-feeding, hand feeding and automatic feeding gave similar and satisfactory growth in small (50-100 gr) and large fish (700-900 gr). The self-feeding indicated an increased feeding period at lower light intensities. A total of 50% of the stomach content had been transferred to the rest of the digestive channel after 8 hours. Sub-task 20.3.3 on two feed distribution methods in cages was initiated. Similar growth was obtained in cages fed during the day and night. Feeding methods that delivered feed at the surface and at the bottom of the cage were compared and the data is being analyzed. The experimental work for sub-task 20.3.4 to compare automatic and demand feeding in tanks has been completed. During an entire year the growth was similar between fish feed with automatic and demand feeders. Demand feeding fish feed during the entire 24 hour period throughout the year. Together these results indicate that cage feeding can be automated with feed delivery early and late in the day when light intensity if lowest.

Details for each Task

Task 20.1 Methodology to avoid size variability in meagre juveniles (led by IRTA, Alicia Estévez and Neil Duncan**)**

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 20.1. Methodology to avoid size variability in meagre juveniles***

Task 20.2 Effect of rearing environment (led by HCMR, Nikos Papandroulakis**)**

The technologies and practices used currently for **meagre** grow out are the same as those used for gilthead sea bream and European sea bass, although this fish presents significant differences in growth rates, feeding and spatial behaviour in the cage. Meagre presents a distinct feeding behavior and has a tendency to stay in the bottom of the cage, feed low in the water column and take time to rise towards the surface to feed. As fish are not very visible to the farmer, feeding may often not be adequate for maximum growth, resulting in large size dispersions.

Objective: **the modification of existing methodologies for cage culture related to volume and light conditions, in order to maximize the performance**

Sub-task 20.2.1 Effect of cage depth (HCMR, Nikos Papandroulakis).

This task was completed and reported during reporting period 2.

Sub-task 20.2.2 Effect of light intensity in the cage (ARGO, Tasos Raftopoulos; HCMR, Nikos Papandroulakis)

The trial is implemented as scheduled. The objective is to test cage rearing with and without shading at the installations of ARGO applying standard commercial procedures for 2 rearing periods. Two cages were used for the 1st rearing period with groups of 11.000 individuals each with an individual weight of 230±75 g. The cages used were rectangular of 10x10x10 m. One of the cages is covered by net of 90-95% shading while the second is covered only with a bird protecting net.

The second period started in October 2016, with the following experimental conditions. The cage size was 10x10x8 (V= 800 m3) and the initial fish groups were 10,940 and 10,200 in each cage. The initial weight was 270 and 240 g respectively. One cage was covered with shading net as in the first trial while both were protected with net against birds. Groups were fed manually. Weigh samples were regular as well as the behavioral monitoring with echo sounders. The trial was completed in July 2017. The results are being analyzed.

Task 20.3 Development of feeding methodology (led by HCMR, Nikos Papandroulakis).

Sub-task 20.3.1 Test of different feeding stimuli (HCMR, Yiannis Papadakis).

A) Description of the work

Test of different feeding stimuli (mechanical, optical etc) were used for the rearing procedure of meagre. The experimental groups were created from two different individual sizes (50-100 and 700-900 g) at different tank sizes (500 and 5000 l respectively) that were used for testing mechanical and optical feeding stimuli. Monitoring with video recordings was implemented, for the definition of the optimal feeding stimuli.

B) Materials and methods

B1) Individual size of 50-100 g (in 500 l tanks)

The experiments that were carried out focused on the effect of different stimuli on the feeding behaviour of meagre. Two repeated experiments (Exp1, Exp2), of 40 days duration each, with an intermediate pause of 1 month, were performed. The stimuli that were applied were light (L), air bubbles (A) and a combination of the two at different times of day. Each stimulus lasted 45 sec. Five seconds before the stimuli were stopped, an automatic electric feeder was activated providing a constant amount of food pellets.

B2) Individual size of 700-900 g (in 5000 l tanks)

The experiments focused on the effect of different stimuli on the feeding behaviour of meagre, in bigger fish (700-900 g), kept in outdoor 5000 l tanks, in duplicate. One experiment with 40 days duration was performed. The stimuli that were applied were light (L), air bubbles (A) and an experimental condition without stimuli (control). Each stimulus lasted 45 sec. Five seconds before the stimuli were stopped, an automatic electric feeder was activated providing a constant amount of food pellets.

B3) Histological analysis of meagre eye

Additional studies were performed which focused on the histological analysis of the meagre eye and especially on the retina. This analysis was considered necessary to be performed for the reason that the eye is the organ that is related with the optical stimuli that were performed in the previous studies. It is known that fish feeding behaviour is modulated by the processing in the brain of set optical, mechanical and

chemical stimuli that are received from the respective sensory organs, such as the eyes, lateral line, olfactory epithelium and gustation system. Vision is considered as the main sensory system in fish, since it is required for orientation, feeding, learning and avoidance of predators. Knowledge about the function of the visual system will offer the necessary information about the behavioural studies that are related with the applications of different optical stimuli, in order to attract the fish population into a specific feeding area.
For the histological analysis, 3 pairs of eyes were removed from three individuals of meager that had mean weight of 200 ± 20 gr. The eyes, before being embedded in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany), were dehydrated in gradually increasing ethanol solutions (70-96%). Serial sections of 3 μm were obtained with a microtome (RM2245, Leica, Germany). Sections from the retina were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA).
Six different areas from the retina of each eye were used for the quantitative determination of the different cell types in the retina. Photographs were taken from histological sections using a digital camera mounted on a microscope at different magnifications. Thereafter, using an image-analysis software (Image J, National Institutes of Health), a number of different 100-μm regions of the retina were examined.
The parameters that were measured were: the number of cones at the photoreceptor layer (PL), the rods that were counted based on the number of nuclei of the rods (RN) at the outer nuclear layer (ONL), the cells at the inner nuclear layer (INL) and the ganglia cells (GC) at the ganglion cell layer (GCL), in 100-μm sections of the retina. Using the number of rods and cones, the number of cells in the INL and ganglia cells were calculated. Also calculated the ratios of rods to cones, rods to ganglia cells, ganglia to INL, rods to INL and cones to ganglia cells.

C) Results

C1) Individual size of 50-100 g (in 500 l tanks)

In Exp 1 the stimulus of light had a direct positive effect on the population since it was shown that during its implementation, fish gathered in the feeding area. During Exp 2 the population also responded positively to the combined stimuli (group 3, **Fig. 20.3.1.1**). Furthermore, the population of Exp 2 in which the air stimulus was applied, responded in this case positively to the stimulus, in contrast to the population of Exp 1.

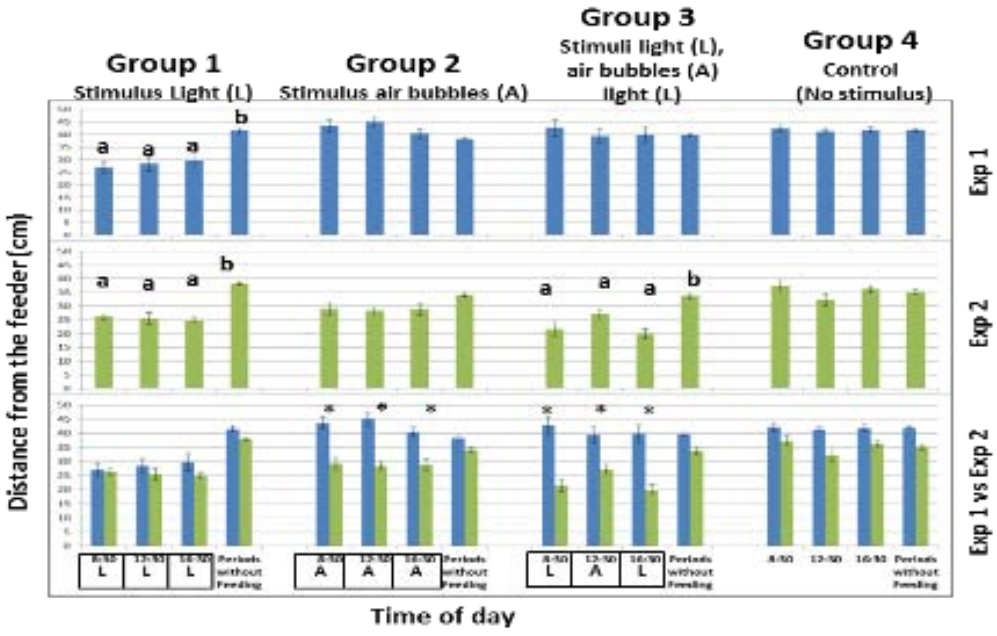


Figure 20.3.1.1. Evaluation of the distance from the feeder during the different periods of the day and different stimuli applications, in the experiments (Exp1 and Exp 2) that were performed (values are mean ± SE). Latin characters (a, b) indicate differences between the different times of day and asterisks (*) indicate differences between experiments (Two Way ANOVA, Duncan test, $P<0.05$).

C2) Individual size of 700-900 g (in 5000 l tanks)

An important result in this experiment was the effect of weather conditions and especially the sunlight intensity on meagre feeding behaviour. The constant daily movement of sun during the day had a large impact on the different parts of the experimental tanks. It created differentiations in the light intensity within the tank environment. There were dusky areas and light areas in the tank. During sunny days the fish in all replicates preferred to stay in the dusky area than in the light area of the tank (**Fig. 20.3.1.2)**. Especially whilst feeding at mid-day (12:30 am), the above phenomenon was very intensive during sunny days.

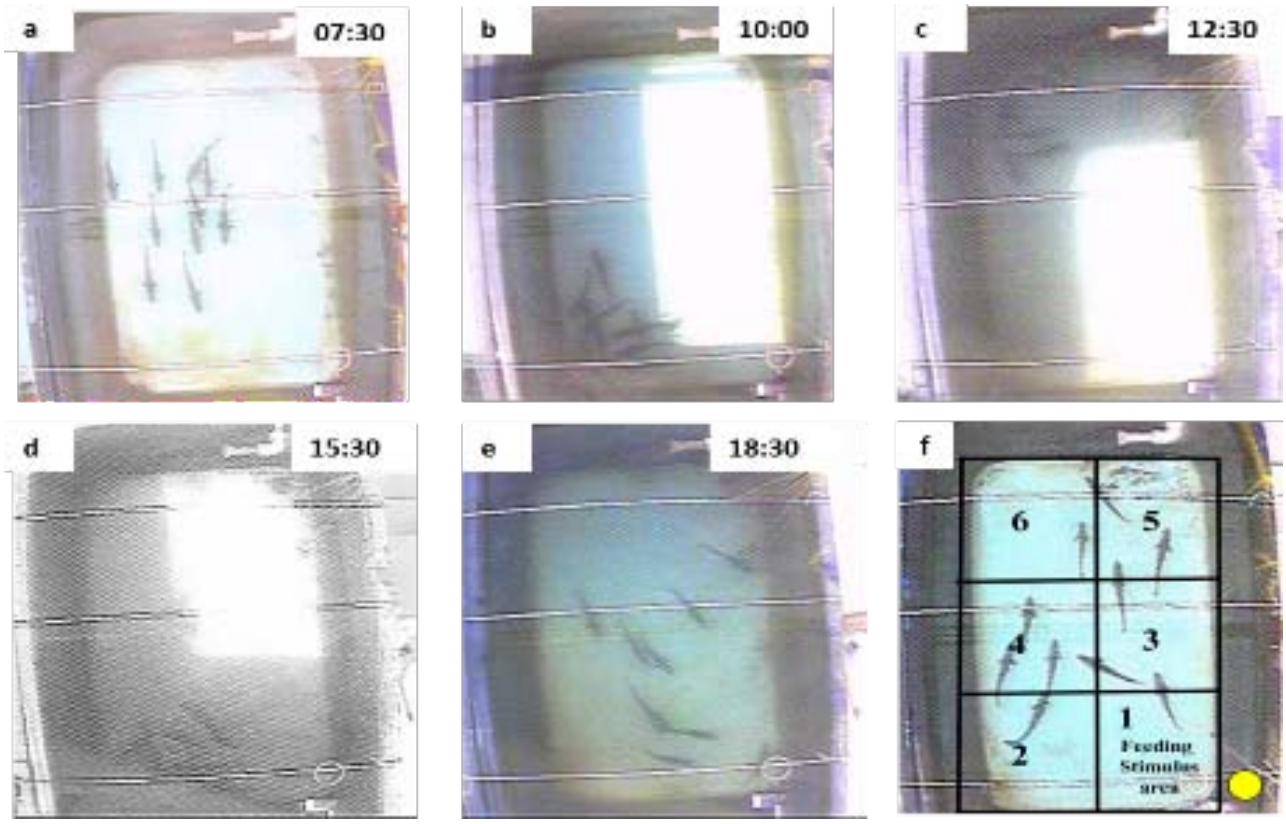


Figure 20.3.1.2. The distribution of the fish in the different areas of the tank during the different periods of the day (a, b, c, d, e). In picture (f) the different areas that the tanks were divided into, are presented. Area 1 included both the stimulus and feeding area, whilst the yellow circle represents the position of the feeder.

The condition with the air stimuli attracted the rearing population (which previous preferred to remain at the dusky area) at the feeding area. This was more intensive than the light stimuli, even though the feeding area was at the light part of the tank. Concerning the effect of the different stimuli that were performed, the stimulus of air bubbles had a direct, positive effect on the population (**Fig. 20.3.1.2)**. This is because it was shown that during its implementation, the fish were attracted to the feeding area during all of the feeding periods of the day.

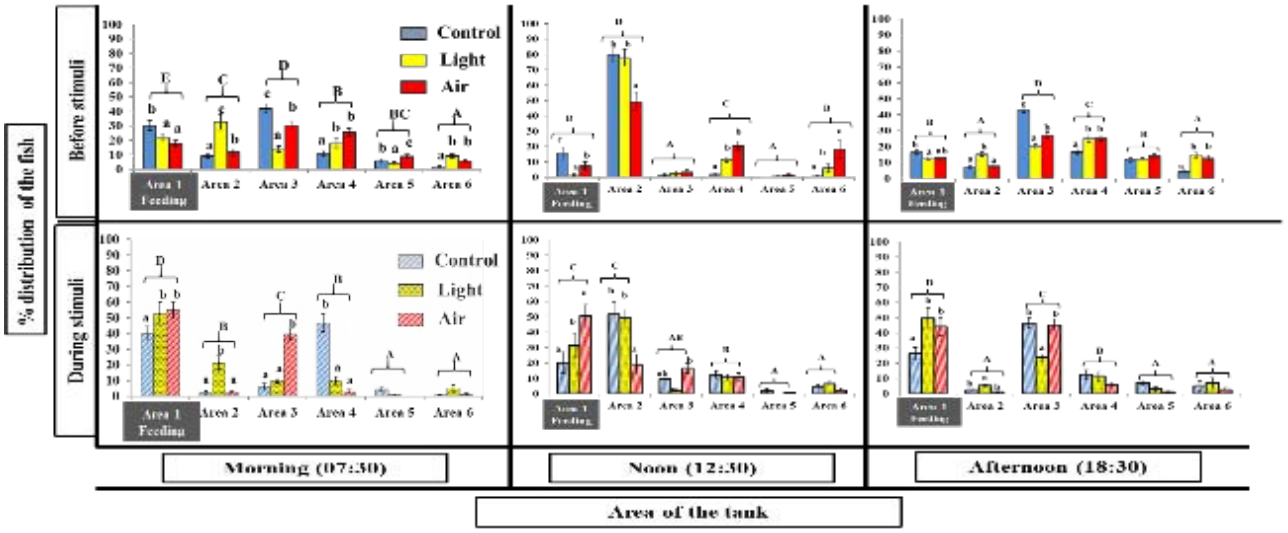


Figure 20.3.1.2. The distribution (%) of the fish in the different areas of the tank during the different periods of the day. The graphs presents the periods with stimuli and without stimuli applications (values are mean ± SE). Latin characters with uppercase letters indicate differences between the different areas and with lowercase, the differences between conditions for each area (Two Way ANOVA, Duncan test, $P<0.05$).

In comparison, the fish populations to which the light stimulus was applied, responded to its application with 5 days delay and they moved to the feeding area only in the morning and in the afternoon feeding times. At noon, the light stimulus overlapped from the high sun light intensity and the stimulus became invisible to the fish.

C3) Eye histology

The relative cell densities in different layers of the retina are considered as a comperative indicator for the classification of a species as nocturnal or diurnal (**Fig. 20.3.1.3a**). The high number of rods that eventually covered the whole surface of the retina in meagre, meant that the cells in the INL were able to capture data from an increasing number of rods. Additionally in meagre, the number of rods, the number of cells in the INL and the ratio of rods, characterized the fish as more nocturnal than diurnal (**Table 20.3.1.1**) Therefore, it is expected that the nocturnal sensitivity of meagre is significantly higher in comparison with other rearing species that means that meagre is more able to identify prey items under low light intensities. For the above reason, the fish selected the most dusky areas during noon. Their motility and the distibution in the tank were also higher when the environmental ligth intensity was low (morning or afternoon).

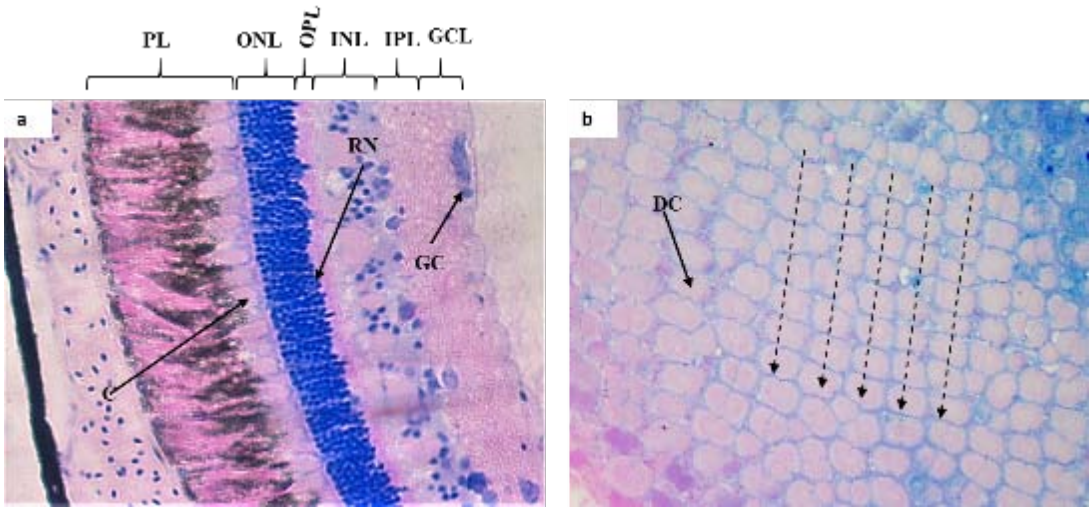


Figure 20.3.1.3. Microphotographs of histological sections of meagre eye 700 ± 50 gr, (a) longitudinal section, (b) transverse section at the surface of the meagre larvae retina. The five dashed arrowhead lines are placed on the common contact surface of the members single cone that constitutes the pair of double cones. GCL = ganglia cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, PL = photoreceptor layer, GC = ganglia cells, RN = rod nucleus, C = cones, DC= double cone.

The arrangement of double and single cones on the retina surface followed a specific pattern, which was clearly visible. Pairs of double cones were placed in series (**Fig. 20.3.1.3b**). The type of cones and the way they are arranged on the retina surface has an impact on the ability to capture images from the environment, which is further associated with the specific behavior of each species. The arrangement of double cones in rows, as was observed in meagre, has been also observed in species that exhibit a schooling behavior. They perceive their prey in a two-dimensional environment along a horizontal axis and exhibit less aggressive predatory behavior. Indeed, meagre is a fish that tends to swim close to the bottom, and in aquaculture they can frequently be found at the bottom of the tank or sea cage. These fish also tend to wait for the feed to drop to their level of swimming rather than very actively swimming to the surface as soon as feeding begins.

Table 20.3.1.1. Values (Means and Standard deviations) for 100μm length of meagre retina for the different structures that retina consist of, as the cones, rods, cells in the inner nuclear layer, ganglia cells and the ratios between them. The size of fish that the measurements were taken from were 200 ± 20 g (n = 6).

	Mean	Standar deviation
Rods	164.03	19.75
Cones	6.25	0.99
INL	35.53	4.97
Ganglia	4.72	1.10
Cones/Rods	0.04	0.00
Ganglia/INL	0.14	0.04
Rods/Ganglia	37.27	14.00
Rods/ INL	4.68	0.73
Cones/Ganglia	1.44	0.67

D) Conclusions: Sub-task 20.3.2

- The results clearly show that meagre was able to learn, to be trained and to remember specific stimuli which are associated with feeding time.
- Light was an acute stimulus in which the fish respond very quickly (from the second day of its application).
- Small fish (50-100 gr) need to be trained in aeration stimuli, but for big fish (700-900 gr) the response was evident from the second day of its application.
- The environmental conditions like light intensity affected the meagre feeding behaviour.
- Both of the stimuli (mechanical and optical) can be used on an industrial scale as they can be manufactured, implemented and managed easily with existing technologies in sea cages.
- The structure of the visual system is closely connected to the development of different behavioural patterns of meagre, that are related to the optical parameters in the rearing environment.

Sub-task 20.3.2 Test of different feeding methods (HCMR, Yiannis Papadakis).

A) Description of the work

The aim of the experiments was to evaluate three different feeding methodologies (Self-feeder, automatic feeding and hand feeding that were performed three times per day) for the rearing of the meagre. The different feeding methodologies were tested in two experimental populations of meagre. The fish used for these experiments were obtained from a brood stock that reproduced in captivity at the Institute of Marine Biology, Biotechnology & Aquaculture Hellenic Center for Marine Research.

A1) <u>First experiment (EXP1). Individual size of 50-100 g (in 500 l tanks)</u>

The first experimental trials were conducted in black indoor tanks of 500-l, connected to an open circuit system with a total water renewal of 400% per hour. The water used was natural sea water (38 psu) pumped from a littoral well. The water temperature was 19°C while oxygen saturation was above 75%. The photoperiod was under natural light conditions corresponding to a geographic width of 35°N from May to August. An additional light coming from fluorescent lamps (450 lx) was used from 08:00 to 18:00. The fish density was 12 fish per tank. Fish were sampled every 4 weeks, and individual measurements of weight (g) and total length (cm) were taken.

A2) <u>Second experiment (EXP2). Individual size of 700-900 g (in 5000 l tanks)</u>

The second experimental trials were conducted in black outdoor tanks of 5000-l, connected to an open circuit system with total water renewal of 400% per hour. The water used was also natural sea water (38 psu) pumped from the same littoral well. Once again, water temperature was 19°C while oxygen saturation was above 75%. The photoperiod was under natural light conditions corresponding to geographic width of 35°N from May to August. The fish density in this case was 11 fish per tank. Fish were sampled every 4 weeks, and individual measurements of weight (g) and total length (cm) were taken.

B) Materials and methods

B1) Feeding methodologies

Three different ways of providing food were studied. Each experimental condition had three replicates. In the first experimental condition, feeding was applied by hand and the fish were fed 3 times a day (8:30, 12:00 and 15:30) ad libitum each time. For the second experimental condition the feeding procedure was performed with automatic feeders that were activated 3 times per day (08:30, 12:00 and 15:30). The released quantity of food in each feeding corresponded to 0.5% of the total biomass in each tank. The third

experimental condition was studied by using self-feeders. The self- feeders consisted of a lever that was immersed in the tank at a depth of 30 centimeters from the surface (total tank depth 60 centimeters). When the lever was moved from its equilibrium position, it immediately sent an electrical signal which activated an automatic feeder and the food (pellets) were released. At the same time of activation of the lever – feeder, wireless signals were sent to a central logger which recorded the number of activations across the 24-hour period, throughout the experiment.

B2) Calculations

For the evaluation of fish growth and feed performance, these indices were used: specific growth rate (SGR), feed conversion ratio (FCR), daily feed consumption (DFC) and condition factor (CF).

- Specific growth rate = $100 \times (\ln W_f - \ln W_I)/T$
- Feed conversion ratio = $W_{TFS}/(W_f - W_I)$
- Daily feed consumption = $W_{TFS} \times 100/[((B_I + B_f)/2) \times T]$
- Condition factor = $W_b/L_b^3 \times 100$

Where W_b is the body weight and W_I, W_f are the initial body weight and final body weight (g) respectively and L_b is the total body length (cm). B_I and B_f are the initial biomass and final biomass (g) respectively, while W_{TFS} is the weight of total dry feed supplied (g) and T is the duration of the experiment (days).

B3) Feeding activity

For the purpose of studying the feeding activity of the fish in the tanks, were used the data from the self-feeder activations that were collected wirelessly in a special recording system. The data were extracted at three-hour intervals and processed in Microsoft Excel.

B4) Digestive evacuation rates

For the study of the evacuation rate of the digestive canal, an additional experiment was performed with fish whose weight ranged between 160 ± 20 g, at 19 °C. Before the experiment the fish had been acclimated for 1 month at a steady temperature of 19 °C. Initially the fish were anesthetized and after that a force-feeding was performed. The fish were weighed and then a number of pellets were introduced with a special piston directly in to the stomach of each fish. The total weight of the pellets corresponded to 0.5% of the body weight of each fish.

C) Results

C1) Feeding activity

As concerning the data that were analyzed from the self-feeder recorder, the feeding activity of the fish in both experiments was significantly statistically lower during the morning hours and until noon, rather than during the rest of the day. More specifically in both experiments (EXP1 and EXP2) between 06:00 and 12:00 the fish had lower a feeding activity, while this activity had increased during the rest of each day's time period. This phenomenon was more intense in outdoor tanks, where they were exposed to direct sunlight and the intensity of light was much higher.

C2) Growth and feeding performance.

EXP1

All of the feeding methodologies used for meagre rearing during EXP1 provided satisfactory growth results. Moreover, the DFC was higher (P<0.05) when using the hand feeding method in comparison with the other methods. However, these differences did not significantly reflect on the FCR values between the different feeding methodologies that were used (**Table 20.3.2.1**).

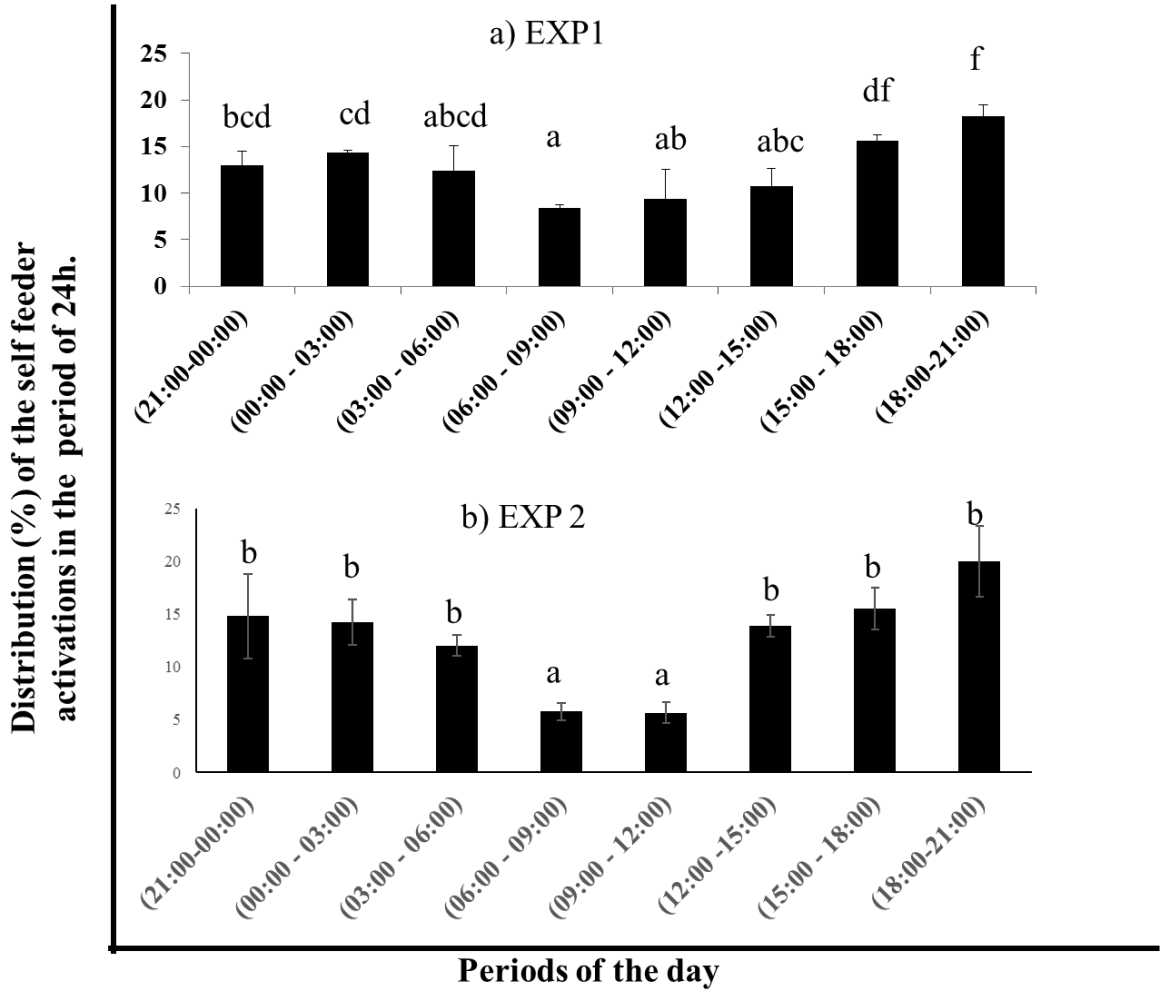


Figure 20.3.2.1. The percentage of distribution of the self-feeder activation in different periods of the day during the 24h period for the EXP1 and EXP2. The columns that share different Latin characters indicate the appearance of statistical differences between the different time periods (Two Way ANOVA, Student – Newman – Keuls Method, P<0.05).

EXP2

Experimental populations that were fed by hand and automatic feeders showed the highest SGR in comparison with the self-feeder methodology (P<0.05). Similar, statistically significant differences also appeared in the DFC index, where lower feeding rates were presented in the group that used the self-feeder methodology (P<0.05). Although there were no important statistically significant differences between the feeding methodologies for FCR, higher values were obtained at the methodology with the automatic feeder (**Table 20.3.2.1**).

Table 20.3.2.1. Evolution of productive indexes during the EXP1. Latin characters indicate differences between the different feeding methods (One Way ANOVA, Duncan test, P<0.05).

	Self feeder	Hand feeding	Automatic feeder
Weight (initial)	64.71 ± 1.96	62.23 ± 2.90	64.58 ± 0,79
Weight (final)	132.48 ± 11.60	133.59 ± 5.22	138.24 ± 1.63
SGR	0.80 ± 0,08	0.86 ± 0,02	0.86 ± 0,01
FCR	1.07 ± 0.11	1.25 ± 0.10	1.15 ± 0.03
DFC	0.73 ± 0.07^{a}	0.92 ± 0.07^{b}	0.84 ± 0.01^{a}
CF (initial)	0.99 ± 0.04	1.02 ± 0.02	0.96 ± 0.01
CF (final)	0.92 ± 0.04	0.93 ± 0.02	0.94 ± 0.02

Table 20.3.2.2. Evolution of productive indexes during the EXP2. Latin characters indicate differences between the different feeding methods (One Way ANOVA, Duncan test, P<0.05).

	Self feeder	Hand feeding	Automatic feeder
Weight (initial)	739.88 ± 23.38	775.95 ± 57.39	673.04 ± 79.35
Weight (final)	927.72 ± 4.50	1090.00 ± 95.14	905.90 ± 112.49
SGR	0.32 ± 0.05^{a}	$0.49 \pm 0,02^{b}$	0.42 ± 0.01^{ab}
FCR	1.27 ± 0.27	1.42 ± 0.23	1.83 ± 0.19
DFC	0.40 ± 0.02^{a}	0.68 ± 0.09^{b}	0.77 ± 0.06^{b}
CF (initial)	0.95 ± 0.00	0.94 ± 0.00	0.93 ± 0.04
CF (final)	1.10 ± 0.02	1.06 ± 0.07	1.02 ± 0.00

C3) Evacuation rates of the digestive canal

According to the results of the evacuation rates of the digestive canal, in order the stomach to become empty at 19 °C, 24 hours from the initial food intake was necessary (**Fig. 20.3.2.2)**. Also, after the first 8 hours, the stomach lost 50% of the initial amount of food that was inserted in it. After that point, the evacuation rates of the stomach were relatively slower. Contrary to this, the intestine reaches its maximum filling 12 hours from the first feeding, but within 24 hours negligible food residues were identified in the intestine.

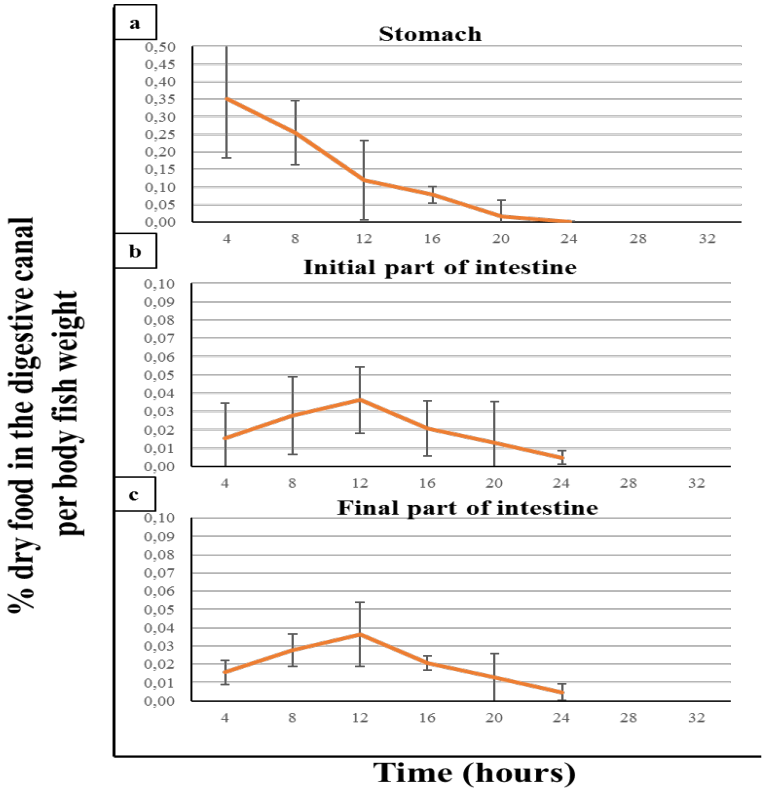


Figure 20.3.2.2. Evacuation rates of a) stomach, b) initial part of intestine and c) final part of intestine.

D) Conclusions: Sub-task 20.3.2

- The ability of meagre to use the mechanical self-feeder with a lever, offers useful information related to its feeding activity.
- The feeding activity of the meagre was higher during periods of the day where low light intensity occurred.
- The rearing in indoor tanks of meagre (50 -100g), all three of the methodologies that were applied offered satisfactory results as concerning the growth performance.
- Scheduled automatic feeding and feeding by hand can provide satisfactory results for meagre growth in larger individuals (700-900 g), in outdoor tanks.
- At 19 °C, 50% of the stomach content had been transferred to the rest of the digestive channel in the meagre after 8 hours. This fact is connected with the feeding activity of meagre and especially with the fact that there is an interval of time, where the fish reduce the feeding activity due to its digestion procedure.

E) Conclusions Sub-task 20.3.1 and Sub-task 20.3.2

The above results from Sub-task 2.3.1 and Sub-task 2.3.2, clearly indicate that the development of a feeding methodology should consider the knowledge of the various functions associated with feeding behavior of any species and that related to the digestive and visual systems. These two systems are involved in the detection of food and in the utilization of food elements, that are considered necessary for the designing of a feeding methodology for any species. The feeding method for meagre can be based on the use of various stimuli (light or air bubbles) in order to attract the rearing population to the specific fish feeding area. The stimuli may be either light under low light intensities or bubbles under high light intensities. The development of a combinational, automatically programmed feeding system using various types of stimuli (depending on the environmental light intensity) on the growing population, can be used for the creation of

the most appropriate feeding methodology. The shadowing of the sea cages is proposed as an additional process that will increase the effectiveness of an automated feeding system for meagre.

Sub-task 20.3.3 Test in cages of 2 feed distribution methods (HCMR, Nikos Papandroulakis).

As planned, instead of performing the trial with the different feed distribution methods with two size classes, this test will be performed only with one size class. An additional trial will be implemented to test in detail the night feeding of meagre in cages.

Test of daily vs night feeding in the performance of meagre

In June 2016 a trial was organised to test whether meagre rearing could be implemented with night feeding only and the potential differences with the daily feeding. Cages of 290 (6x6x8) m^3 at the HCMR pilot farm in duplicates were used, indicated as Daily and Night.

Fish origin was the hatchery of HCMR. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery of the institute. Juveniles of 2 gr were transferred at the cage facility and they were reared under similar conditions until the beginning g of the trial. Four groups were created, of ~1,820 each cages. The wet weight at the beginning of the trial was 500 ±20 g. The duration of the trial was planned to be 8 months and was completed on March 2017.

During the experimental period, growth performance was estimated with monthly samples. Every month blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation.

Blood sampling

Fish were netted (10 fish per group), anaesthetized (Phenoxy-ethanol), total length and body weight were measured and blood was drawn from the caudal vessel, using a sterile syringe, and placed in tubes containing heparin. After the determination of hematocrite and hemoglobin, blood was centrifuged (2000× *g*, 4◦C for 10 minutes) and plasma aliquots were stored at −20◦C for further analysis of cortisol, glucose and lactate.

Behavioral monitoring

The vertical distribution of the populations in cages has been monitored using an echo integrator. The system used is the CageEye 1.3, (Lindem Data Acquisition AS, Norway).

Results

Biological performance

Although analysis is not completed we present here some preliminary results. In **Figure 20.3.3.1** the growth performance is presented.

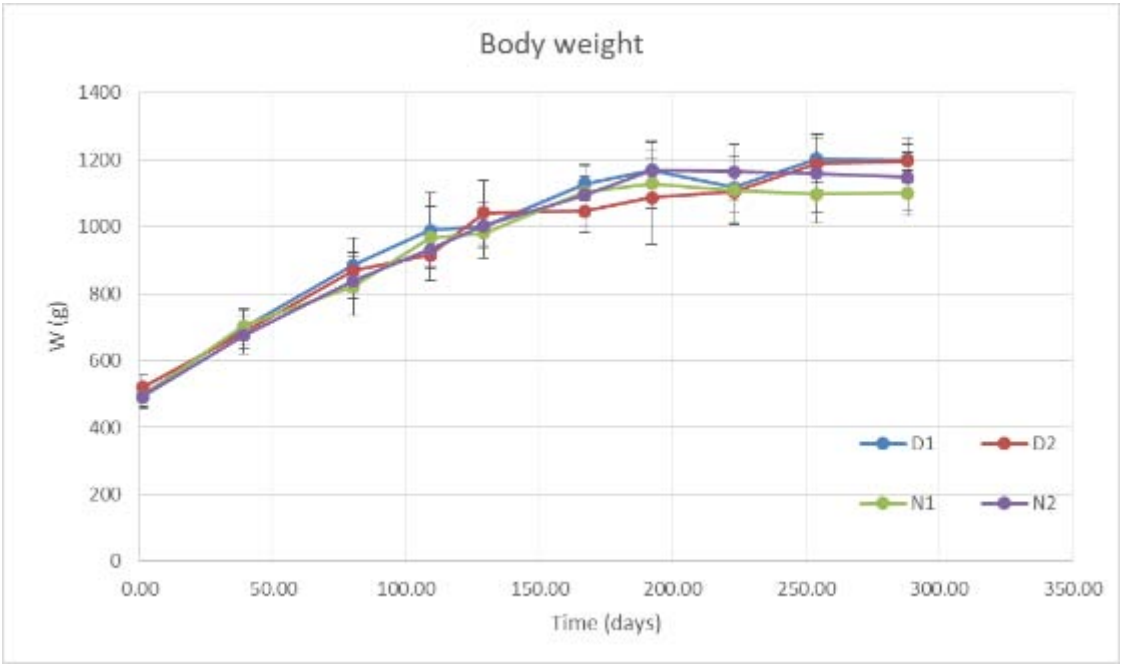


Figure 20.3.3.1. Growth performance, mean weight, of meagre. Cages fed during the day are D1 and D2, and cages fed during the night are N1 and N2. Error bars are the standard deviation (n=10).

During the experimental period, the growth rate was ~2.5 g d^{-1} without significant differences between the experimental groups.

Table 20.3.3.1. Performance indicators during the two experimental phases. Cages fed during the day are D1 and D2, and cages fed during the night are N1 and N2.

	D1	D2	N1	N2
Mortality (%)	4.8	3.5	5.4	2.6
FCR	2,6	2,6	3,0	2,7

Regarding other performance indicators, in **Table 20.3.3.1.** the mortality (as %) and the food conversion ratio are presented. No significant differences were presented.

The remaining of the collected data are still under analysis and will be presented at a later stage.

Test of two feed delivering methods in the performance of meagre

In May 2017 a trial was organised to test two different feeding methods and their effect on the performance of the reared groups.

The standard feeding with feeders located on the surface of the cage were compared with submerged feed distributed. For the second feeding was performed by transferring feed together with seawater through a flexible tube from the surface. An electric pump located on the platform pumped water into the cage while an electric dosing mechanism delivered the required feed quantity (**Fig. 20.3.3.2**).

Figure 20.3.3.2. Feeder installed during the trial for the submerged feeding

The feeding pipe was installed at the centre of the cage. A rotating S-form ending ensure an even distribution of the pellets (**Fig 20.3.3.3**).

Figure 20.3.3.3. The S-form ending of the submerged feeding system

Cages of 290 (6x6x8) m^3 at the HCMR pilot farm in duplicates were used, indicated as Normal and Submerged. Fish origin was the hatchery of HCMR. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery of the institute. Juveniles of 2 gr were transferred at the cage facility and they were reared under similar conditions until the beginning g of the trial.

Four groups were created, of ~2,720 individuals for each cages with a mean wet weight of 290 ±20 g. The duration of the trial was planned to be 8 months and will be completed on November 2017.

During the experimental period, growth performance was estimated with monthly samples. Every month blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. The data is being analyzed.

Sub-task 20.3.4 Comparison of automatic and demand type feeding in tanks. (IRTA, Neil Duncan and Alicia Estevez)

This sub-task has been completed as described in the amended DOW.

There was a delay of three months to start the task. During the three-month period the fish grew from 10 g to 50 g. The delay was due to time taken to modify and develop a demand feeding mechanism that worked with meagre. Various mechanisms were tested and the mechanism that worked was a pendulum that was positioned exactly at the water's surface. When the pendulum entered deeper into the water column, the meagre over demanded and feed was wasted.

The experiment started on the 4 October 2017. Prior to initiating the experiment 1200 juvenile meagre were "trained" to use the pendulum demand feeding system. The fish were randomly distributed into six 1500 L tanks, 200 fish per tank. The tanks were connected in pairs to a recirculation system (IRTAmar) (**Picture 20.3.4.1**). As the fish grow, biomass was removed from the tanks by reducing the number of fish per tank. On the 10 January 2017 the number of fish per tank was reduced to 150 and on 7 June 2017 the number of fish per tank was reduced to 75. All tanks were fitted with a net to avoid fish jumping from the tank. The photoperiod was natural and the temperature regimen simulated the temperature of the seawater on the Mediterranean coast of Spain where meagre are grown out in sea cages. Three tanks were set up with the demand feeder. The feeder delivered 5 g of feed when the pendulum was triggered, a demand. The food fell from the feeder within 0-5 seconds after the demand when the pendulum was triggered. The time of each feed demand was registered. Each day the feeder was filled with a pre-weighed excess of feed. Before filling the feeder, the previous days uneaten feed was removed and weighted to determine the exact amount of feed that was demanded. Three tanks were set up with programed feeders. The programmed feeders delivered 3 feeding periods (09:00, 13:00; 17:00) of 1 hour when the fish had a mean of 50-100 g and two feeding periods (09:00, 17:00) of 1 hour when the fish had a mean of 100-400 g. This follows cages farming feeding practices in Spain. The programed amount of feed delivered followed manufactures feeding tables. The pellet size for both programmed and demand feeders was adjusted to fish size following manufactures recommendation.

Picture 20.3.4.1. Experimental set up with six tanks connected in pairs to a recirculating aquaculture system (RAS, IRTAmar). Each pair of tanks was a programmed feeding regimen (Prog) and a demand feeding system (AD).

Each tank had two movement sensors that registered when a fish passed within ~35 cm in front of the sensor. One sensor was positioned 20 cm below the water´s surface and a second 80 cm below the water´s surface and 20 cm from the bottom of the tank. Video recording was made during 1 week in February 2017 with red night illumination to observed demand feeding during the night period. Videos were recorded of the fish behaviour at 3 time points during the trial. Every month a sample of 60 fish from each tank were weighed, length measured and fin condition scored. A scale of fin condition was used where 1 = perfect fins with no damage, 2 = light damage, 3 = excessive damage and 4 = no fin. The trail finished on 6 September 2017. Two weeks after the end of the trail the effect of night illumination of different intensities on circulating melatonin was measured using the experimental animals.

Results

The data is being analysed. The fish in all replicas of the two treatments grow from a mean of 58.3±12.4g on the 4 October 2016 to 331.3±112.6g on the 6 September 2017 (**Fig. 20.3.4.1**). There were no significant differences in mean weight amongst replicas at the beginning of the experiment (4 October 2017) or at the end (6 September 2017). During the experiment, the meagre demanded feed over the entire 24-hour period as shown in the first three months of the experiment (**Fig. 20.3.4.2**). There were no differences in size variation or fin condition. Close to all fish exhibited slight fin damage during the entire experiment. The data, videos and blood samples for the entire experiment are being analysed.

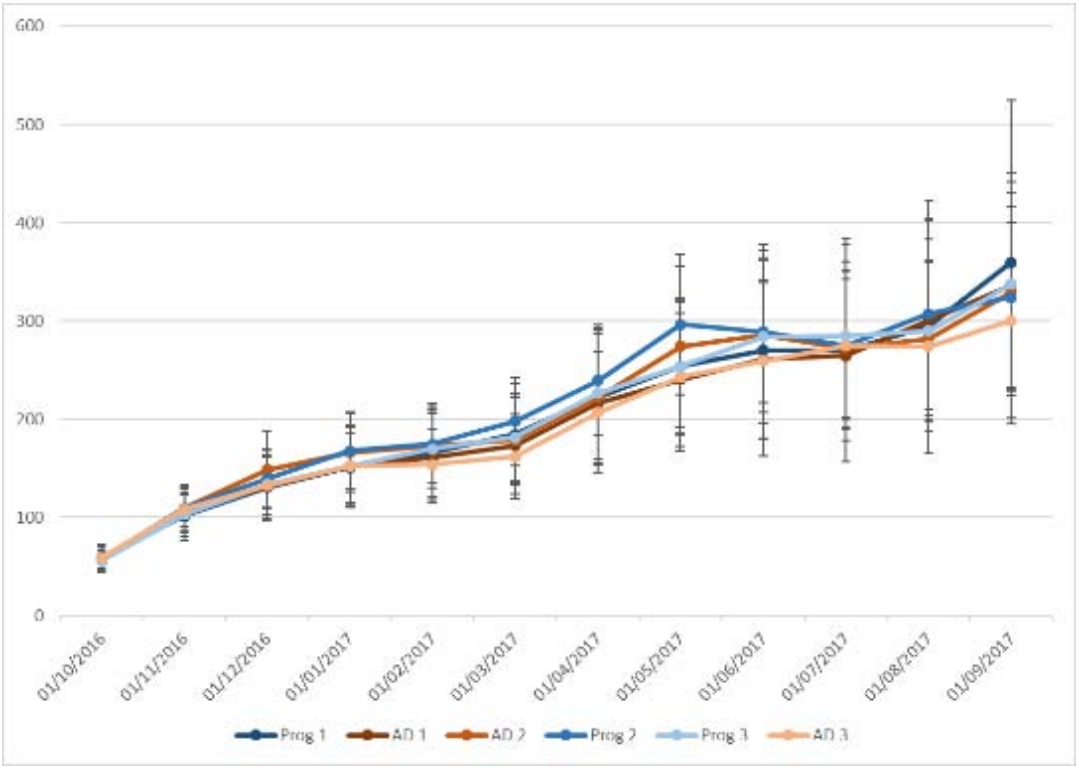


Figure 20.3.4.1. Mean growth (g) of six groups of meagre (*Argyrsomus regius*). Three were feed with a demand feeding system AD1, AD2 and AD3. Three groups were feed with a programmed feeding regimen, Prog 1, Prog 2, Prog 3, following feed manufactures feeding tables. The y axis is wet weight (g) and x axis is date.

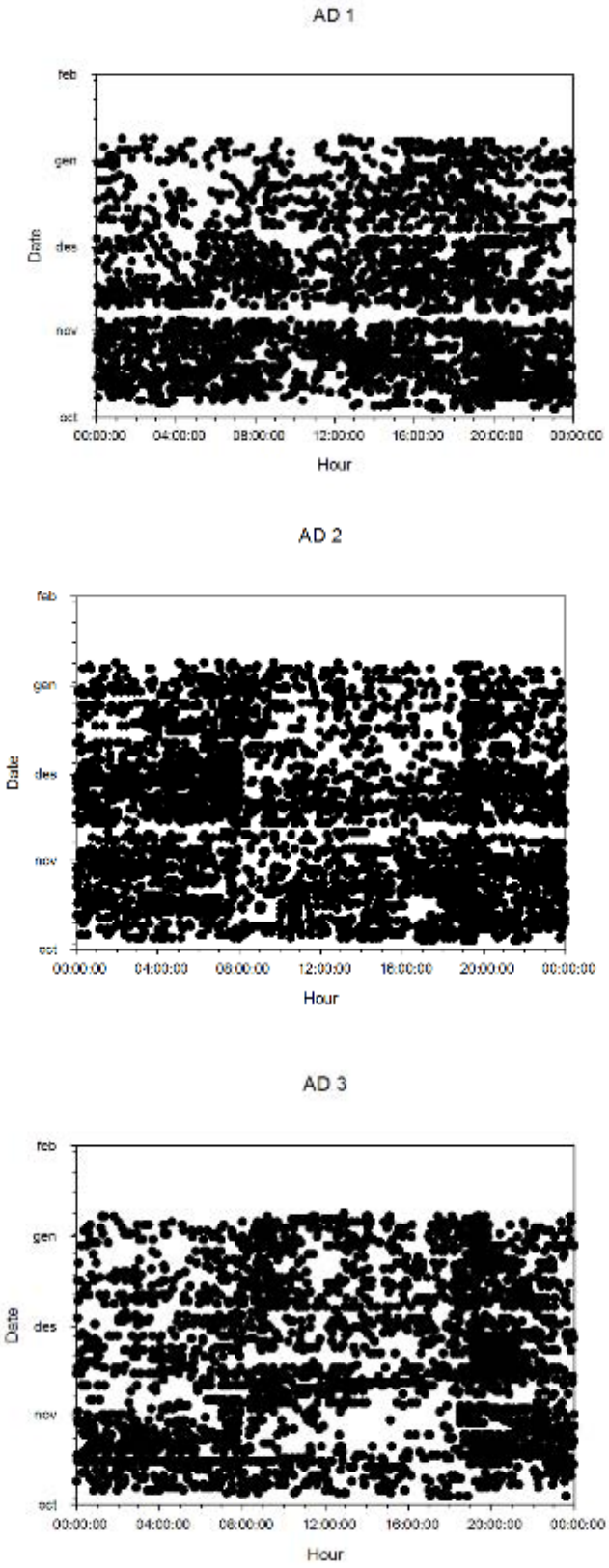


Figure 20.3.4.2 Time that meagre (*Argyrosomus regius*) demanded feed during the day (x axis) during three months October, November and December (y axis).

Sub-task 20.3.5 Development of feeding system for industrial application (HCMR, Nikos Papandroulakis).

This task has not started yet.

Deviations from Annex I and their impact:

There is no deviation from the DOW after the Amendment 2.

WP 21 Grow out husbandry – greater amberjack

WP No:	21	**WP Lead beneficiary:**		P1. HCMR
WP Title (from DOW):	Grow out husbandry – greater amberjack			
Other beneficiaries (from DOW):	P2. FCPCT	P8. IEO	P15. ULL	P27. FORKYS
P28. CANEXMAR				
Lead Scientist preparing the Report (WP leader):	Nikos Papandroulakis			
Other Scientists participating:	Lidia Robaina(P2), Salvador Jerez, Virginia Martín, Marta Arizcun, Elena Chaves, Veracruz Rubio Eduardo Almansa (P8), José Pérez (P15), Ioannis Diakogeorgakis (P27)			

Objectives

1. Development of appropriate rearing methods for cages including rearing volume and type of cage,

2. Development of feeding methods for fry and juveniles by identifying daily rhythms and feeding frequency.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The WP (21) for the greater amberjack is targeted on the study of the husbandry and environmental requirements during on-growing. In particular the rearing methodologies of the greater amberjack will be studied with emphasis on (a) the cage technology (depth and type), (b) the feeding method and (c) the husbandry practice (temperature, stocking density). During the first reporting period preparatory activities took place for the various trials.

During the second period four experiments were implemented for (1) the definition of feeding pattern for 5 g, and (2) for 200 g individuals, (3) the determination of minimum-maximum temperature ranges for juveniles fish and, (4) Definition of optimal stocking density for juveniles of 5g. The main results achieved so far can be summarized as follows. For the feeding pattern of juveniles, one meal daily resulted in lower growth and higher FCR compared to those fed 3 or 4 meals per day. For bigger individuals (200g), the better results in growth and feed conversion rates have been obtained with 7 meals daily. The greater amberjack juveniles are able to adapt to the different feeding frequencies which however could influence the health status of fish.

Environmental temperature significantly affects the performance of g. amberjack juveniles. Fish held at 26º C showed significantly higher body weight compared with fish held at 22ºC while fish held at 17ºC showed the lowest final body weight. The analysis showed that the increase of temperature led to elongated shape of fish body, especially of the head, differencing clearly the specimens reared between 17ºC and 26ºC.

Regarding the stocking density, for juveniles, the results showed that stocking density affects growth rates and feed intake. Fish maintained at High density presented lower specific growth rate and condition index than the other groups. Further to this, feed intake along overall period was significantly lower in fish at high densities.

Details for each task:

Task 21.1 Development of rearing method in cages (led by FCPCT, Lidia Robaina)

In order to develop appropriate rearing methods for the cage rearing of the greater amberjack two trials were performed during the reported period as described below.

Action 21.1.1 Effect of rearing volume (depth) on performance.

Effect of rearing volume (depth) on performance. A first trial was implemented in the commercial cages of FORKYS for a period of 12 months starting from September 2016 until the July 2017. Growth performance was estimated every second month.

A group of 29.300 individuals of 0.5 g mean weight were delivered to the pregrowing facility of FORKYS on July 22 2016. Of them 12.000 were transferred to cages on August 16 at a mean weight of 10g.

Groups were transferred in two cages with net of different depth. The cage with the deep net accommodated 6.500 individuals while the cage with the shallow net 5.500.

During the first month in the cages the groups exhibited a high growth rate reaching on October 4 a mean weight of 218 ± 56 g and 205 ± 65g for the deep and shallow cage respectively. Furthermore, significant mortality accounting of 25% and 34% at the shallow and deep cage respectively was also observed without any particular pathology.

Apart from the high mortality it was also observed high variability within groups.

In order to continue the trial applying appropriate husbandry practises, the fish were selected and re-organised in two groups again with the following characteristics:

A group of 6,130 individuals with a mean weight of 460±20g and a second group of 3,500 individuals of mean weight 263±19g.

Following the above, the objectives of the trial were modified and were mostly concentrated in gathering all possible information that could help in the definition of appropriate husbandry practices in cages.

In January 2017, mortality was again observed that for the group with the larger individuals were apx 25% while it was significant lower (apx 6.5%) for the group with the smaller fish.

The mortality was associated with the presence of gill warms. The incident was confronted with repeated baths with hydrogen peroxide that efficiently eliminated the warms.

Efficient methodology was developed for the application of the peroxide bath that was repeated when required i.e. in case warms were observed during sampling.

The rearing continued until June 17 when 4.900 individuals with a mean weight of 914±150g and 2.900 with mean weight of 631±120g were remaining in the cages.

During the rearing additional evaluation of the performance was done with haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid), and hormonal (cortisol) analysis.

This stock remained in the facility of the farm and is the first to be commercialized. The first group of fish was in the market in November 2017. The results related both to the quality and the growth of the individuals are as follows: A small number of individuals (5%) were deformed, 78% were at the range between 2.0 and 3.0 Kg, 15% were between 3.0 and 3.5 Kg and 2% were more than 3.5 Kg in body weight.

For the implementation of Task 21.1.1 for the definition of rearing parameters in cages for the greater amberjack it was planned a second trial to start in October 2017. However FORKYS decided that it is not possible for them to proceed due to administrative issues in their cage farm.

A contingency plan for this includes the transfer of this partner's activities to another partner that has the required infrastructure and has shown also the will to contribute in the DIVERSIFY project. Task 21.1.1 has been allocated to partner ARGO, which has the required facilities. The second trial, in ARGO fish farm was organized and started in September 2017 again with apx 28.000 individuals.

The trial is under implementation.

Action 21.1.2 Effect of cage type on performance.

After amberjack spawning and the subsequent rearing from larvae to juveniles at the GIA laboratories (FCPCT), fish from 52.92 ± 23.86 g were transported to the Taliarte harbour for their reception by Canexmar company and stocking in their experimental cages, according to the previously developed protocol.

Fish transport scheme from lab to the sea: A track prepared with 500L fish transport boxes, each with cover, was used. Fish density during transport was around 20-22 kg/m^3 and oxygen level maintained close to 6.5 mg l^{-1} using an oxygen bottle.

Timing for the transport: initial fish picking from the tanks at the FCPCT (11:00 am) and arriving to the port (13:30).

Fish boxes were received by Canexmar and immediately transported with their boat to the experimental sea cages.

Once fish stocked, a protocol for the parameters to be daily controlled, and a scheme for the subsequent sampling along the year was accorded between CANEXMAR and the FCPCT trials responsible (every 3-4 moth sampling was agreed) (see tables below).

The feeding method, feed company; feed type; feed diameter according to fish weight; feeding time / h /days a week, all of them are being shown in the correspondent daily control tables by the company. Same for the husbandry practices: temperature, stocking density, net changes, medicals or so.

An example of the daily-recorded data for fish rearing in the cages is shown below (**Table 21.1.1.)**

Fish were sampled every 2-3 month depending on the sea conditions with the subsequent sampling schedule according to the DOW objectives: 1) Weight 3 batches of fish at the cages and determine medium weight and size; 2) Take 15-20 fish to the FPCT-GIA laboratories- for the individual fish sampling; 3) Weight; length; growth; observations & photos; 4) Parasites observations; 5) Eviscerate & fish biometric parameters; 6) SGR; TGR; FCR; 7) Different tissues samples for health status analysis.

Table 21.1.1. Daily-recorded data for fish rearing in cages.

Day	Dead fish	ANTI-birds	CAGES appearence	FOULING RED %	TEMP °C	SEA overview	WATER CURRENT
11/01/17		ok	ok		19	3	2
12/01/17		ok	ok		19	3	2
13/01/17	2	ok	ok		19	3	2
14/01/17		ok	ok		18	3	2
15/01/17		ok	ok		18	3	2
16/01/17		ok	ok		18	3	2
17/01/17		ok	ok		18	3	2
18/01/17		ok	ok		18	3	2
19/01/17		ok	ok		18	3	2
20/01/17	3	ok	ok		18	3	2

CAGE N°	*kg TEORETHICAL*	*KG REALES*	*FEED TYPE*	*FEED LOTE N°*	*FISH RESP.*	*INITIAL (time)*	*FINAL (time)*
9×	2	2	1,9		3	18:48	18:54
	2	2	1,9		3	17:55	18:08
	2	2	1,9		3	18:04	18:10
	2	2	1,9		3	13:22	13:26
	2	2	1,9		3	13:46	13:51
	2	2	1,9		3	18:15	18:19
	2	2	1,9		3	17:35	17:39
	2	2	1,9		4	18:44	18:47:00
	2	2	1,9		4	18:11	18:15
	3	3	1,9		3	18:40	18:43

Also, to better define the state of the sea during working – feeding the amberjack, a scale was used and recorded according to specific numbers: SEA OVERVIEW 1 Calm; 2 Wind, 3 Heavy, 4 Heavy + wind, 5 Storm; WATER CURRENT 1 No current, 2 Some current, 3 Heavy current.

The next 3 tables show a summary of the results for the individual fish sampled at the FPCT and the different samplings periods established; **Tables 21.1.2**, for the growth and **21.1.3** and **21.1.4** for the fish biometric parameters.

Table 21.1.2. Data from the amberjack growth in the CANEXMAR cages.

	Temperature	Sampling	weight (g)	Initial wt	Growth (g)	Growth (% initial)
MEDIA	18	ene-17	52.91			0
MEDIA	19	abr-17	119.00	52.91	66.09	124.91
MEDIA	21	25-jul	293.70	52.91	240.79	455.10
MEDIA	23	28-nov	521.82	52.91	468.91	886.24
SD	18	ene-17	23.86			
SD	19	abr-17	25.44	23.90	25.44	48.08
SD	21	25-jul	57.35	23.90	57.35	108.40
SD	23	28-nov	103.73	23.90	103.73	196.05

	Sampling	Total length (cm)	K	Furcal length /cm)	Std length (cm)	Evisc weight (g)	VSI
MEDIA	ene-17						
MEDIA	abr-17	21.01	1.27	18.48	17.80	105.83	11.33
MEDIA	25-jul	29.66	1.11	26.37	25.25	275.70	9.19
MEDIA	28-nov	35.31	1.16	29.81		546.75	7.14
SD	ene-17						
SD	abr-17	1.46	0.07	1.46	1.34	15.04	0.94
SD	25-jul	1.72	0.07	1.47	1.38	55.72	2.47
SD	28-nov	2.68	0.08	0.24		91.22	1.65

Table 21.1.4. Data from the amberjack biometric parameters (fillet and liver) under cage culture conditions.

	Sampling	Right fillet (g)	Left fillet (g)	Total fillet (g)	Fillet (%)	Liver weight	HSI
MEDIA	ene-17						
MEDIA	abr-17	26.33	25.94	52.28	55.77	2.14	1.82
MEDIA	25-jul	48.92	56.20	102.53	65.80	3.50	1.11
MEDIA	28-nov					9.20	1.55
SD	ene-17						
SD	abr-17	6.09	4.74	9.68	3.75	0.50	0.41
SD	25-jul	11.01	14.73	21.91	3.00	1.89	0.42
SD	28-nov					2.80	0.35

Representative pictures during sampling of the amberjack at the CANEXMAR cages are shown below (**Photo 21.1.1 and 21.1.2**)

Photo 21.1.1. Intermediate sampling for the Amberjack at Canexmar cages.

Photo 21.1.3. Fish sampled at GIA labs for amberjack growth, biometric and health parameters controlling (intermediate sample).

All the results for the amberjack growth in cages together with their respective biometric and feed efficiency will be analyzed in relation with the natural temperatures recorded, and also with those previously obtained for the growth of the fish in tanks. Health status samples continue to be under analysis and will be also discussed with the whole obtained results to better achieve the objectives and deliverable for the development of the rearing methods of the amberjack in cages.

Task 21.2 Development of feeding methods (led by IEO, Salvador Jerez).

Different feeding methods including estimation of daily rhythm and frequency (continuous vs. fixed ratios) were tested with individuals at different developmental stages of juveniles (5 g and 200 g individuals). Experimental trials were performed at P2 FCPCT and P8 IEO for 5 and 200 g greater amberjack, respectively.

The full description of the work and results have been provided in ***Deliverable D21.1 "Definition of optimum feeding methods for greater amberjack grow out***".

Task 21.3 Development of appropriate husbandry practise (led by HCMR, Nikos Papandroulakis)

Action 21.3.1 Determination of minimum-maximum temperature ranges (led by HCMR, **Nikos Papandroulakis).**

This study evaluated the optimal temperature for growth performance maximization and its effects on key physiological parameters in order to determine the most appropriate conditions for this species. Three water

temperatures were tested (16ºC, 21ºC and 26ºC) in juveniles of *S. dumerili* during 98 days and individuals were sampled for blood and growth parameters three times throughout the experimental period.

21.3.1.1 Experimental design

Experiments were carried out at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Center for Marine Research (HCMR), Iraklion, Crete from March to June 2017. A total of 108 juveniles of greater amberjack (mean ± SD body weight: 325.6 ± 24.2 g) were brought in from HCMR's net-pen cage facilities in Souda Bay, northwest Crete and randomly distributed among 9 circular 500L indoor rearing tanks (n = 12 per tank) at an initial temperature of 15 ºC (ambient). Over the following week, seawater temperature was gradually adjusted from ambient to temperature regimes: 16ºC (Group A), 21ºC (Group B) and 26ºC (Group C).

The experiment was performed in triplicates, where each temperature regime composed a semi closed recirculating water system with mechanical and biological filtration (see **Figure 21.3.1**) in order to maintain the physico-chemical parameters of the water within an adequate range. Seawater was pumped directly from the coast (salinity 38 psu, pH ranging from 7.6 – 8.0). Temperature, pH and dissolved oxygen were monitored daily using digital probes, whilst NH_3 and NO_2 were measured once a week with a photometer. Water renewal per system ranged from 25 – 50% day^{-1}, depending on conditions in order to maintain temperatures constant and low levels of NH_3 and NO_2. Water recirculation within each tank was kept at 300% $hour^{-1}$ throughout the experimental period while the photoperiod was set at 12L/12D.

All groups were fed standard extruded commercial diet (IRIDA, S.A., Greece). Fish were fed manually *ad libitum* twice a day (09.30 h and 12.30 h) and, in addition, a simple automatic belt-feeder was used to distribute the food between approximately 14.00 h and 20.00 h, when the artificial lights switched off. Leftover food in the tank was collected the following morning using a siphon and a 1,000 µm net and subsequently oven-dried overnight at 90 ºC, in order to quantify feed intake per tank. Fish mortality was monitored daily and any dead individuals were immediately removed and taken to HCMR's pathology laboratory to be checked for bacterial or parasitic diseases.

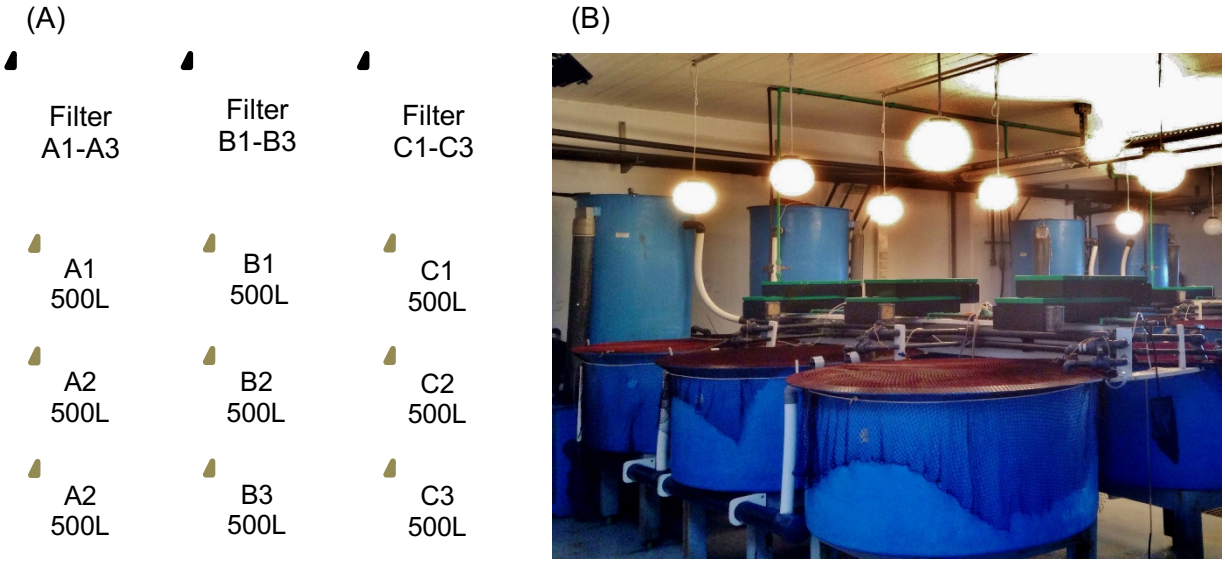


Figure 21.3.1. (A) Schematic representation of experimental tanks (A1-A3: 16 ºC, B1-B3: 21 ºC and C1-C3: 26 ºC). (B) Experimental room equipped with the filters, net-covered tanks, artificial lighting and automatic feeders.

21.3.1.2 Sampling procedure

Samplings were performed on a monthly based. At the beginning of the trial, all individuals were measured and weighted and the same procedure was also followed during the 3 samplings (April, May and June 2017) implemented until the end of the project.

For the physiological measurements, blood samples were collected. In order to minimize any possible diurnal fluctuations and impact of food and/or digestion on the estimated blood parameters, fish were starved 24h prior to sampling and all procedures were performed between 10.00 a.m. and 12.00 p.m.

One tank at a time, all individuals were quickly caught with a fish trap and transferred to a 60-litre bucket containing 2-phenoxyethanol (Ethylene glycol monophenyl ether) at a concentration of 250 mg L^{-1}. Once the fish were lightly anaesthetized (*i.e.* swimming slowly in an upright position due to equilibrium loss), weight and length measurements were done. Simultaneously, three specimens per tank were randomly chosen for blood sampling, except on the last sampling (*i.e.* June) where blood collection was performed in all individuals (n = 91).

In order to avoid clogging, blood was collected from the caudal vein by 1 ml heparinized syringes with 27G x 1/2 needles, immediately transferred into 1.5 ml heparinized eppendorf tubes and then centrifuged at 5,000 rpm for 3 min at room temperature. Resulting plasma was stored at -20 ºC until analyses were performed. A total of 145 blood samples were collected throughout the experimental period.

21.3.1.3 Analytical procedures

Hematocrit and hemoglobin were quantified immediately after sampling procedures, before blood clotted. Hematocrit values were determined with standard capillary tubes, which were then centrifuged at 4,000 rpm for 3 min. Hemoglobin was quantified calorimetrically following Drabkin's method by using a Spinreact kit (Girona, Spain).

Plasma was used for the analysis of the remaining physiological parameters: cortisol, glucose, lactate, total proteins, triglycerides, fish insulin-like growth factor 1 (IGF-1) and growth hormone (GH). All analyses were performed using commercially available kits, detailed on **Table 21.3.1.** In particular, IGF-1 and GH were quantified only for the samples collected only at the end of the experimental period, by using commercially available kits (Cusabio, Wuhan, China), according to the manufacturer's instructions (not tested for cross-reactivity or interference with all analogues).

Table 21.3.1. Analytical methods used to quantify the physiological parameters analyzed.

Parameter	**Method**	**Kit**
Cortisol	ELISA	Neogen (KY, USA)
Glucose	Enzymatic calorimetric	Biosis (Athens, Greece)
Lactate	Enzymatic calorimetric	Spinreact (Girona, Spain)
Total Proteins	Biuret assay	Biosis (Athens, Greece)
Triglycerides	Enzymatic calorimetric	Biosis (Athens, Greece)
IGF-1, GH	ELISA	Cusabio (Wuhan, China)

2.4 Statistical analysis

Effects of temperature on growth performance were analysed by the general regression model of the form: $Y = a_0 + a_1 \cdot t + a_2 \cdot D + a_3 \cdot t \cdot D$, where Y represents the dependent variable (*i.e.* weight or length), t the time, D is a dummy variable (with 0 and 1 values for each condition tested) and α_i (i = 1, 2, 3) are constants. The coefficient α_1 indicates the linear growth rate, whereas α_2 and α_3 indicate the effects of the coupling of the tested condition and the time. This method tests the hypothesis that the constants α_2 and α_3 are zero. Time

series have the same slope when constant α_3 is zero, and the same initial value when α_2 is zero. When both constants are zero, time series describe similar dependent variables.

Feed conversion ratio (FCR) was calculated as the ratio between total feed consumption (g) and total biomass gain (g) during a same period of time. These values were estimated both on a monthly basis and for the entire the experimental period. Mortalities were deducted from the final biomass and one-way ANOVA was used to identify significant differences in FCR between treatment groups.

Regarding the physiological data, statistical analyses were performed using SigmaStat 3.5 and all results are presented as mean ± SD. In order to eliminate outliers, 5% of the data at the upper and lower extremes was identified by direct estimation of the percentiles and subsequently excluded from further analyses. This procedure was performed for all physiological parameters, except cortisol due to its known high inter-individual variability. Data were tested for normality using the Kolmogorov-Smirnov test and Levene's test for homogeneity of variance. Any parameter that failed either test were then log transformed to meet the assumptions. Two-way ANOVA was used to determine statistically significant effects of temperature and sampling time (*i.e.* 1st, 2nd and 3rd sampling) on physiological parameters, followed by Holm-Sidak's multiple comparison *post hoc* test. To check the effects of temperature on IGF-1, GH and on growth performance parameters, one-way ANOVA was performed also followed by Holm-Sidak's *post hoc* test. Finally, Pearson's product-moment correlation was used to identify significant associations between all measured parameters.

21.3.1.2 RESULTS

21.3.1.2.1 Growth performance

Starting from 325.6 ± 24.2 g body weight and 29.5 ± 0.9 cm total length in all groups, individuals reached 395.1 ± 67.7 g, 483.7 ± 64.3 g, 441.7 ± 95.6 g and 31.6 ± 1.3 cm, 34.2 ± 1.4 cm, 32.5 ± 1.9 cm for groups reared at 16°C (Group A), 21°C (Group B) and 26°C (Group C) respectively (Figure 2). A summary of the biological performance of each reared group is presented on **Table 21.3.2**. According to the multiple regression analysis, estimated exponential growth rates for both body weight and total length differed between the tested conditions and were respectively 0.69 day^{-1} and 0.02 day^{-1} for Group A, 1.65 day^{-1} and 0.05 day^{-1} for Group B and finally 1.17 day^{-1} and 0.03 day^{-1} for Group C. Although some low intra-group variability was observed, there was no significant differences between replicates (see **Table 21.3.3**). Moreover, there were some differences between the coefficients of variation for body weight (Group A = 17.2 ± 6.7%; Group B = 13.3 ± 3.9%; Group C = 21.7 ± 0.7%), although not statistically significant (One Way ANOVA, $p = 0.15$). Differences were also observed in survival rates between treatment groups, where values registered for Group A were 94.4 ± 4.8%, 83.8 ± 8.3 % and 75.0 ± 14.4% for Groups B and C, respectively.

Table 21.3.2. Detailed biological performance of groups in each temperature regime. Different letters indicate statistically significant differences ($p < 0.05$).

	A 16°C	**B 21°C**	**C 26°C**
Initial Body Weight (g)	327.8 ± 25.8	322.3 ± 22.7	326.7 ± 24.5
Final Body Weight (g)	395.1 ± 67.7^{a}	483.7 ± 64.3^{b}	441.7 ± 95.6^{a}
Initial Body Length (cm)	29.6 ± 0.7	29.4 ± 1.0	29.5 ± 0.9
Final Body Length (cm)	31.6 ± 1.3^{a}	34.2 ± 1.4^{b}	32.5 ± 1.9^{a}
Feed Conversion Ratio (*)	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.3
Survival Rate (%)	94.4 ± 4.8	83.8 ± 8.3	75.0 ± 14.4

(*) during the whole experimental period

Feed intake varied significantly among experimental groups (One Way ANOVA, $p < 0.001$), with the lowest values observed in group A (35.8 ± 3.9 g day^{-1}) and the highest in group B (64.2 ± 3.4 g day^{-1}). Furthermore, the C-group consumed an average of 58.1 ± 6.9 g of feed per day, although this value differed significantly only when compared to the A-group (Holm-Sidak, $p = 0.02$), hence there was no differences in feed intake between groups B and C. The calculated feed conversion ratio (FCR, in dry food basis) varied throughout the experimental period within groups B and C (**Table 21.3.4**) and remained relatively constant for the groups reared at 21°C (0.4 ± 0.1), however, a statistically significant difference among experimental groups was not observed (One Way ANOVA, $p = 0.09$).

Table 21.3.3. Coefficients of exponential growth rate (α_1) obtained through regression analysis for each experimental condition as well as between replicates. Different letters or asterisks indicate statistically significant differences in body weight or total body length, respectively ($p < 0.001$). BW = body weight, BL = total body length.

	α_1 (BW)	**α_1 (BL)**		**α_1 (BW)**	**α_1 (BL)**		**α_1 (BW)**	**α_1 (BL)**
A1	0.00224	0.00062	**B1**	0.00405	0.00143	**C1**	0.00346	0.00103
A2	0.00173	0.00046	**B2**	0.00436	0.00164	**C2**	0.00255	0.00081
A3	0.00128	0.00063	**B3**	0.00349	0.00186	**C3**	0.00122	0.00073
A	0.00178[a]	0.00062*	**B**	0.00413[b]	0.00144**	**C**	0.00256[a]	0.00086*

Table 21.3.4. Average weight gain (g), food consumption (dry weight) and food conversion rate (FCR) for each treatment group measured at different time points throughout the experimental period.

	Days of rearing	**Biomass gain (g)**	**Food consumed (g)**	**FCR**
A	1-35	-17.8	1711.8	-96.2
	36-70	887.6	2718.9	3.1
	71-98	1056.6	2554	2.4
	1-98	**1926.4**	**6984.7**	**3.6**
B	1-35	1457.6	3212.9	2.2
	36-70	1784.1	5076.3	2.8
	71-98	1607.4	4234.8	2.6
	1-98	**4849.1**	**12524**	**2.6**
C	1-35	-41.5	2832.5	-68.3
	36-70	1073.6	4707.5	4.4
	71-98	2128.5	3916.1	1.8
	1-98	**3160.6**	**11456.1**	**3.6**

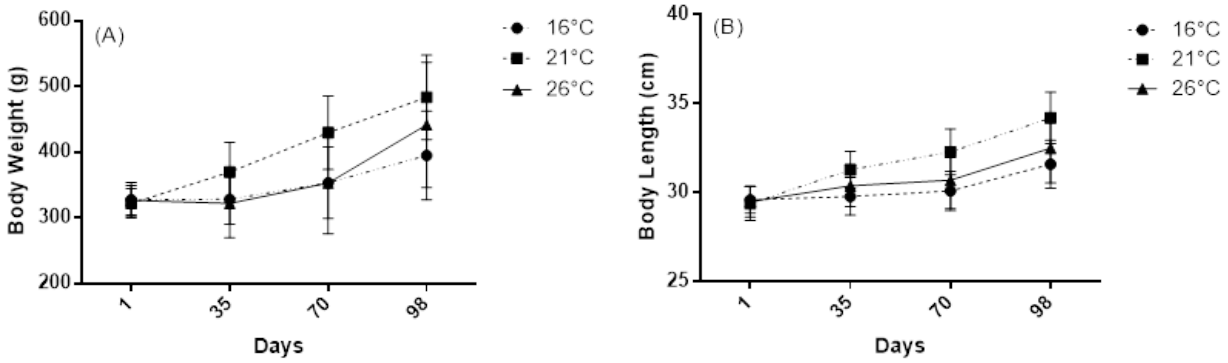


Figure 21.3.2. Growth performance of the experimental groups reared under different temperature regimes. Represented in (A) the body weight gain for each group throughout the experimental period, whilst in (B) the increase in total body length for the same period.

21.3.1.2.2 Physiological parameters

Significant changes were observed on the majority of the measured parameters either in relation to temperature, sampling time or both variables. The results of the analyses of each parameter for the different temperature regimes and sampling points throughout the experimental period are exhibited on **Table 21.3.5**.

Cortisol levels averaged 14.1 ± 29.3 ng ml^{-1}, 27.8 ± 36.9 ng ml^{-1} and 41.7 ± 43.3 ng ml^{-1} for Groups A, B and C respectively (**Figure 21.3.3a**). Plasma cortisol showed a high inter-individual variability, illustrated by high standard deviation values and consequently high coefficients of variation, which ranged from 97.2 ± 41.3% for Group B to 157.3 ± 41.3% for Group A and 119.7 ± 46.1 for Group C. Although these values varied significantly in relation to both sampling time and temperature, main effects cannot be properly identified due to significant interactions between factors (Holm-Sidak's method, $p < 0.001$).

Temperature did not have an effect on lactate, however values measured on the 2nd sampling were significantly higher in all groups (**Figure 21.3.3b**), going from 3.4 ± 0.7 mmol l^{-1} on the 1st sampling to 6.4 ± 0.3 mmol l^{-1} on the 2nd and then dropping to 2.8 ± 0.6 mmol l^{-1} on the final sampling. Triglycerides and total proteins were also only affected by sampling time and highest values were registered for the 3rd sampling in both cases (2.4 ± 1.1 mmol l^{-1} for the former and 3.9 ± 0.1 mg dl^{-1} for the latter) (**Figures 21.3.3c and 3d**). Although hemoglobin levels were also not affected by temperature, higher values were registered for the 1st sampling (8.5 ± 1.2 g dl^{-1}) and lower ones for the final sampling (6.77 ± 0.7 g dl^{-1}) (**Figure 21.3.3e**).

Plasma glucose was affected by both factors tested, although no interactions were determined. Concentrations increased with temperature and sampling time (**Figure 21.3.3f**), hence, highest values were registered for individuals in Group C (8.3 ± 0.5 mmol l^{-1}) and the lowest ones in Group A (5.7 ± 1.4 mmol l^{-1}). For all treatment groups, highest values were obtained on the final sampling. Regarding the remaining parameters (*i.e.* hematocrit, insulin-like growth factor 1 and growth hormone) there were no significant variations.

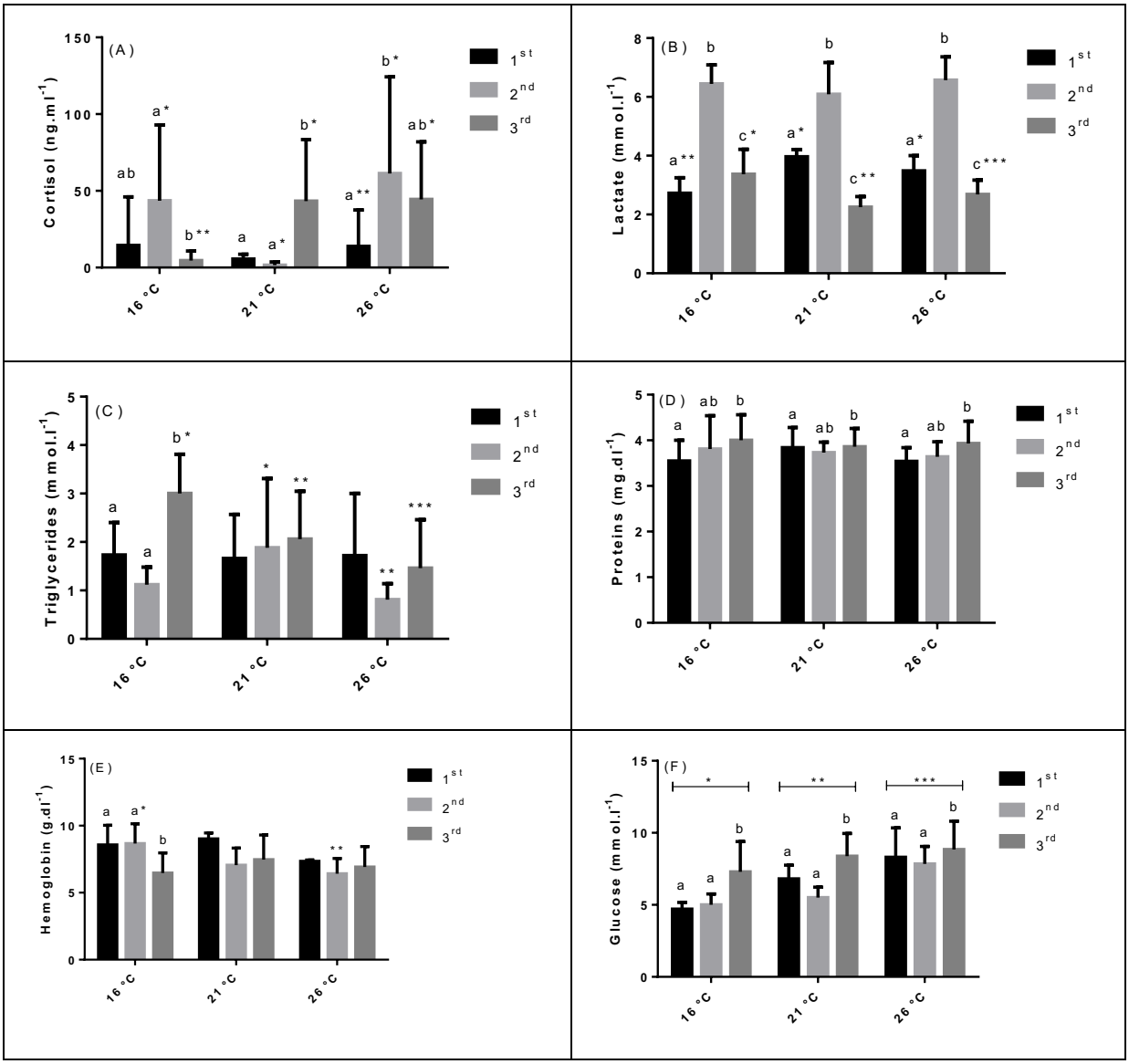


Figure 21.3.3. Changes in plasma cortisol (A), lactate (B), triglycerides (C), total proteins (D), hemoglobin I and glucose (F) concentrations between and within treatment groups and sampling points. Different letters indicate statistically significant differences between sampling points within the same temperature treatment, while asterisks indicate significant differences between treatment groups within the same sampling point ($p < 0.05$).

Correlations were found among some of the parameters analyzed in this study. Body weight showed a positive correlation with cortisol ($r = 0.185$, $p = 0.04$), glucose ($r = 0.275$, $p = 0.002$) and proteins ($r = 0.306$, $p < 0.001$). A correlation was also found between cortisol and glucose ($r = 0.220$, $p = 0.02$).

Table 21.3.5. Summary of physiological parameters of greater amberjack reared in different temperature treatments and sampled once a month throughout the experimental period. Values are exposed as mean±SD.

	Sampling Time											
	1st			2nd			3rd			2-way ANOVA		Holm-Sidak
	16 °C	*21 °C*	*26 °C*	*16 °C*	*21 °C*	*26 °C*	*16 °C*	*21 °C*	*26 °C*	*T (°C)*	*Time*	*T(°C) x Time*
Cortisol (ng.ml^{-1})	14.4±31.7	5.5±3.2	13.8±23.7	43.5±49.9	1.6±2.2	61.3±63.0	4.6±6.3	43.3±40.2	44.4±37.5	p=0.02	p=0.02	p<0.001
Glucose (mmol.l^{-1})	4.7±0.5	6.8±0.9	8.3±2.0	5.0±0.7	5.5±0.7	7.8±1.2	7.3±2.1	8.4±1.9	8.8±2.0	p<0.001	p<0.001	ns
Lactate (mmol.l^{-1})	2.7±0.5	4.0±0.2	3.5±0.5	6.4±0.6	6.1±1.1	6.6±0.8	3.4±0.8	2.2±0.4	2.7±0.5	ns	p<0.001	p<0.001
T-proteins (mg.dl^{-1})	3.5±0.4	3.8±0.4	3.5±0.3	3.8±0.7	3.7±0.2	3.6±0.3	4.0±0.6	3.9±0.4	3.9±0.5	ns	p=0.01	ns
Triglycerides (mmol.l^{-1})	1.7±0.7	1.7±0.9	1.7±1.3	1.1±0.4	1.9±1.4	0.8±0.3	3.0±0.8	2.1±1.0	1.5±1.0	p=0.02	p<0.001	p=0.002
Hematocrit (%)	45.3±2.9	45.3±2.5	38.0±4.8	40.9±4.1	43.6±3.4	42.4±5.8	41.4±5.1	41.8±5.1	41.7±5.7	ns	ns	ns
Hemoglobin (g.dl^{-1})	8.6±1.5	9.0±0.4	7.3±0.1	8.7±1.5	7.1±1.3	6.4±1.1	6.5±1.5	7.5±1.8	6.9±1.5	ns	p=0.04	p=0.03

										1-way ANOVA		
IGF-1 (pg.ml^{-1})	-	-	-	-	-	-	157.7±35.1	180.5±37.8	167.3±42.8	ns	-	-
GH (ng.ml^{-1})	-	-	-	-	-	-	1.2±0.3	1.2±0.2	1.2±0.3	ns	-	-

21.3.1.2.3 Effect of temperature on nutrient digestibility of greater amberjack

On the completion of the growth trial fish were transferred in digestibility tanks for the determination of the effect on rearing temperature on energy, protein, fat and dry matter digestibility of feed. For each temperature (16ºC, 21ºC, 26ºC) three experimental cylindroconical tanks of 250 L (9 tanks in total) were used -connected to a recirculation system supplied with borehole water- and in each tank 5 fish were placed. The tanks had a faecal collection device attached to the bottom. For the determination of apparent digestibility coefficients, the indirect acid-insoluble ash (AIA) method was used. The fish were fed on the same commercial feed as in the growth trial after grinding and mixing with water and addition of 1% Celite® (Fluka, St. Gallen, Switzerland). The paste was formulated into pellet with a mincing machine and dried at 40 ºC overnight. For acclimation fish were fed two times a day for 10 days. Subsequently, the faecal collectors were placed, and faeces were collected overnight for another 10 days. The collected feaces were centrifuged, lyophilized and analysed for dry matter, energy, crude protein and crude fat. Dry matter was determined after drying in an oven at 90ºC until constant weight, energy was determined by bomb calorimetry (Parr 6200), crude lipids were extracted with methanol/chloroform and crude protein was determined by Dumas method. The apparent nutrient digestibility was calculated by the following formulae:

ADC of nutrient (%) = 100-100 ((%dietary marker/ %faecal marker) x (%faecal nutrient/ %dietary nutrient))

The results were analysed by one way analysis of variance following by Tukey post hoc test using an SPPSS software.

The fish kept at 21 ºC apparently developed a non-identified disease and exhibited reduced appetite. Their faeces were loose and develop a greenish colour. The other two groups kept at 16 ºC and 26 ºC had normal health. The results of the digestibility experiment are presented in the following table

Effect of water temperature on apparent nutrient digestibility coefficient (%) (mean±stdev).

Temperature (ºC)	Energy	Fat		Protein	Dry matter
16	89.2 ± 1.9	91.6 ± 1.1	a	91.4 ± 0.8	76.2 ± 2.4
21	61.5 ± 29.3	72.5 ± 14.7	b	69.3 ± 22.2	64.5 ± 25.0
26	89.9 ± 0.7	90.5 ± 0.9	a	92.0 ± 0.5	78.6 ± 0.7

Values within a column with a different letter differ statistical (p<0.05)

Nutrient digestibility values of amberjack were in line with the observations made in earlier studies in *Seriola dumerili* (Dawood et al., 2015) and *Seriola lalandi* (Miegel et al., 2010). Overall, the digestibility coefficients were high indicating the good quality of the diets. Although temperature is one of many parameters affecting gut transit time (fish size, food quality meal size and feeding frequency to name a few) it did not affect energy fat, protein and dry matter digestibility of amberjack. This was also the case in past experiments with *Seriola lalandi* (Miegel et al., 2010) and other species like rainbow trout *Oncorhynchus mykiss* (Windell et al., 1978), salmon S*almo salar* (Ng et al., 2004) and sea bass *Dicentrarchus labrax* (Moreira et al., 2008). On the other hand, a low temperature may reduce the digestibility of saturated fats when alternative ingredients are used in feeds (Ng et al., 2003).

Due to their disease the fish kept at 21 ºC exhibited the lowest digestibility values. This study provides evidence that disease has an impact on nutrient digestibility in addition to the usually observed decrease of appetite.

21.3.1.3 **Effect of the temperature on the amberjack digestive characteristics and capacity during their ongrowing stage.**

Once presented the growth and the gut transit time for the different assayed temperatures, to fulfil the objectives of this task, the effect of the temperature on the fish digestive capacity was analysed. The results are mostly focussed on the protein side, as this species require high protein diets. To better understand the digestive characteristic of the amberjack, the activity and total reaction time for the hydrolysis in stomach and also in the intestine was determined. To achieve the objectives, the routine digestive enzymatic techniques were adapted to specific species conditions to latter evaluate the effects in the experimental fish under the different temperature test. After 105 days feeding, stomach and intestine from final fish from the different temperature tested (17, 22, 26) were taken and subsequently analysed according to the showed figures below (**Fig. 21.3.1.1.**). Specific management protocols for the stomach and the intestine samples were defined for the latter on analysis.

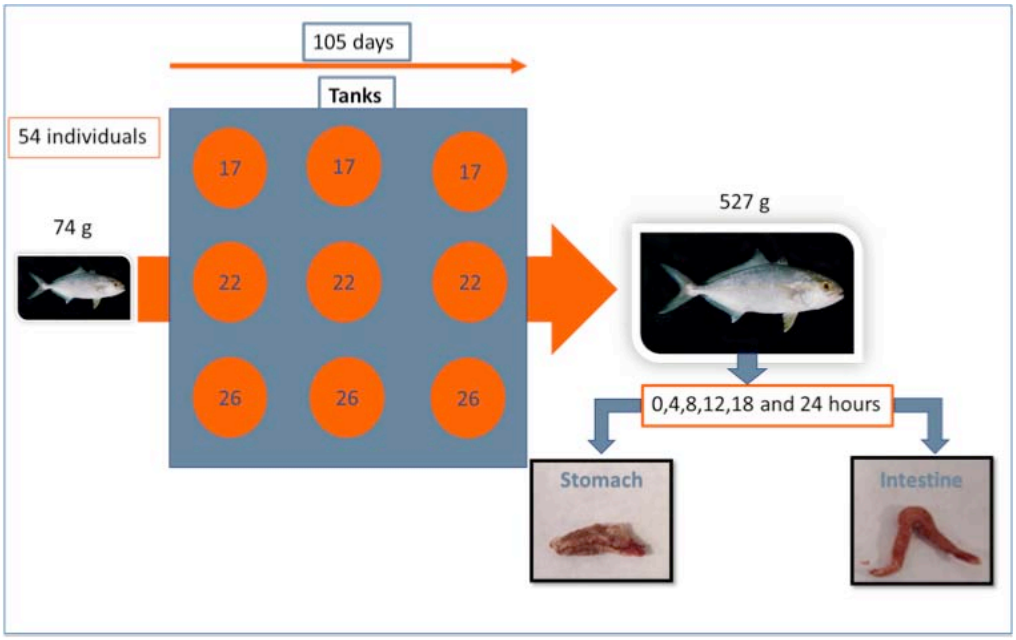


Fig. 21.3.1.1. Detailed scheme for the stomach and intestine (N=54) obtained for the analysis.

Having these information, it was decided to execute an experimental design according to the response surface model called Box-Behnken design. This type of design is used to evaluate the effect of multiple factors on a response variable minimizing the number of trials. For this, a range is established in which the minimum and maximum values of the factor to be evaluated are sought and a series of random combinations of factors are generated by MiniTab 17 software using the design and analysis of the experiments. The ranges used to define factors were based on the results of the previous physiological study: a) Temperature: between 17 and 26°C; b) E:S stomachic relationship: between 0.14 and 0.2U/mg protein; c) pH intestinal: between 6.5 and 7.5.

The *in vitro* hydrolysis assays were carried out using a membrane bioreactor modified from that described in Morales and Moyano (2010). The device consists in two chambers separated by a semipermeable membrane of 3500 MWCO (ZelluTrans/Roth®) (Figure 6). The upper part of the reaction chamber contains the mixture of the desired substrate and the enzyme extract dissolved in buffer (0.2 M phosphate buffer in different concentrations, pH = 6.5, 7 and 7.5 simulating the intestine) while the lower part of the reaction chamber only contains water. The protein substrate used in the assays was a comercial feed (Alterna XL, Skretting 8mm) whose content is 38% protein and 24% lipid. A magnetic stirrer maintains the upper mixture under

continuous agitation (280 rpm) and the small molecules released during the hydrolysis pass across the membrane, being recovered in the lower chamber at different moments during the desired reaction time. The complete arrangement was maintained within a thermal chamber at the desired temperature (17, 21.5 or 26ºC).

Results for the intestine pH did not show any significant variation pattern post intake, neither were affected by temperatures (**Fig. 21.3.1.2.**). Stomach pH was reduced at a maximum of 4.0. Slow acidification was observed in all cases, being about 18h in those fish maintained at 17 & 22ºC and much quicker (8h) in those maintained at 26ºC, which would correspond with a higher velocity for the gastric evacuation in the latest. In any case, stomach pH did not reach values below 4, did not reduce enough to transform significantly quantities of pepsinogen into pepsine, which denote a limited gastric protein hydrolysis in this specie (**Fig. 21.3.1.2.)**.

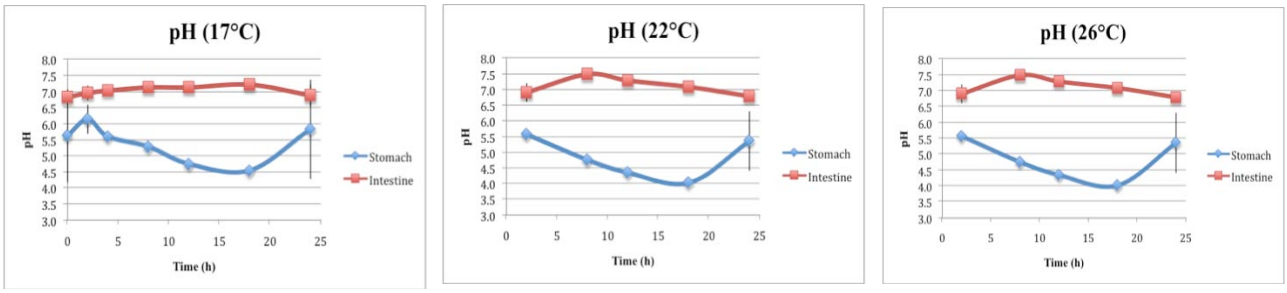


Fig. 21.3.1.2. Results for pH variation in the stomach and the intestine for the 3 temperatures assayed.

Results showed, as a conclusion from these preliminary assays, that the optimal range for the digestion of the amberjack is between 22ºC and 26ºC and the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the enzymatic activity in that range is maximum, while in the intestine the maximum activity range between 12 and 18h.

The activity of stomachic acidic protease (pepsin) was always markedly lower than that detected in the intestine (Figure 10, 11 and 12). This would be explained considering that it was measured at pH 4.5 which was the actual pH found in the stomach and at this value most of the pepsinogen has not been activated to pepsin (**Fig. 21.3.1.3**).

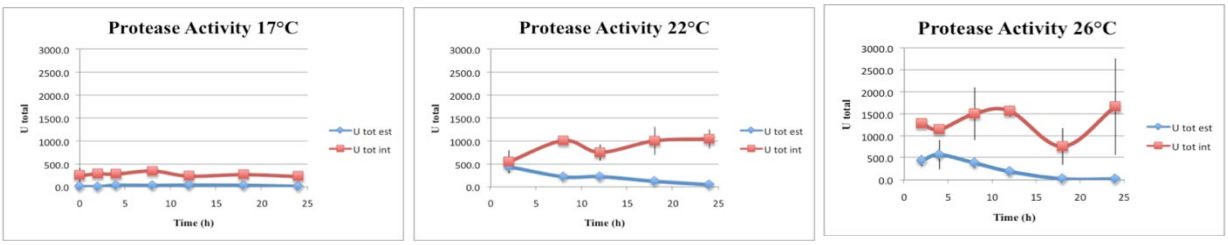


Figure 21.3.1.3. Protease activity variation both in the intestine and the stomach after the food intake at the 3 different temperatures assayed.

The values of total activity increased significantly with fish size, but when normalized in 100g of fish, no significant differences were observed in alkaline or acid proteases (**Figure 21.3.1.4**.), except for the low values detected in the fish kept at a lower temperature.

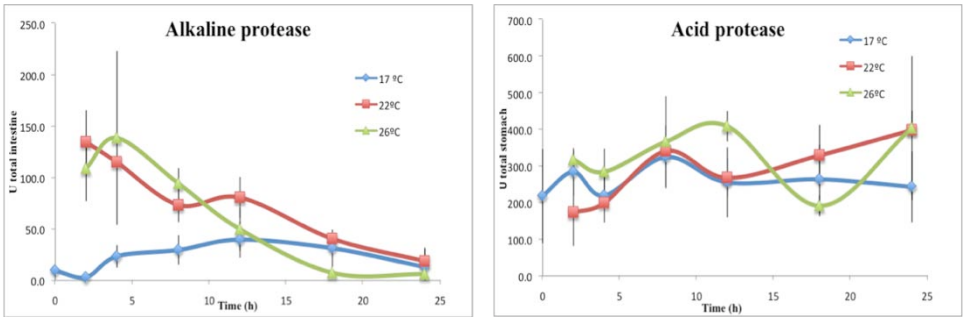


Figure 21.3.1.4. Alkaline and acid protease activity variation (normalized to 100g fish) both in the intestine and the stomach, after the food intake at the 3 different temperatures assayed.

These measurements have allowed us to apply a mathematical model based in the Response Surface Methodology and the in vitro digestibility assays, for which it was necessary to run the previous study of the *S. dumerili* specific digestive conditions. In order to reduce the number of assays needed, the Split-Split Plot Design was applied, giving us reliable results while a less number of samples to be run. A summary of the results obtained applying the model are presented below (**Figure 23.1.3.5, 23.1.3.6.** and **23.3.1.7.**).

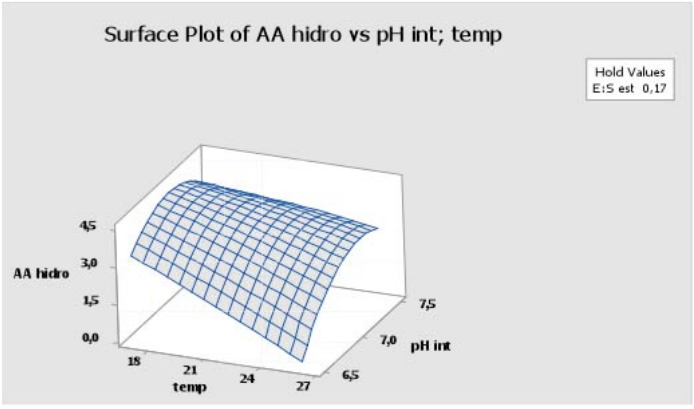


Figure 21.3.1.5. Surface model that predicts the hydrolysis of AA from the protein used in the function of the pH intestinal and temperature

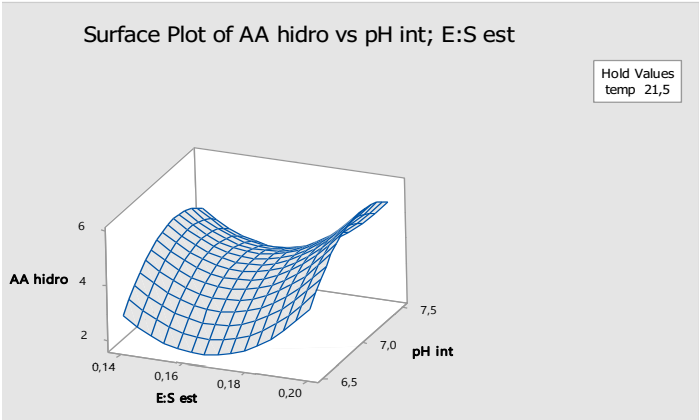


Figure 21.3.1.6. The response surfaces showing the variations in protein hydrolysis as a function of pH intestinal and in the stomach.

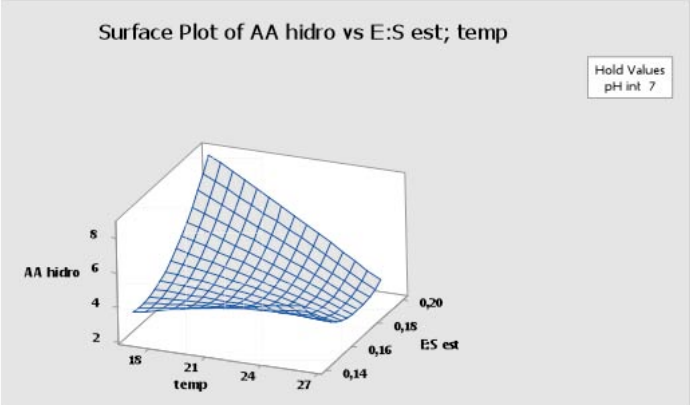


Figure 21.3.1.7. Surface model that predicts the hydrolysis of AA from the protein used in the function of the temperature and the E:S in the stomach.

Action 21.3.2 Definition of optimal stocking density (led by IEO, Salvador Jerez, Virginia Martín, Eduardo Almansa).

Stocking density has been shown to affect behavioral interactions in several fish species and may ultimately affect growth rates. To achieve and increase the profitability of fish commercial culture, it is necessary to establish the appropriate grow out practices, and the optimal density that produces the highest growth rates without compromising fish health and welfare. In this study, differences in growth performance of juvenile greater amberjack with initial size of 5g and 150 g held at different stocking densities have been examined with the aim of define the optimum stocking density considering the following parameters: growth rate, fish condition, feed efficiency, and quality including morphological aspects and haematological, histological, biochemical, immunological and oxidative stress studies.

This Action will contribute to deliverable D21.2 Definition of optimum conditions for cage culture of greater amberjack.

Experimental conditions

To achieve this objective, rearing trials at 3 different stocking densities (9 groups) were performed with individual size of **5 g in 500 l tanks** during 2015, and at 4 different stocking densities (12 groups) with fish initial size of **150 g in 4000 l tanks** during 2017, for a period of 4 months.

Juveniles of *Seriola dumerili* born in captivity in IEO facilities were maintained in indoor tanks with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu) and temperature. Fish were fed daily with a commercial pellet for turbot (Skretting Ltd, Norway; composition in % dry weight was: 52% crude protein, 20% crude fat, 8.7% ash, 1.7% crude cellulose and 1.4% total phosphorus), supplied *ad libitum* at feeding frequency accord to fish size. Feed left uneaten was recovered from the bottom of the tank 30 minutes after its administration to quantify the daily feed intake (FI).

Dead fish during the trial were recorded daily, measured and observed to check the presence of parasites or other pathologies. The level of parasitation by monogenean was also monitored weekly by dish traps (1.5 mm mesh net) placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014). Mesh traps were placed every Friday and retired every Monday to count the eggs entangled in the dish traps.

At the beginning (day 0), and at 60, 90 and 120 days, all fish in each tank were anesthetized with 2-phenoxiethanol and measured for weight and length. At each sampling time, five fish per tank were then selected randomly for blood collection from the caudal vessels using heparinized syringes. Plasma samples were separated after centrifugation at 1400 rev min^{-1} for 20 minutes and stored at -80°C until analysis.

A total of five fish at the beginning (0 day) and six fish per treatment at the end of the trial (120 days), were sampled to determine biometric parameters (viscerosomatic and hepatosomatic indexes) and obtain samples

of muscle, liver, brain and gill. Tissue samples were frozen in liquid nitrogen and stored at -80°C until analysis.

During the study, specific growth rate (SGR, % day^{-1}), coefficient of variation for weight (CV, %), condition factor (CF, g cm^{-3}), Viscerosomatic index (VSI, % body weight), Hepatosomatic index (HSI, % body weight), survival (S, %) and feed intake (FI, % body weight) were calculated as below:

SGR=100 x (ln final Body weight (g) - ln initial Body weight (g)) x $days^{-1}$

CF=100 x (Body weight (g) x Total $length^{-3}$ (cm))

VSI=100 x Visceral weight (g) x $Body\ weight^{-1}$ (g)

HSI=100 x Liver weight (g) x $Body\ weight^{-1}$ (g)

S=100 x final fish number x $initial\ fish\ number^{-1}$

FI=100 x feed consumption (g) x $average\ biomass^{-1}$ (g) x $days^{-1}$

Results

Optimal stocking density for 150 g greater amberjack in 4000 l tanks

The trial with the 150 g individuals was performed at 4 different stocking densities maintained in fiberglass 4m^3 square tanks in IEO facilities during 2017. A total of 480 *Seriola dumerili* juveniles born in captivity (average weight of 175.7±56.4g and size 20.2±2.3cm) were divided into 4 homogeneous groups, by triplicate, stocked at four different initial densities of 1.3, 1.7, 2.4 and 3.2 kg m^3 for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively. The final stocking density reached for the different treatments at the end of assay (120 days) were 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively.

The Specific Growth Rate (SGR) of the fish stocked at HD was significantly higher in the periods 30-60 and 60-90 days (P<0.05). In the period 90-120 the tendency changed and the SGR decreased with the increasing of the fish density. Thus, although the SGR tended to rise with the increasing of the fish density, not significant differences were observed in the overall period (0-120 days) (**Figure 21.3.2.2).**

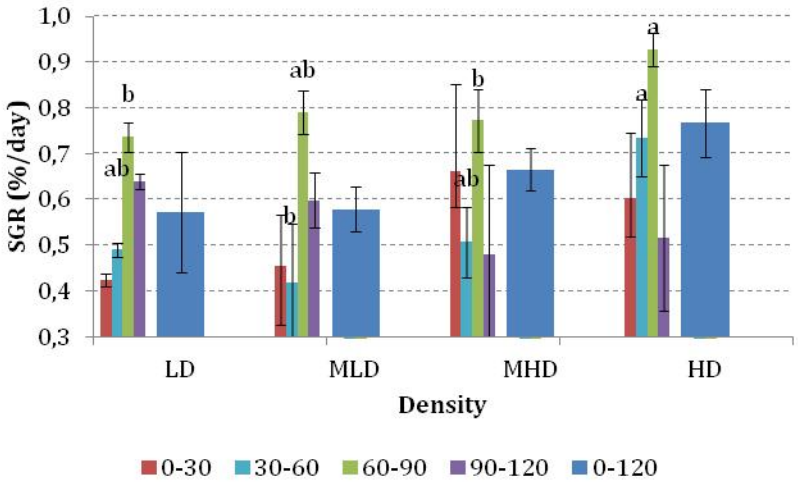


Figure 21.3.2.2. Specific growth rate (SGR, % day^{-1}) at the different periods and overall trial (120 days) of fish stocked at different densities (kg m^{-3}). Different letter indicates significant differences among treatments (P<0.05).

Condition Factor (CF) of fish decreased along the trial in all groups (**Figure 21.3.2.3**). Fish stocked at different densities showed similar CF within each period. Hepatosomatic index (HIS) and Viscerosomatic index (VSI) tended to rise with increasing stocking density at the end of assay (120 days) but not significantly **(Fig. 21.3.2.4).**

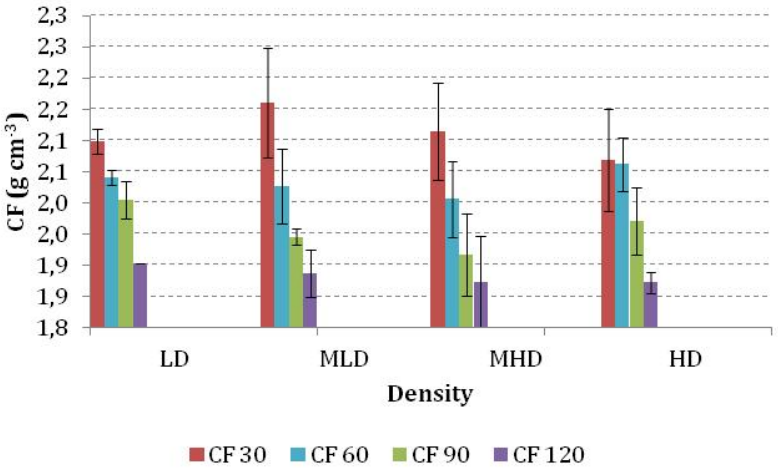


Figure 21.3.2.3. Condition factor (CF) (g cm^{-3}) of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) at the different periods.

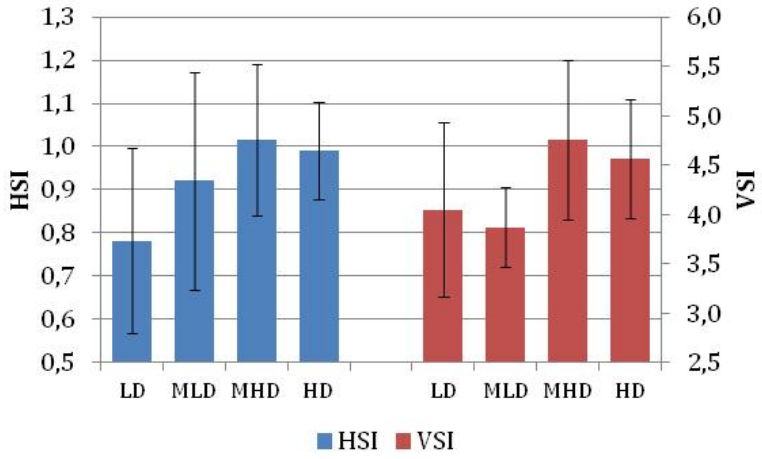


Figure 21.3.2.4. Hepatosomatic (HIS) and Viscerosomatic Index (VSI) at the end of the study of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) density.

Feed intake (% body weight day^{-1}) decreased significantly during the first three months of experimental period in all stocking density assayed (**Figure 21.3.2.5)**. Results of two-way ANOVA showed that both factors time (month) and stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four month during which the feed intake decreasing with the increase of density, although no significantly.

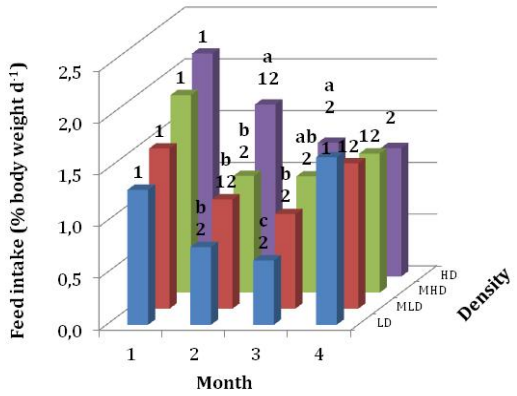


Figure 21.3.2.5. Feed intake (% body weight day^{-1}) of fish stocked at Low (LD), Medium low (MLD), Medium high (MHD) and High (HD) density during the trial. Different letter indicates significant differences among different treatments at each period. Different number indicates significant differences among each periods at the different treatment (P<0.05).

The daily feed intake at the different feeding times was higher in fish stocked at High density during the first three months of the trial, although significant differences were registered only during month 2 (at 8:00, 14:00 and 18:00 feeding times) and month 3 (14:00 and 18:00 feeding times). During the following month the feed intake at the different feeding times was similar irrespective of the culture density assayed. **(Figure 21.3.2.6)**.

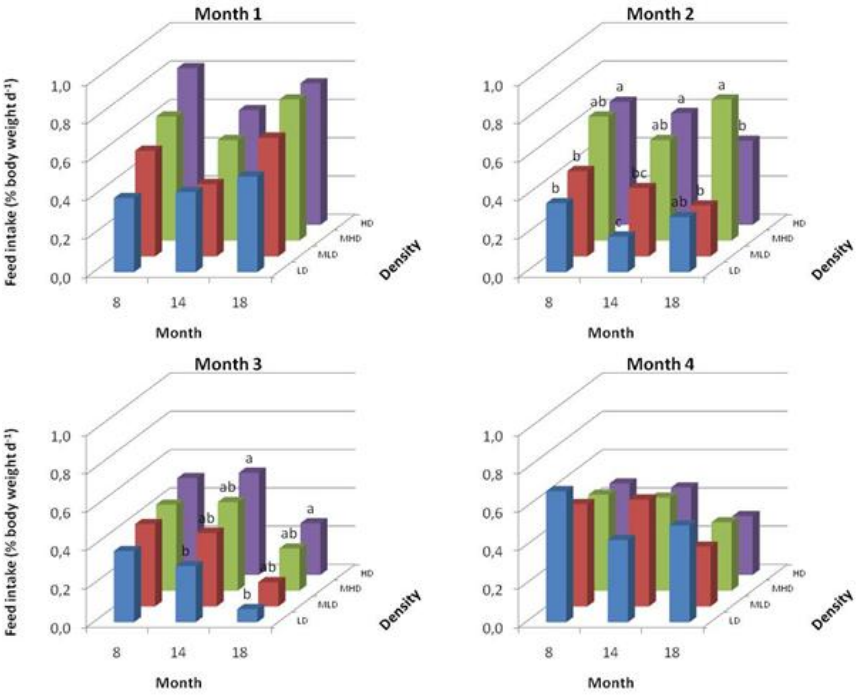


Figure 21.3.2.6. Feed intake (% body weight day^{-1}) of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) density, during the trial and at the different time of day. Different letter indicates significant differences among different stocking densities (P<0.05).

Immunological parameters (IEO) and oxidative stress enzymes (ULL) corresponding to trials of 5 g in 500 l tanks and 150 g in 4000 l tanks, are being analyzed.

Deviations from Annex I and their impact:

Project Coordinator comment: Although no deviation has been reported by the WP leader, I see in the report submitted that subtask 21.1.2 (lead beneficiary P28. CANEXMAR) has not been implemented as described in the DOW. Specifically, the trial was supposed to last for 2 years and include a comparison between floating and submersible cages (so 2 cages for 2 years). At the end, only a single floating cage was used and the study lasted for only 1 year. This was the result of several reasons, one being licensing problems that the company faced to deploy the submersible cages resulting in a delay to start the subtask. Another delay was caused due to heavy juvenile mortalities during transfer from the hatchery to the cages. Lastly, another delay was caused by a breakage of the cages due to a storm and a loss of 80% of the juveniles from the cages. So, at the end a trials was done for only 1 year and only 1 cage, so only 25% of the work has been carried out. As this study can not be repeated now with one year remaining, the deliverable "*D21.2 Definition of optimum conditions for cage culture of greater amberjack*" will not include all the data expected based on the DOW.

WP 22 Grow out husbandry – pikeperch

WP No:	22	**WP Lead beneficiary:**		P16. FUNDP
WP Title (from DOW):	Grow out husbandry – pikeperch			
Other beneficiaries (from DOW):	P9. UL	P21. DTU	P29. ASIALOR	
Lead Scientist preparing the Report (WP leader):	Patrick Kestemont (P16)			
Other Scientists participating:	Mandiki Robert (P16), Baekelandt Sébastien (P16), Fontaine Pascal (9), Ledoré Yannick (P9), Ivar Lund (P21), Jiri Bossuyt (P39)			

Objectives

1. Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status,
2. Characterization of pikeperch growth, immune and physiological status in farm conditions,
3. Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity.

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the first reporting period, it was necessary to perform two preliminary experiments in order to better define the methodological requirements of the multifactorial stress screening since there is limited information on stress response for pikeperch. As grading manipulations are unavoidable during the early developmental stage of pikeperch, the first refinement experiment compared control groups to fish submitted to single or repeated emersion stress challenge (mimicking grading) in order to determine the stress sensitivity of pikeperch juveniles to such stress, and to standardize some methodological aspects, especially concerning the physiological and immune analyses. The results from this experiment indicated that pikeperch juveniles are highly sensitive to aquaculture manipulations such as grading. They also indicate that only long-term application of grading manipulations may alter growth rate and food utilization.

A second preliminary experiment was conducted in order to determine the optimal time for sampling. The specific objective was to minimize as much as possible the β error during the multifactorial experiment since it was planned to sample at once various organs from 16 experimental conditions for the analysis of stress and immune parameters. Serial blood samplings were applied within 8 hours to controls and fish submitted to emersion stress once a week during one month. The results showed that the stress response culminated 30 min after emersion stress, and was sustained more than 3 h depending on the stress indicator. Such result support the interest for using various stress indicators to account for the stress responsiveness in pikeperch. As for the first preliminary assay, stress indicators confirmed a high sensitivity of pikeperch juveniles to emersion stress but no marked effect on lysozyme was observed.

Apart from the time needed for the refinement experiment, it was also necessary to adapt the rearing conditions of the UL facilities to the multifactorial protocol requirements. Indeed, it was planned to start this experiment around the end of the 1st year of the project in close collaboration with the UL partners (P9) that have suitable RAS for such type of experiments. Finally, that multifactorial experiment started on June 2015.

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the second reporting period, the aim was to characterize the effects of multiple variables on stress, immune response and growth performance by a multifactorial experiment. We then performed a fractional multifactorial trial testing 8 relevant husbandry and environmental factors. Each experimental unit represented a combination of 8 factors in two modalities including grading, stocking density (15 *vs* 30 kg/m^3), feed type (sinking *vs* mid-floating), light intensity (10 *vs* 100 lux), light spectrum (red *vs* white), photoperiod (long *vs* short), dissolved oxygen (60 *vs* 90 %) and temperature (21 *vs* 26 °C).

The results showed a clear effect of the feed type and light intensity on husbandry variables. Furthermore, stress and immune markers were affected by several interactions between feed type, light spectrum, temperature, photoperiod and oxygen saturation. Combining the results on husbandry performances and on stress and immune status, three combinations of modalities were selected as suitable for improving performances of pikeperch in intensive culture. Apart from the sinking feed, these experimental conditions were mainly characterized by their light characteristics.

Summary of progress towards objectives (31-48 Mo):

The first objective (Task 22.2) during the third period was to characterize growth, immune and physiological status of pikeperch in order to validate the effects of the best-identified variables in farm conditions. The ongrowing experiment from juveniles to marketable size started in April and will finish around January 2018. Since light characteristics may be an important factor in pikeperch culture (see Task 22.1), it has been decided to maintain fish under the two, defined as, optimal experimental modalities but testing only red vs. white light spectrum, since other factors modalities induced less variability. Several samplings of organs are done along this *in vivo* experiment and the last one is planned around January 2018. Then, the analyses for stress and immune markers will be performed from February to April 2018. A complementary *in vivo* experiment was conducted in FUNDP facilities in order to further understand some effects of the light intensity (10 vs 100 lux) and two light spectra (white and red) on stress status, humoral innate immune response and expression profile of immune-relevant genes in pikeperch. While light spectrum had little influence on tested variables, the use of a high light intensity was followed by long-term stress associated to an immune suppression. Several immune variables also followed a day-night variation. Since the secretion of the melatonin hormone by the pineal gland follows a circadian rhythm by being produced only during the dark phase of the photoperiod, it is thought to be a crucial immunomodulatory component. However, this hypothesis needs further investigations.

The second objective (Task 22.3) during the third periodic report was to assess the effects of pikeperch domestication level and geographical origin on growth and stress sensitivity (Task 22.3). For this task, the *in vivo* experiment conducted in the URAFPA facilities (Nancy, France) has just started in October 2017 and will last 3 months. Fish will be examined for physiological stress responses and immune competence. This task will allow to establish basis knowledge for future selection studies of pikeperch strains according to the rearing conditions of commercial fish farms.

Details for each Task

Task 22.1. Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status (led by FUNDP, Patrick Kestemont).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 22.1. Effects of multiple variables on stress, immune response and growth performances and recommendations of optimal conditions for pikeperch grow out.***

The work has also been published as Baekelandt, S., Redivo, B., Mandiki, S. N. M., Bournonville, T., Houndji, A., Bernard, B., El Kertaoui, N., Schmitz, M., Fontaine, P., Gardeur, J-N., Ledoré, Y., Kestemont, P. (2017). Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pikeperch *Sander lucioperca*. *General and Comparative Endocrinology (in press)*.

Task 22.2. Characterization of pikeperch growth, immune and physiological status in farm conditions (led by F2B, Jiri Bossuyt).

Farm validation experiment

Objectives

Based on the results from the multifactorial experiment (see Task 22.1), growth and physio-immunological status of pikeperch at different developmental stages (from 10 g to about 500 g) are compared, in farm conditions (Fish2Be), between standard husbandry conditions usually applied in routine by the SME. From this task, the expected results will help to recommend the best conditions applicable in pike perch farming for reducing stress level and supporting maximal growth performances.

Experimental design

A stock of 7,000 pikeperch juveniles was produced by Fish2Be farm. The fish (mean weight 9 g) were then distributed into 6 indoor 2 m³-tanks. After a 2-week acclimation, the white spectrum was replaced by a red spectrum (610 nm) for half of the tanks. The other parameters were kept constant (10-lux light; constant photoperiod 12:12; 21 °C; sinking feed). In order to assess the effects of sorting procedures on stress and immune status, we sampled fish 1 hr before and 30 min after the grading process. Since high size heterogeneity observed in pikeperch culture may highly influence the physiological stress response, each sampling consisted in capturing 4 of the smallest and 4 of the biggest fish in order to collect blood and organs.

The experiment started in April 2017 and is still ongoing. It will end once the fish reach about 500 g bodyweight. From the beginning, fish sorting and subsequent samplings were performed at days 43, 76, 122 and 174. The fifth and last sampling will be held in January.

Various biomarkers including husbandry parameters (survival and relative growth rate), stress indicators (plasma cortisol and glucose levels) and immune parameters (lysozyme and peroxidase activity and the expression of immune-relevant genes in the head kidney) will be assessed.

Preliminary results

The individual body weight reached around 90 g at day 76 (Fig. 2.2.1). However, from day 0 to day 76, no difference in individual body weight (Fig 2.2.1), specific growth rate (Fig 2.2.2) and weight heterogeneity (Fig 2.2.3) was observed between the two light conditions.

As regards to stress indicators, a significant increase of cortisol level from 50 to 500 ng.ml^{-1} was observed due to grading manipulations, irrespective of the day of sampling and the light spectrum (Fig. 2.2.4; 2.2.5). Glucose levels followed the same pattern as observed for cortisol (Fig. 2.2.6; 2.2.7). The size class of the fish has also not influenced the cortisol and glucose levels in plasma.

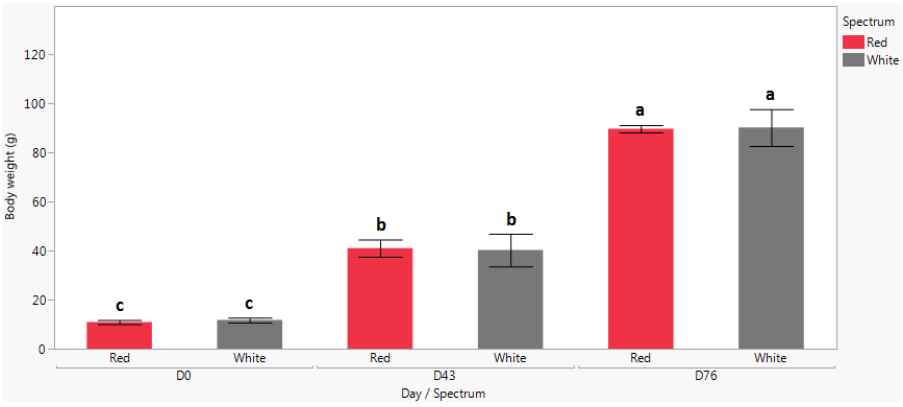


Fig. 2.2.1: Effects of the light spectrum on individual body weight (g) at days 0, 43 and 76. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

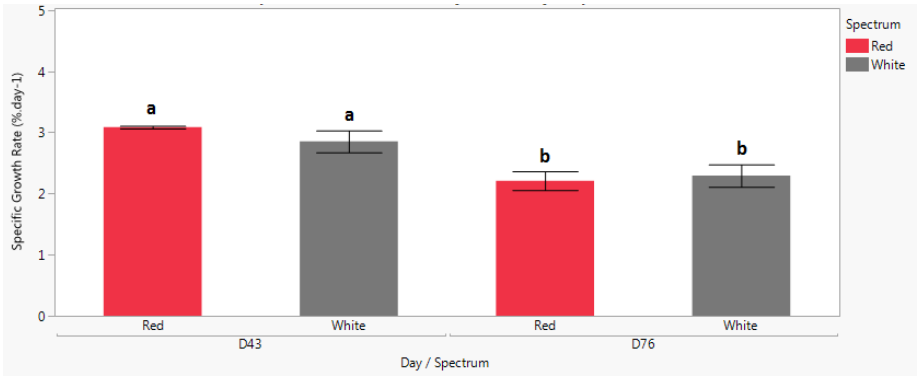


Fig. 2.2.2: Effects of the light spectrum on specific growth rate (%.day^{-1}) at days 43 and 76. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

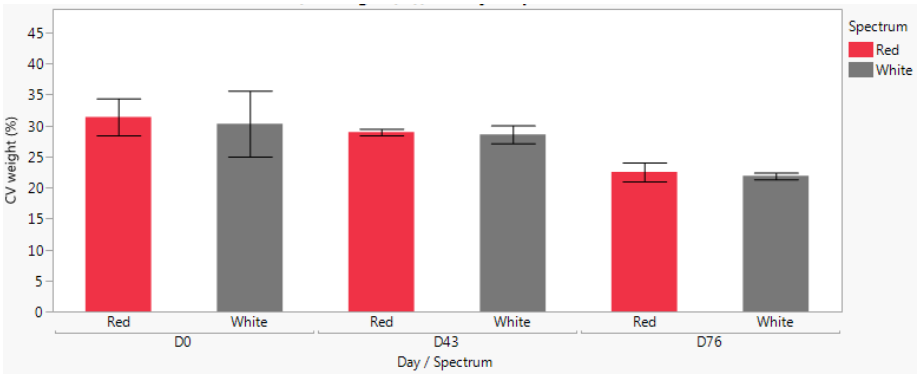


Fig. 2.2.3: Effects of the light spectrum on weight heterogeneity (CV, %) at days 0, 43 and 76. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

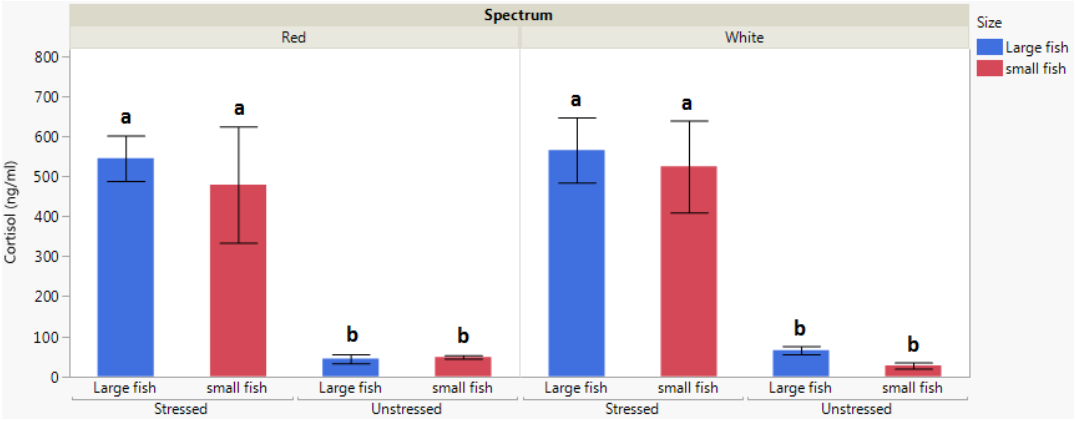


Fig. 2.2.4: Effects of the light spectrum on plasma cortisol (ng.ml^{-1}) at day 43. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

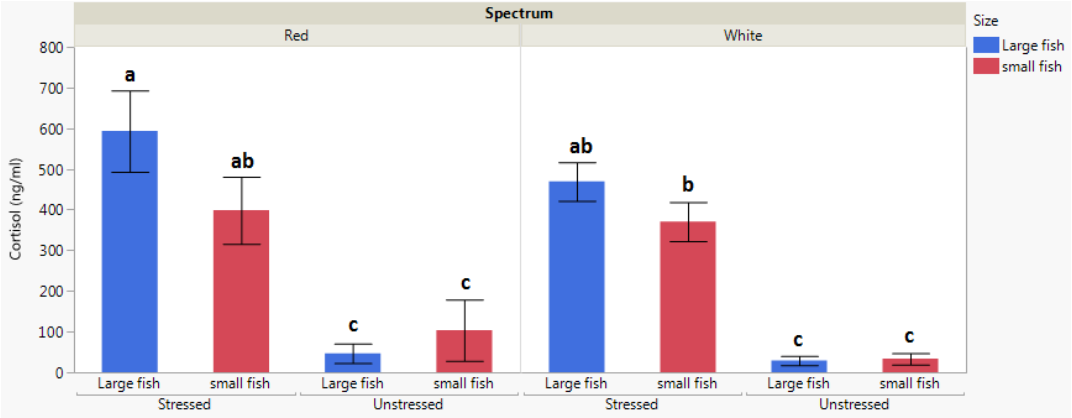


Fig. 2.2.5: Effects of the light spectrum on plasma cortisol (ng.ml^{-1}) at day 76. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

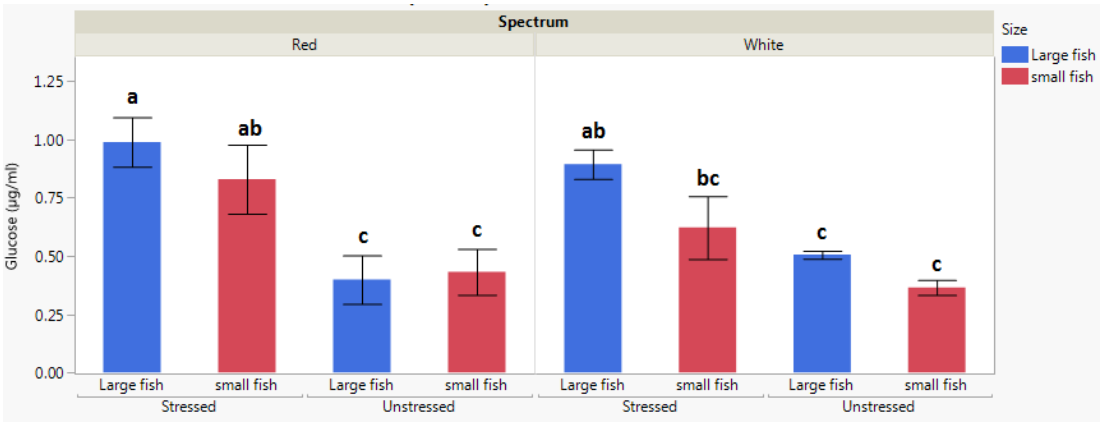


Fig. 2.2.6: Effects of the light spectrum on plasma glucose (µg.ml^{-1}) at day 43. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

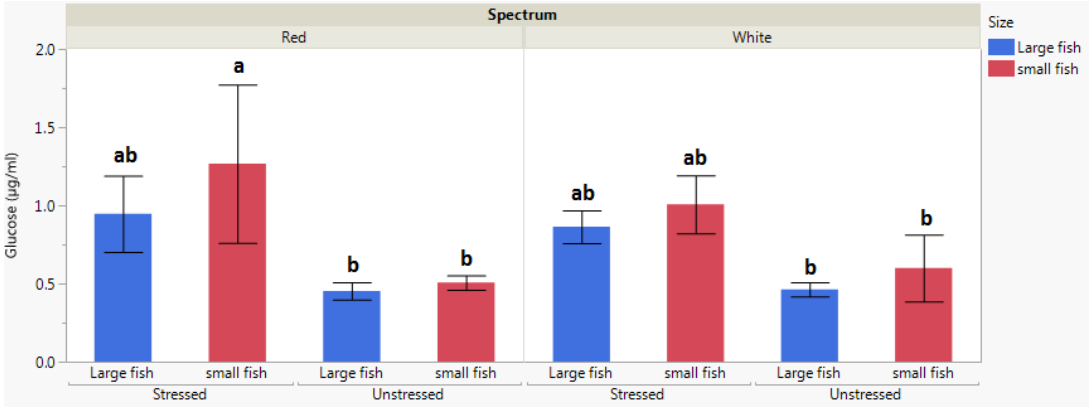


Fig. 2.2.7: Effects of the light spectrum on plasma glucose (µg.ml^{-1}) at day 76. Data are presented as mean ± 1 s.d. (n = 3). Statistical differences are indicated by different letter annotations.

Laboratory validation experiment

Description and objective

Light is one of the environmental factors that profoundly affect the life of fish and unsuitable light characteristics may induce high stress which may negatively affects immunity and growth of reared species. Environmental colours may affect the vision of fishes, influencing food intake, reproduction, growth and even survival, while light intensity can affect many behavioural and biological processes, mainly foraging and learning ability. Light-induced stress may be of particular concern in pikeperch. Indeed, this species possesses a *tapetum lucidum* that is a specific anatomo-histological tissue of the retina which greatly amplifies the eye sensitivity to light.

In the experiments of Task 22.1, it was demonstrated that unsuitable light characteristics could influence physiology and immune status of pikeperch, and by the way, an impact on the circadian rhythm of endocrine and immune activities can be hypothesized.

Experimental design

Pikeperch juveniles (mean weight 25 g) were randomly distributed into 24 tanks and maintained for 4 weeks under a 10-lux white light with constant photoperiod (LD 12:12). New light conditions, including 2 light spectra (white *vs* red) and 2 intensities (10 *vs* 100 lux) were then applied. To avoid stress artefact of nocturnal fishing on diurnal samplings, the number of tanks was doubled. Each treatment group had 3 replicates and 4 individuals were sampled for each treatment. Samplings occurred during scotophase (4 am) and photophase (4 pm), at both days 1 and 30. Growth as well as stress and immune variables were measured.

Results and discussion

Any of the light conditions led to higher cortisol level at D1 or D30 (Fig. 2.2.8). Plasma cortisol reached about 80 ng/ml during the dark phase and significantly dropped to 20 ng/ml during photophase, irrespective of the day of sampling and the light condition (p<0.05). A similar day-night variation was also observed for immune parameters, including plasma lysozyme and peroxydase activities in plasma and expression of immune-relevant genes in the head kidney (Fig. 2.2.9 and 2.2.10). While the change of light spectrum did not affect stress level and immune variables, the increase of light intensity from a 10-lux to a 100-lux light was followed by an increase of the serotonergic activity and a long-term immune suppression (Fig. 2.2.8 and 2.2.10). For this crepuscular predator living in dark environments, the use of a high light intensity in culture conditions should be avoided.

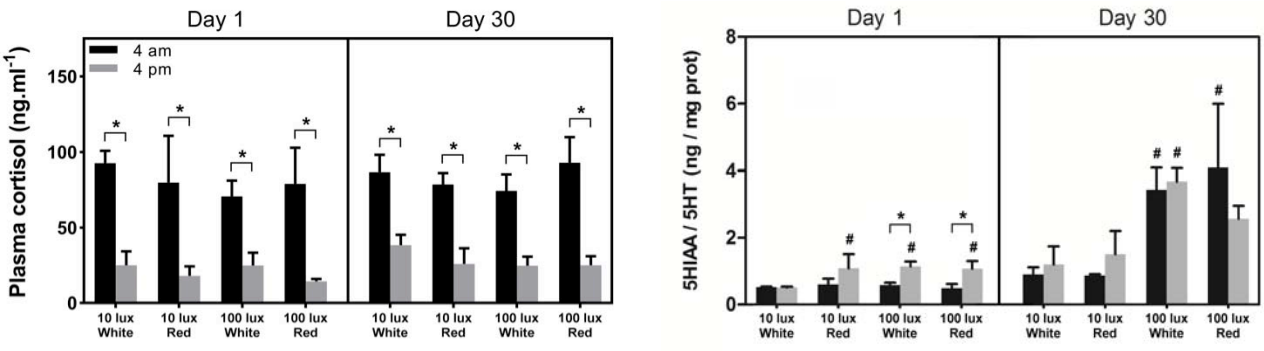


Fig. 2.2.8: Effects of tested factors on (left) plasma cortisol and (right) serotonergic activity in the whole brain on days 0 and 30. Data are presented as mean ± SEM (n = 3). (*) indicates a significant day-night variation at p < 0.05; (#) indicates a significant difference at p < 0.05 with the control group (10 lux white) at the same day.

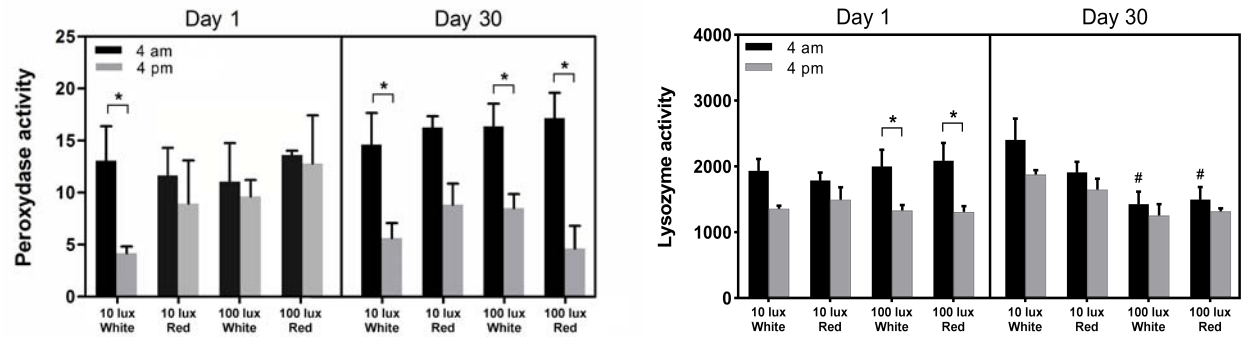


Fig. 2.2.9: Effects of tested factors on (left) peroxydase and (right) lysozyme activities in plasma on days 0 and 30. Data are presented as mean ± SEM (n = 3). (*) indicates a significant day-night variation at $p < 0.05$; (#) indicates a significant difference at $p < 0.05$ with the control group (10 lux white) at the same day.

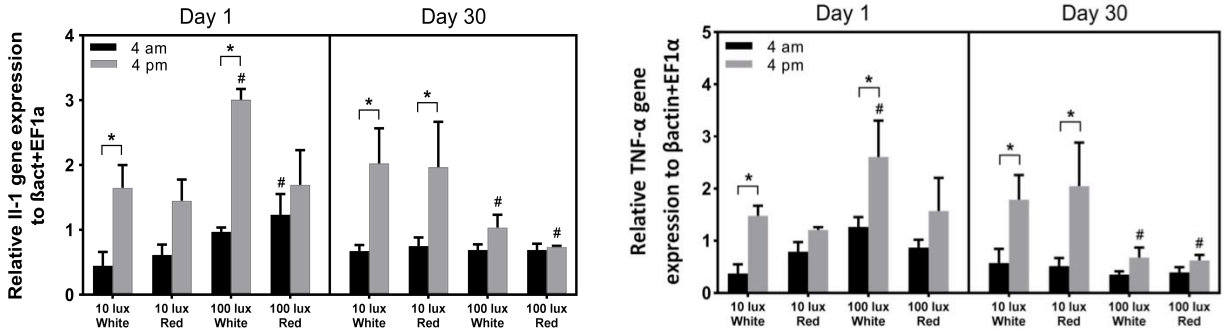


Fig. 2.2.10: Effects of tested factors on (left) interleukine-1 and (right) TNF-α gene expression in the head kidney on days 0 and 30. Data are presented as mean ± SEM (n = 3). (*) indicates a significant day-night variation at $p < 0.05$; (#) indicates a significant difference at $p < 0.05$ with the control group (10 lux white) at the same day.

Task 22.3. Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity (led by FUNDP, Patrick Kestemont).

This experiment has just started on October 2017. Based on results from the microsatellite characterization of domesticated pikeperch broodstock (see Deliverable 4.1 Genetic analysis of domesticated pikeperch broodstocks), three populations were collected according to their domestication level and geographical origin: (*a*) a French strain, F0; (*b*) a Czech strain, F0; and (*c*) a Czech strain, F4.

After being reared in the same conditions in URAFPA facilities, these three batches will be submitted to manipulations mimicking grading. Samplings will occur before and after this stress event, as done in Task 22.2. Stress markers and immune variables will be assessed.

Deviations from Annex I and their impact:

For Task 22.2 (delivery date: week 42)

This task was supposed to start in May-June 2016 in Asialor farm but the work study was transferred to Fish2Be facilities in November 2016. So, the ongrowing experiment started in April 2017 and will finish around January 2018. Considering all the analyses and the manuscript, the deliverable report will be done at the end of May 2018.

For Task 22.3 (delivery date: week 48)

The experiment for this task was delayed because it was not possible to have juveniles of different geographic origins and domestication levels due to a total loss of larvae by a Rhabdovirus occurrence in April 2016 in the URAFPA facilities. Therefore, the *in vivo* experiment for this task has just started in October 2017, and will last 3 months. Considering all the analyses and the manuscript, the deliverable report will be done at the end of May 2018.

WP 23 Grow out husbandry – grey mullet

WP No:	23	**WP Lead beneficiary:**		P4. IOLR
WP Title (from DOW):	Grow out husbandry – grey mullet			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P18. CTAQUA	P25. DOR
P26. GEI	P31. IRIDA			
Lead Scientist preparing the Report (WP leader):	Bill Koven			
Other Scientists participating:	Yannis Kotzamanis (P1), Alicia Estevez, Enric Gisbert (P3), Rocio Robles (P18), Hagay Sarusi (P25), Evangelos Geitonas (P26), Nikos Papaioannou (P31)			

Objectives

4. Evaluating the geographic range for grow-out of grey mullet in the Mediterranean basin,
5. Determine the cost-benefit of different weaning diets on the performance and health status of juvenile grey mullet.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The objectives of WP 23 is the study of some parameters during the grow-out of grey mullet. The first study is related to the definition of an optimal weaning diet. The second study is a multifactorial comparison of different stocking densities and rearing systems with individuals of different origin (wild VS F1) fed an improved diet. During the period preliminary actions took place related to the collection of the required wild juveniles but also the definition of the optimal diet that will be tested. It included the collection of wild grey mullet post-larvae (about 300 mg) which in September 2014, in order to carry out Task 23.3.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In the grey mullet, studies determined the cost-benefit of different weaning diets on juvenile grey mullet performance as well as evaluating the effect of stocking density on the grow out of grey mullet as a function of geographic region. Task 23.1 concluded that it is possible to replace at least 75% of the fish meal dietary component with plant based meals without compromising growth, survival or body composition. Although the feeding trials are on-going in Israel (Task 23.2), Greece (Task 23.3) and Spain (Task 23.4), the picture emerging is that increasing stocking density markedly reduces average fish weight while having little effect on survival resulting in a skewed size distribution to smaller fish.

Summary of progress towards objectives (31-48 Mo):

P4.IOLR in Israel compared the densities of 4 and 6 mullet/m^2 that were fed the **P4.IOLR** mullet grow out diet and demonstrated that the average wet weight (WW) gains in these two density treatments were not significantly different from each other. On the other hand, the effect of higher stocking densities (10 and 12 fish/m^2), which were fed the **P31.IRIDA** extruded diet, did have an effect on average wet weight (WW) and size distribution where the lower density demonstrated significantly higher weight gain. In addition, the FCR was improved suggesting that the extruded **P31.IRIDA** diet, which also replaced poultry meal with fish meal in the diet, was superior to the **P4.IOLR** pelleted diet. Moreover, the fish at the higher density (12 fish/m^2) revealed an FCR of 3.5 while the FCR for fish in the 10 fish/m^2 treatment was 3.0. **P18.CTAQUA** in Spain conducted a pond trial that also demonstrated the effect of density on the final wet weight of the fish. SGRs

for the 0.5 and 1.0 fish/m^2 were 0.83% and 0.73%/d, respectively, after a growing period of 533 days. A greater percentage of smaller fish was found in the 1.0 fish/m^2 treatment compared to 0.5 fish/m^2 while a higher percentage of larger fish was found in the 0.5 fish/m^2 treatment compared to 1.0 fish/m^2. There was a generalized lymphocyte infiltrate in mucosa and lamina propria of the intestine of grey mullet reared at low density while moderate congestion of blood vessels was found in 50% of the samples from the high density treatment. **P1.HCMR** in Greece tested the density effect of stocking wild fry at 4 and 6 fish/m^2 and that weighed *ca* 21 g per fish. This group found no significant differences between treatments in survival, growth performance and size distribution. Although these results largely agreed with **P4.IOLR** trial of the same densities, the Greek fish exhibited only a 30 g/fish gain over the 14 month feeding period.

Details for each Task

Task 23.1. Determine the cost-benefit of different weaning diets on the performance and health status of wild juveniles (led by IRTA, Enric Gisbert).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D23.1 Cost effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status***.

Task 23.2 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of F1 juveniles stocked at two different densities in cement and earthen ponds (led by IOLR, Bill Koven).

Introduction

In the previous periodic report F2 8.23 ± 0.23 g mullet were stocked in two cement (19.0 m^2) and one polypropylene (3.5 m^2) tanks at densities of 55, 29 and 286 fish/m^2, respectively. Unfortunately, due to unexpected Israeli customs bureaucracy and demands, the extruded mullet feed from P31.IRIDA did not arrive in time for the growth trial. However, the fish were fed with a similar formula pelleted IOLR feed (IRIDA feed formula is based on the IOLR formula but used fish meal instead of poultry meal). Fish were fed a ration at 2% of tank biomass that was divided into two daily feedings. There was a clear indication of stocking density on size distribution. At all density levels over 50% of the population was below the average weight, which is an indication of poor growth of the majority of fish. Nevertheless, as density decreased there was concomitant increase in average weight. These results have implications for the monoculture of grey mullet and suggest a significant delay in fish growth and consequently a poor overall FCR in the farmed fish population. Although feeding trials of the Greek and Spanish studies were on-going at the time of this report, the initial growth results exhibited a tendency of higher weight with lower density which agrees with the studies in Israel. In the current report, two studies were carried out using the extruded mullet feed from P31.IRIDA. In experiments 1 and 2 the effect of feeding the extruded diet to 4 and 6 fish/m^2 of F2 fish (108 ± 0.35 g) and to 10 and 12 fish/m^2 of wild fish (44.8 ± 1.44 g) on fish growth and size distribution was tested.

Methods and Materials

Experiment 1

On 28.2.16 four 5m^3 polypropylene tanks, which were in a flow through filtered (8 μm), ambient sea water (40 ‰) system, were stocked with F2 grey mullet juveniles weighing an average of 108 ± 0.35 g. Two of the tanks were stocked with 29 fish each representing a density of 4 fish/m^2 while the other two tanks were stocked with 42 fish each or 6 fish/m^2 . The fish were fed pelleted feed produced at the P4. IOLR for grow-out diets for grey mullet. The fish were fed a ration size according to feed tables developed at P4. IOLR. At the end of about 6 months on 4.9.16, all fish were individually live weighed.

Experiment 2

On 4. 5. 16, two 18 m^3 cement tanks were stocked with 245 and 200 fish (44.8 ± 1.44 g), which allowed for the testing of densities of 12 and 10 fish/m^2, respectively. The tanks were in a flow-through filtered (8 µm) ambient sea water (40 ‰) system and the fish were fed a ration size according to feed tables developed at P4.IOLR. The feed used in this study was extruded and produced by P31.IRIDA. This diet also replaced the poultry meal with fish meal as poultry meal is prohibited in fish feeds in Europe. The study was terminated on 25.12.16 or after almost 8 months where all fish were individually live weighed.

Results and discussion

The fish at the end of the experiment that were stocked at 4 individuals/m^2 had an average weight of 285.34 ±14.0 g which was similar to that (294.65 ± 15.9 g) of fish from the 6 individuals/ m^2 treatment and represented a growth increase of 164.2% and172.8%, respectively. Although the wet weight gain was not significantly (P>0.05) different in both density groups (**Figure 23.2.1**), **Figure 23.2.2** shows that at the end of the study, the fish from the 4 fish/m^2 were more concentrated in the weight range of 200-350 g which represented 77.6% of the population while this range represented only 48.8% of the population of fish stocked at 6 fish/m^2. On the other hand, larger fish (350-550 g) from the 6 fish/m^2 treatment represented a larger part of the population (29.1 %) than the lower density of 4 individuals/m^2 which was only 12.1 % of the population (**Figure 23.2.2**). Moreover, the 4 fish/m^2 treatment demonstrated an FCR of 3.26 whereas fish in the higher stocking density showed a FCR of 3.78. Overall, the results suggest that average growth rate was not markedly affected by these low stocking densities although the size distribution and FCR may be influenced.

On the other hand, the effect of higher stocking densities did have an effect on average wet weight (WW) and size distribution. At the end of experiment 2, the average WW of fish in the 12 individuals/m^2 was 219.5 ± 5.0 g (390% increase), which was significantly less (P<0.0001) than fish from the 10 individuals/m^2 that weighed 276.4 ± 8.7 g (517% increase) (**Figure 23.2.3**). In the higher density treatment, 71.2 % of the fish population was represented by smaller fish ranging from 100-250 g whereas the percent of these smaller individuals represented less than half (44.7%) of the population from the lower density of 10 fish/m^2 (**Figure 23.2.4**). In contrast, larger fish (250-550 g) from the lower density treatment represented 52.3% of the population while only 27% of the population from the high density treatment were in this size range (**Figure 23.2.4**). In addition, the FCR was improved with a decrease in stocking density. Fish at higher density (12 fish/m^2) demonstrated an FCR of 3.5 while the FCR for fish in the 10 fish/m^2 treatment was 3.0.

Although there was not enough tank replicates to come to robust conclusions, the higher FCR of the P4.IOLR diet compared to the FCR of P31.IRIDA diet may be expected. The P31.IRIDA diet was extruded which gave it better water stability which made it available to the fish over a longer period than the pelleted P4.IOLR diet. Moreover, the P31.IRIDA diet replaced poultry meal with fish meal, which may have provided other nutrients or a more favorable amino acid composition that promoted growth.

It is well documented that increasing the fish stocking density can lead to decreased growth in an increasing segment of the population resulting in larger numbers of smaller fish as well higher stress and disease among cohorts. This is often attributed to increasing competition for the same food source. However, a recent study carried out on juvenile grey mullet at the P4.IOLR (not listed as a task in the DOW), showed that the effect of increased stocking density on the percent of smaller fish in the population can be significantly reduced if the ration size is doubled and distributed over a higher number of meals. Omnivores and herbivores are constantly grazing resulting in continuous consumption. Carnivores, in contrast, consume prey in discreet meals. Although grey mullet was shown to be an omnivore in this project, the percent biomass used to feed the mullet in the present studies ranged from approximately 1.3 to 2% and was provided in discreet meals, according to the P4.IOLR mullet feeding table. In future studies, the effect of increased ration size and the number of meals per day (simulate continuous feeding), on the reduction of the population of slower growing (high FCR), smaller fish may be a worthwhile avenue of research to increase the efficiency of grow-out. As feed represents a significant cost in the production of grey mullet, pond management that would produce a population with a normal size distribution would be more efficient and profitable.

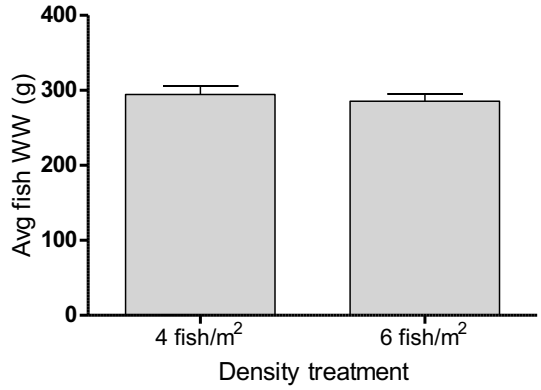


Figure 23.2.1 The effect of two stocking treatments (4,6 fish/m2) on the average fish weight. N=86, 58 of the 4 and 6 fish/m^2 treatments, respectively. ANOVA of values was found not significant (P>0.05).

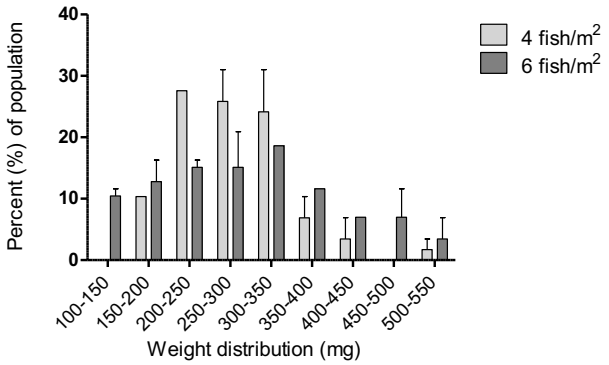


Figure 23.2.2 The effect of the two stocking treatments (4 and 6 fish/m^2) on the weight distribution in the population.

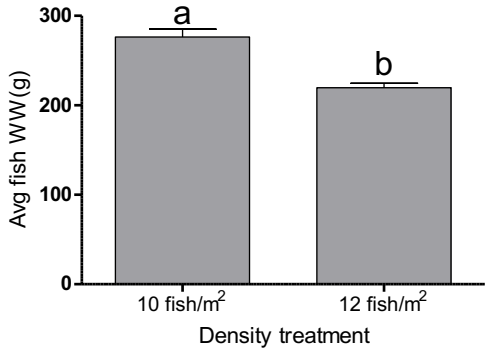


Figure 23.2.3 The effect of two stocking treatments (10 and 12 fish/m2) on the average fish weight. N=200 and 177 of the 12 and 10 fish/m^2 treatments, respectively. 2 tailed T test was performed and was found significant (P<0.0001).

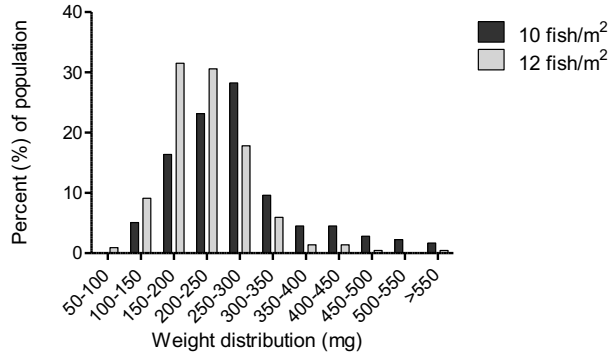


Figure 23.2.4 The effect of three stocking treatments (10,11,12 fish/m^2) on the weight distribution in the population.

Task 23.3 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild caught juveniles stocked at two different densities in cement ponds in Greece (led by HCMR, Yannis Kotzamanis).

Introduction

The feeding trial was started in GEI's farm and was described in the previous periodic report F2. Briefly, on July 27, 2015, 600 wild-caught mullets (*Mugil cephalus)* weighing 21 ± 1.4 g were distributed into six 14 m^3 grow-out rectangular cement ponds at the predefined densities (4 and 6 individuals per m^2, 80 and 120 fish per pond, respectively) using three replications for each density. Fish fed a species specialized extruded diet formulated by IOLR and produced by IRIDA SA.

Methods and Materials

The cement ponds were continuously supplied with artesian bore water with a dissolved oxygen level around 8 ppm, which is considered optimum and water temperature at 18-22°C. The photoperiod followed the natural cycle of the season. The feeding trial started using the experimental extruded feed (IRIDA mullet 1.5mm) provided by IRIDA SA. The feeding ration was 2% of tank biomass and was performed 2 times per day (09:00 & 15:00 h), six days a week and the daily feed intake was recorded. Feeding was not performed when the farm staff observed feed wastes at the bottom of pond from the previous feeding. Monitoring of fish health and feed consumption as well as a recording of water physicochemical parameters were performed daily. The feeding trial was completed on 4.11.16 (**Picture 1**), and it lasted in total 14 months. At the end of the trial, all fish from each pond were anesthetized and individually weighed.

Results and discussion

There were no significant differences in survival and growth performance of wild fry between the two densities treatments at the end of the experiment. The survival of fish was similar in both density treatments, with values of 72.1 ± 11.3% and 75 ± 5.3% for 4 and 6 fish/m^2 treatments, respectively. Fish that were stocked at 4 individuals/m^2 had an average weight of 51.6 ±11.2 g, which was similar to that (51.2 ± 8.5 g) of fish from the 6 individuals/ m^2 treatment and represented a growth increase of 143% in both treatments (**Figure 23.3.1**). The frequency analysis showed that the weight range of 20-80 g represented the dominant weights of the fish population in both density treatments (**Figure 23.3.2**). On the other hand, larger fish (80-180 g) from the 6 fish/m^2 treatment represented a somewhat larger part of the population (14 %) than the lower density of 4 individuals/m^2, which was 12 % of the population. Conclusively, there wasn't any clear effect of stocking density on size distribution.

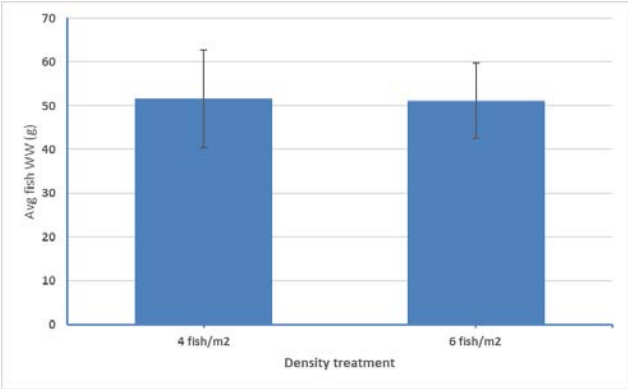


Figure 23.3.1 The effect of two stocking treatments (4,6 fish/m^2) on the average fish weight. N=80, 120 of the 4 and 6 fish/m^2 treatments, respectively. ANOVA of values was found not significant (P>0.05).

Picture1. Final sampling of mullet fish by HCMR's staff in GEI fish farm.

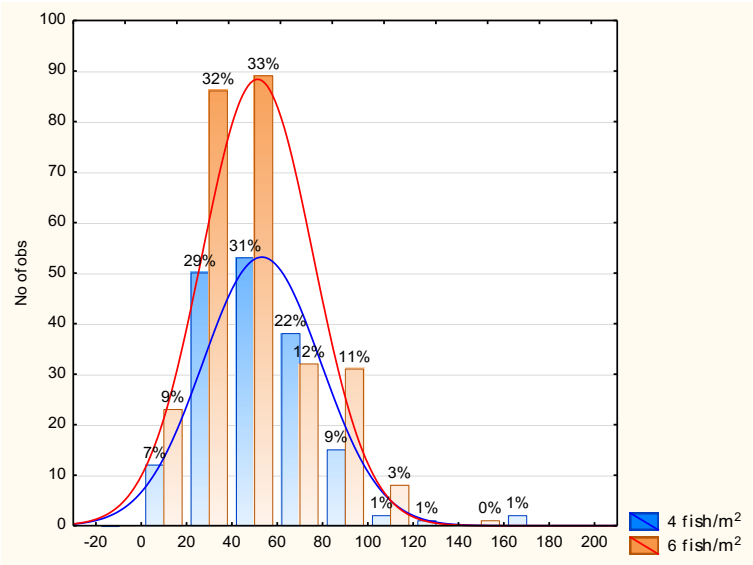


Figure 23.3.2 The effect of the two stocking treatments (4 and 6 fish/m^2) on the weight distribution in the population.

Fish in all ponds were fed the same amount of feed, which was represented a ration at 2% of pond biomass that was divided into two daily feedings. At both density levels, a very poor growth of fish was observed (only ~30 g weight gain) over the 14-month feeding period. Several factors may have negatively affected the fish growth such as the unsuitability of cement ponds for mullet culture, which could have led to improper feeding by the fish, the lack of natural food, palatability and potential nutrient deficiencies of feed, or husbandry practices as well.

Task 23.4 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild juveniles at two different densities in ponds in Spain (led by CTAQUA, Rocio Robles).

Introduction

Most of the work of this task has been reported in the 2nd Reporting period. The trial was finalized during the third Reporting Period (December 2016). Wild grey mullet (*Mugil cephalus*) fingerlings of 1g average body weight were received at the facilities of **P18.Ctaqua** and were acclimatized from 10 ‰ (water salinity from origin) to the 35 ‰ of the recirculation aquaculture system (RAS) water where they were first grown until they reached a suitable size to be seeded in the two earthen ponds at the farm where the field trial has been carried out (Trebujena, province of Cádiz) in southern Spain.

The fish were grown in the RAS system, during 4 months until they reached 3.5g average body weight. During the two weeks before moving them to the ponds, grey mullet fingerlings were acclimatized to the lower salinity of the farm water (12 ‰) and to the feed they would receive in the farm. The feed was provided by **P31.IRIDA** and it is a diet specifically formulated for grey mullet (based on **P4.IOLR** formula) of 1.5 mm diameter and 3 mm for the last part of the growing period.

Materials and Methods

A total of 1.344 fish were moved to two different ponds (L3 and L4) following the densities described in the DOW:

- L3: 1100 m^2; in this pond the density of 0,5 indiv./m^2 was used; 544 fingerlings were seeded.
- L4: 800 m^2; in this pond the density used was 1 indiv/m^2; 800 fingerlings were seeded.

The trial lasted from July 2015 till December 2016 (18 months). During the trial the fish were fed the extruded diet provided by the **P31.IRIDA**. Feed has been provided in two pellet size: 1.5mm diameter for the first growing period and 3 mm for the second growing period. Fish have been fed manually once per day at the first time of the morning to check fish feeding behavior and with automatic belt feeders for the rest of the day.

Four samplings have been performed: at stocking (July 2015), two intermediate samplings, February 2016 and June 2016, and the final sampling in December 2016, where all harvested fish were individually live weighed and measured.

Although it was planned after the sampling of the summer 2016 to modify the culture conditions and collect all the fish from pond L4 to move it to the pond L3, it was not possible to harvest completely the pond L4. In this situation, the culture continued in the two ponds and a final sampling was performed in December 2016. It has to be taken into account the difficulty of this type of field samplings since it is very difficult to congregate all the fish in the net without causing high stress (**Figure 23.4.1)**. Likewise, in the final sampling it was not possible to harvest all the fish from the ponds since the pond cannot be completely emptied (**Figure 23.4.2)**.


Figure 23.4.1. Final sampling in the farm (left). It is a labor intensive task and it is very difficult to empty the ponds completely (right) to harvest all the fish.

Figure 23.4.2. Set-up for the field sampling including extra oxygen supply.

To further document the culture of grey mullet in earthen ponds, at the moment of the final sampling, 10 individuals from each pond were dissected and individual samples of distal intestines were preserved in buffered formalin to evaluate the histological status of the epithelium (task not included in the DOW).

Results

A summary with the results of the four sampling of the trial is included in the **Table 23.4.1.** The results obtained during the pond trial reflect the effect of density on the final wet weight of the fish from both density conditions. Pond L3 (0,5 indiv/m^2) yielded fish with an average final body weight of 294,02 ± 138,89 g and in the pond L4 (1 indiv/m^2), fish had an average final body weight of 174,48 ± 55,36 g. These results are in accordance with the findings of **P4.IOLR**. **Figure 23.4.3** presents the final average body weight of the grey mullet reared at the two densities. Condition index was similar for both culture densities as it is presented in **Figure 23.4.4,** which it is an indication of no differences in feeding conditions between the two ponds.

Final specific growth rate (SGR) values from the two culture conditions were 0,83 %/day for the lowest density pond and 0,73%/day for the highest density, for a total culture period of 533 days. Concerning FCR, no reliable data is available since the farm staff did not always load the automatic feeders everyday based on their observation to the fish reaction when feeding manually at the first time in the morning and did not really follow the advice of the scientific staff. Taking this into account and considering an estimate of 30% mortality (survival cannot be better estimated since the ponds were not fully harvested), the available data for calculation provides an overall FCR of 0,46 which is not considered reliable. In any case, this indicates that the grey mullet grown in earthen ponds is able to feed on the food sources present in this type of ecosystem which is their natural environment. A better management of the feed supply could provide a more reliable results in this type of trials.

Table 23.4.1 Data on grey mullet samplings done during the trial.

DATE	POND	INITIAL FISH NUMBER	N (sampled fish)	ABW (g)
01/07/2015	L3	544	-	3,6
	L4	800	-	3,6
04/02/2016	L3	544	57	92,65
	L4	800	88	33,17
21/06/2016	L3	544	28	163,55 ± 28,99
	L4	800	183	62,94 ± 30,78
15/12/2016	L3	544	157	294,02 ± 138,89
	L4	800	97	174,48 ± 55,36

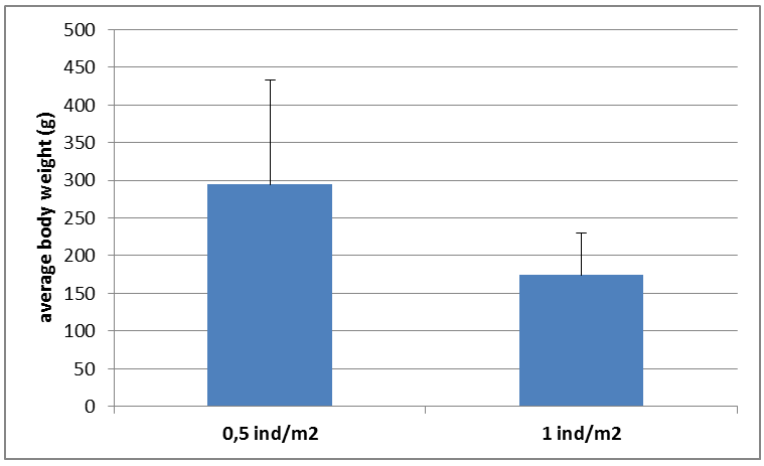


Figure 23.4.3. Final average body weight of the grey mullet reared at the two densities.

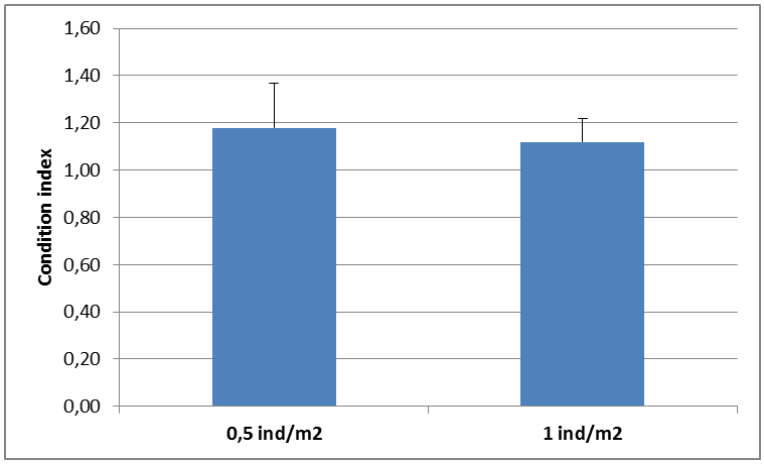


Figure 23.4.4. Condition index of the fish at the final sampling moment.

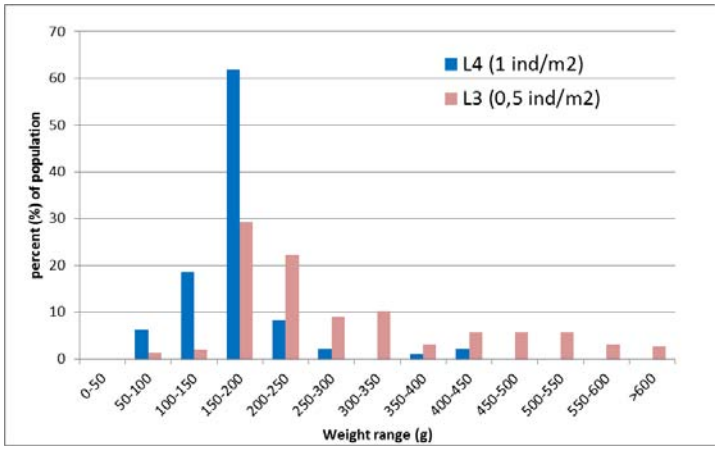


Figure 23.4.5. Weight range distribution of the two culture densities in the earthen ponds.

However, the weight range distribution of the grey mullet at the final sampling (December 2016) shows a skewed distribution of fish size (**Figure 23.4.5**). There is an effect of the culture density on the weight range of the grey mullet. In the higher density treatment, 61.85% of the population is in the range of 150-200 g and only 29.30% of the lower density pond is in that weight range. Moreover, in the high-density treatment only 2.06% of the population is above 400 g average body weight, much lower than the 22.93% of the population of the lower density treatment that is above the 400 g of average body weight. Additionally, from this 22.93%, there is a 11.46% of the fish population above 500 g average body weight.

With regard to the histological analyses of the intestines, the samples from the high density pond were not well preserved during the transport from the farm and could not be sent for histological evaluation. Only the intestines from the lower density treatment were processed and histologically evaluated.

All the intestines samples presented a marked and generalized lymphocyte infiltrate (**Figure 23.4.6)** in mucosa and lamina propria; 50% of the samples showed mild to moderate number of intraepithelial degenerated forms with occasional impact of hydropic degeneration and vacuolation of the mucosal epithelium and few infiltrated eosinophilic granular cells (EGC). Intestinal sub-mucosa displayed mild to moderate mixed cellular infiltrate including mononuclear and EGC infiltrate with focal marked EGC infiltrate and moderate congestion of blood vessels in 50% of the samples (**Figure 23.4.6)**. One of the samples presented in the muscle intestinal layer a mild impact of Myxosporean aggregates (Sphaerospora-like) which is a normal condition in fish cultured in earthen ponds (**Figure 23.4.7)**.

Examined intestines presented marked cellular infiltrate in intestinal mucosa and submucosa. These signs are compatible with chronic enteritis with multifocal degenerative signs. No widespread bacteria, parasite or fungal forms were detected in the examined sections.

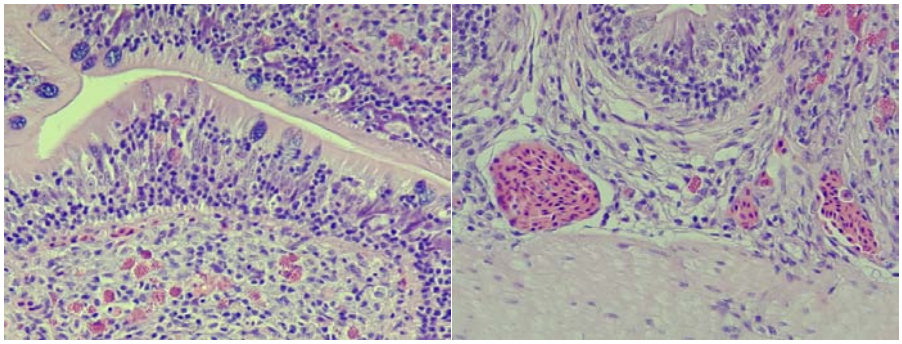


Figure 23.4.6. Left**:** Generalized lymphocyte infiltrate in mucosa and lamina propria of the intestine of grey mullet reared at low density. Right: Image of moderate congestion of blood vessels found in 50% of the samples.

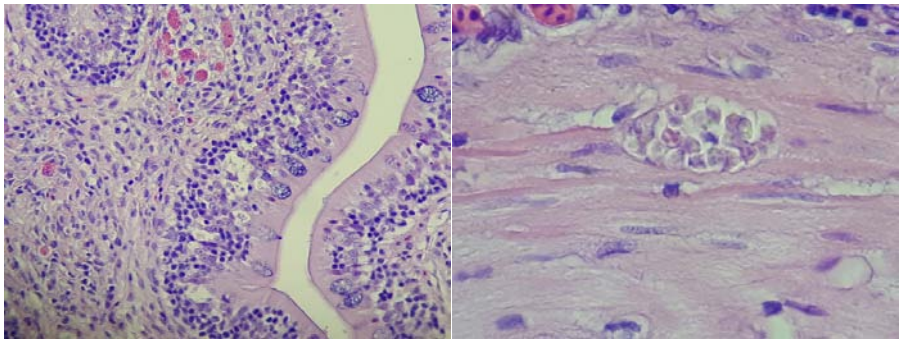


Figure 23.4.7. Left: hydropic degeneration and vacuolation of the mucosal epithelium. Right: Myxosporean aggregates (compatible with Sphaerospora-like parasite) detected in one fish (magnification x100).

Discussion

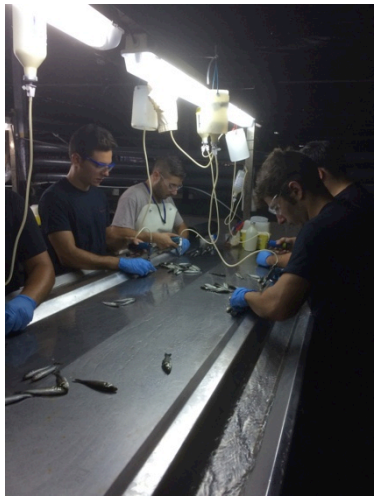
While the trial has been performed with one single replicate of each density condition, the presented results clearly indicate a growth advantage in the lower stocking density (0.5 fish/m^2) and it is consistent with the results from the P4. IOLR in Israel, which also showed a density effect (**Task 23.2**).

Deviations from Annex I and their impact:

Due to the delay in Israeli Customs, Experiments 1 and 2 were postponed for one year. In addition, the extruded IRIDA diet was not used in Experiment 1 and instead the IOLR mullet pelleted diet was fed to the fish. However, the IRIDA diet was fed to the fish in Experiment 2. An experiment investigating the effect of 0.5 and 1.0 fish/m^2 in earthen ponds, using the IRIDA feed is currently running until the spring of 2018. Deliverables D23.2 and D23.3 were scheduled to be delivered in month 30 and 40, respectively. It is planned that they will be delivered in the spring of 2018.

Group Work Packages

Fish health



Good progress has been made in all three WPs relating to Fish Health. In the case of **WP 24** attempts have been made to mitigate against Systemic Granulomatosis (SG) by dietary means. The results from 5 feeding trials show that high inclusion of phosphorus, vitamin C and astaxanthin have beneficial effects concerning the severity of the disease, while plant proteins in the diets have negative effects. The cause of chronic ulcerative dermatopathy has also been studied. In this reporting period analysis of samples obtained from rearing trials in borehole water and natural sea water were finished and indicate the disease can be induced with borehole water. Histology and SEM analysis confirmed that the lesions were limited to the lateral line organ mainly in the head while qPCR analysis showed overexpression of genes connected with specific osteolytic enzymes. Two alternative infestation models were trialled to develop a challenge method for *Scianocotyle pancerii* while another experiment was set up to test the efficiency of cinnamon as an antiparasitic agent against *S. panceri*. The results are currently under analysis. Attempts to identify and isolate *Nocardia* sp. from SG-affected meagre were also undertaken but only a single case of nocardiosis in cultured meagre was found, suggesting it is not the cause of SG. This impacted on our vaccine studies where we have switched to studying a commercially available *Vibrio anguillarum* vaccine rather than try to develop an autogenous vaccine based on *Nocardia* isolates. These trials are scheduled for later this year. Analysis of immune responses have shown that meagre can respond robustly to pathogen derived molecules (PAMPs), and reagents to detect antibody production in meagre have been developed. During the reporting period, two disease outbreaks were recorded, several bacterial strains have been isolated and a challenge test was trialled with *Nocardia*. Such information will contribute to a diagnostic manual for fish health specialists and producers at the end of the programme.

In **WP 25** attempts to identify the causative agent of epitheliocystis have been undertaken. Mesocosm studies gave no clear results but samples collected from fish farms in Greece revealed that, in contrast to the prevalent belief that Epitheliocystis is caused by Chlamydia, at least in Greece the main pathogens causing Epitheliocystis disease are intracellular bacteria that belong in the β- or γ-proteobacteria. Studies to promote resistance to parasitic incidence on greater amberjack trialled two different prebiotics, MOS and cMOS. Positive effects were found for cMOS and prebiotic combination (MOS + cMOS) following challenge with the monogenean *Neobenedenia girellae*. Immune gene expression analysis of skin and gills also showed positive effects with cMOS. Whether cMOS could impact on bacterial load was also studied. After feeding for 90 days the fish were stressed by crowding and prevalence of opportunistic bacteria detected in tissues. Crowding resulted in 100% prevalence for opportunistic bacteria in liver and spleen, with more bacterial species (Vibrio's) present in control diet fed fish vs cMOS fed fish. Studies of an antimicrobial peptide (piscidin) from greater amberjack showed good bacterial growth inhibition against two fish pathogens. The effect of stocking density on parasite (*Neobenedenia melleni*) egg production was also studied and revealed that egg number tended to decrease with stocking density. Several anti-attachment factors were trialled and two treatments (cumin and mannose) showed a reduction in egg number. Mannose in particular looked promising and further optimisation of treatment dose and frequency may lead to potential application in the control of monogenean parasites. These practical applications will be incorporated into a diagnostic manual, to be published at the end of the programme as for WP24/meagre.

Lastly, in **WP 26** attempts to vaccinate Atlantic halibut against nodavirus (VNN) were made. The VNN capsid protein expressed in different expression systems was delivered to halibut larvae by injection or in feed (via *Artemia*). The juveniles were challenged with VNN 10 weeks after vaccination, and samples collected post challenge to assess for effect of vaccination on protection and immunity (analysis on-going).

WP 24 Fish health – meagre

WP No:	24	**WP Lead beneficiary:**		P1. HCMR
WP Title (from DOW):	Fish health - meagre			
Other beneficiaries (from DOW):	P2. FCPCT	P3. IRTA	P5. UNIABDN	P20. SARC
Lead Scientist preparing the Report (WP leader):	Pantelis Katharios			
Other Scientists participating:	Stavros Chatzifotis, George Rigos, Efi Cotou, Marianna Tsertou, Maria Smyrli (P1), Daniel Montero (P2), Ana Roque, Karl Andree (P3), Chris Secombes (P5), Ramon Fontanillas (SARC)			

Objectives

1. Identify the causes of systemic granulomatosis (SG), and chronic ulcerative dermatopathy,
2. Investigate anti-parasite treatments in juvenile meagre,
3. Undertake preliminary characterisation of immune genes and study specific immune responses post-vaccination,
4. Evaluate the occurrence of *Nocardia* infections in meagre and develop an autogenous vaccine,
5. Develop diagnostic-prevention-treatment protocols for diseases in meagre.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Task 24.1 Systemic granulomatosis in meagre. Two of the five feeding trials in HCMR and FCPCT have started and analysis was ongoing. The characterization and the detailed description of the disease has also been commenced. **Task 24.3** Antiparasitic treatments led by IRTA have started with preliminary investigations on the acceptance of medicated feeds by juvenile meagre. **Task 24.4** Nocardia infection in meagre led by HCMR. Isolation attempts for the pathogen have been started and despite intense sampling effort no nocardia-related strain has been recovered. **Task 24.5** First characterization of the immune system led by UNIABDN. Considerable progress was reported with the sampling of fish for obtaining tissues and organs from various stages completed and preliminary results on immune gene characterization on track. **Task 24.7.** Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in meagre led by FCPCT. Bacterial sampling for recording and characterizing pathogens was initiated. Analysis was ongoing.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The progress of the specific WP is in accordance with the provisions of the DoW. **Task 24.1** is dedicated to the study of Systemic Granulomatosis (SG) of meagre. In this task we have foreseen 5 feeding trials where we will test different diets in relation to the development of the disease. Three trials have already finished, two of which submitted in the form of Deliverable. The last two trials are scheduled for this year. From the above task we have obtained significant insights concerning the development of the disease and its pathobiology. In addition, we have seen that both high inclusions of Phosphorus and astaxanthin have beneficial effects concerning the severity of the disease. **Task 24.2** is related to the chronic ulcerative dermatopathy. We have finished the rearing trials in this reporting period and have already obtained the

samples to be analysed. Analysis is still in progress; however preliminary results confirm the hypothesis that the disease is related to the use of borehole water. Further, qPCR analysis has indicated that there is overexpression of the genes, which are connected with the specific osteolytic enzymes showing that the mechanism of the disease involves the activation of the osteoclasts by the increased CO_2 in borehole water. In **Task 24.3** we investigated various antiparasitic drugs against the most significant parasites of meagre. In the reporting period an experiment was performed in order to assess the acceptability of medicated feeds by juvenile meagre. In **Task 24.4** we have made extensive samplings for the isolation of *Nocardia* spp or related bacterial strains. We have not been able to isolate this pathogen even from severely affected fish using selective microbiological media specific for Acid-fast bacteria. However, this task provides significant information concerning the bacterial pathogens of meagre. In the same task we have foreseen to produce an autogenous vaccine based on the *Nocardia* isolates. Since this has not been achieved we have decided to change the direction and use a commercially available *Vibrio anguillarum* vaccine. **Task 24.5** is dedicated to the characterisation of the immune system. The task has been completed successfully and the relative information has been submitted as a deliverable. The results of this task are of great importance not only for the progress of this WP, but also for future studies that will require molecular markers of the immune system. **Task 24.6** is directly linked to the isolation of *Nocardia* and the production of the autogenous vaccine. Since this is going to change, the task will start this year with a *Vibrio anguillarum* vaccine. In **Task 24.7** we have planned specific challenges with bacterial and viral pathogens. In this period the P1. FCPCT team who are engaged in this task have started the fine-tuning of the challenge experiments using juvenile meagre and the analytical techniques that will be used. Finally, in **Task 24.8** we have been recording diseases occurring in our stocks but also in stocks of collaborating fish farms to develop a diagnostic manual for the diseases of the species. Several incidences have been recorded with an outbreak of monogeneans in broodstock and of mycobacteriosis in cage cultured fish being the most significant.

Summary of progress towards objectives (31-48 Mo):

Task 24.1 is related to the study of Systemic Granulomatosis (SG) of meagre. All of the 5 feeding trials have already finished, three of which are submitted in the form of a Deliverable. From the above task we have seen that both high inclusions of phosphorus, vitamin C and astaxanthin have beneficial effects concerning the severity of the disease, while plant proteins in the diets of meagre negatively affects SG. **Task 24.2** is dedicated to chronic ulcerative dermatopathy. In this reporting period we have finished the analysis of samples obtained from the rearing trials in borehole water and natural sea water. The results indicate that the disease can be induced with the use of borehole water. Histology and SEM analysis confirmed that the lesions were limited to the lateral line organ mainly in the head while, qPCR analysis has indicated that there is overexpression of the genes, which are connected with the specific osteolytic enzymes. In **Task 24.3** two alternative infestation models were devised for the development of a challenge method for *Scianocotyle pancerii* while another experiment was set up to test the efficiency of cinnamon as an antiparasitic agent for meagre infested with *S. panceri*. The results are currently under analysis. The main target of **Task 24.4** was to identify and subsequently isolate *Nocardia* sp. from SG-affected meagre. Following extensive samplings, we have identified only one single case of nocardiosis in cultured meagre from a fish farm in West Greece. The conclusions of this task are that nocardiosis is present in Greece; however, it is not the cause of SG. In the same task we had proposed to produce an autogenous vaccine based on the *Nocardia* isolates. Since none were found until after the 2nd periodic report we decided to change direction and use a commercially available *Vibrio anguillarum* vaccine. The trials for this task are scheduled for this year**. Task 24.5** is dedicated to the characterisation of the immune system. The task has been completed successfully and the information has been submitted as a Deliverable. Furthermore, manuscripts have been published in peer-reviewed journals covering description of gene expression analysis of several of the identified immune transcripts from meagre. **Task 24.6** has been completed and the results of the analysis of antibody and cytokine kinetics post stimulation with PAMPs, has been reported in the Deliverable. In **Task 24.7** we have planned specific challenges with bacterial and viral pathogens. During the reporting period, two disease outbreaks were recorded and a challenge test was conducted with the isolated *Nocardia*. Finally, **Task 24.8** is still in progress and runs throughout the lifespan of the WP. Several bacterial strains have been isolated; however, none of these can be considered a primary pathogen and are probably environmental opportunists.

Details for each Task

Task 24.1. Systemic Granulomatosis (led by HCMR, Pantelis Katharios).

Sub-task 24.1.1. Feeding trials (HCMR, Pantelis Katharios)

Trial 1. (HCMR) The effect of 3 levels of dietary vitamin D in the development of SG

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.1 The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre***.

Trial 2. (HCMR) The effect of various dietary Ca/P ratios in the development of SG

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.2 The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre.***

Trial 3. (HCMR)

Meagre of approximately 2g in weight, produced in June 2016 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the feeding trial. In total, 600 fish were weighed and placed into 12 500-l cylindrical tanks at a density of 50 fish per tank. Three replicates were allocated to each diet. Four experimental diets were formulated at SKRETTING Aquaculture Research Centre (SARC) with 60% and 14% fishmeal and increasing levels of phosphorus in the diets with 14% fishmeal. Fishmeal replacement in the diet with 14% fishmeal was achieved using plant proteins derived from corn gluten, wheat gluten and soya concentrate. The feeding trial lasted 3 months (1 August 2016- 1 November 2016). Assessment of fish was made at several different levels including growth rates, feed efficiency and general zootechnical measurements, visual examination of various tissues and organs for the presence and the development of granulomas, hematology and serum biochemistry, histology and histopathology.

The present trial showed that the plant proteins in the diets of meagre negatively affect SG. Fish fed 60% fishmeal were in a significantly better state regarding the total score of granulomas in all tissues **(Figure 24.1.1).** Furthermore, fish of this diet group exhibited a significantly lower percentage of liver and spleen calcification and there was a significantly higher percentage of fish with no granulomas in these organs compared to those fed the plant protein diets **(Figure 24.1.2)**.

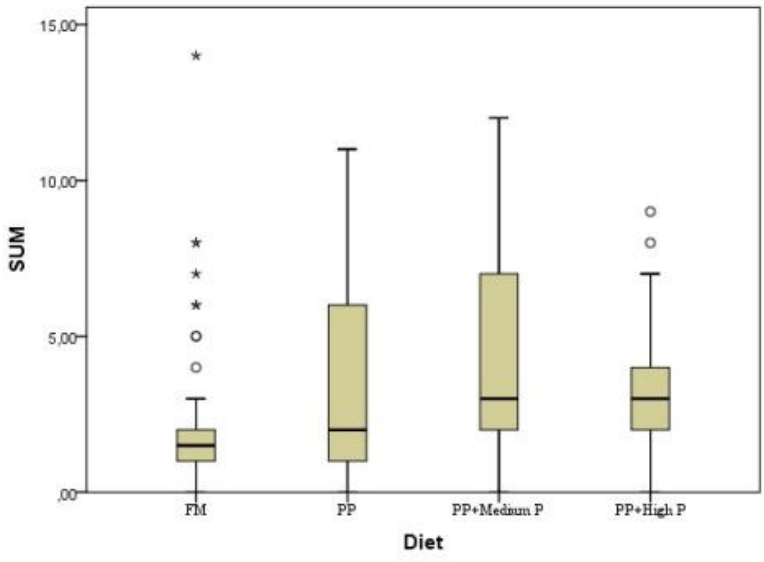


Figure 24.1.1. Boxplots of meagre's total score of granulomas at the end of the experiment fed diets with plant proteins and increasing levels of P at the end of the feeding trial. Outliers are presented as circles and extreme scores as asterisks. The medians of the groups are significantly different (H(3)=41.455, p=0.000)

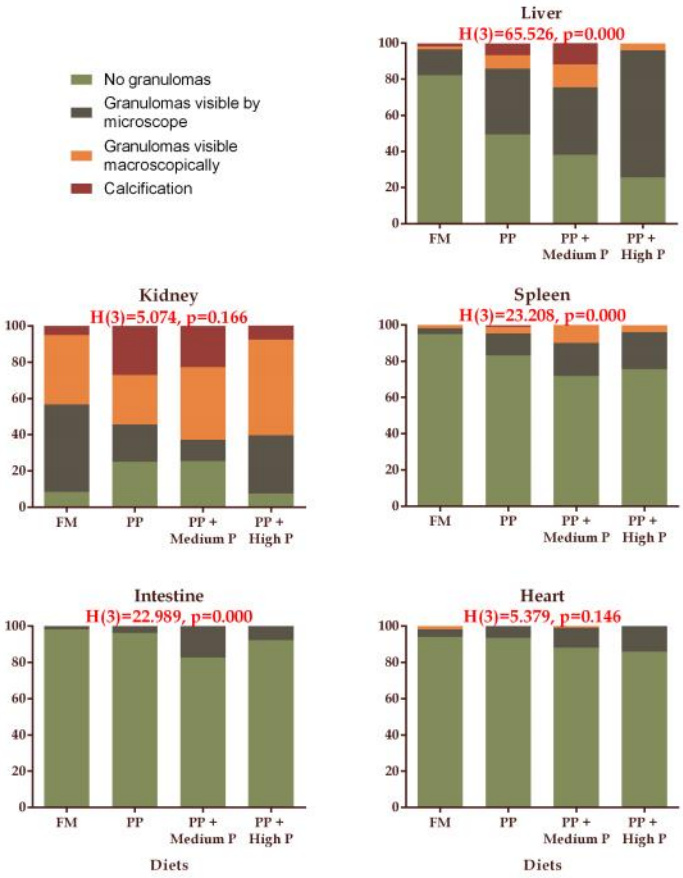


Figure 24.1.2. Percentage of the fish fed diets with plant proteins and increasing levels of P in each of the 4 categories of the granulomas scoring system (no granuloma, granulomas visible only with microscopy, granulomas visible macroscopically and tissue calcification) for every tissue examined. Kruskal-Wallis test results are indicated with red letters.

Furthermore, histological assessment confirmed the results of the visual inspection performed in fresh preparations while another interesting histological finding, which is not directly linked to SG is that high plant protein diets were found to induce excessive vacuolization in the intestinal villi of meagre. Moreover, the results obtained in this trial showed that a reduction of fishmeal from 60% to 14% is possible for juvenile meagre in terms of growth performance, only in combination with high levels of phosphorus supplementation (14 g kg^{-1}).

The full details concerning this task have been submitted in ***Deliverable 24.5 The effect of high plant protein diets in the development of Systemic Granulomatosis in meagre.***

Trial 4. (FCPCT-Daniel Montero) Effects of vitamins E, C, plus astaxanthin.

Results of this trial were reported in the previous report.

Trial 5 (FCPCT) (FCPCT-Daniel Montero). **The effect of minerals in SG prevention.**

The objective of this study was to elucidate the involvement of the dietary vitamins E and C and the addition of dietary Mn, Zn and Se on the appearance and incidence of systemic granulomatosis in meagre. To reach

this objective, diets containing several graded levels of the three vitamins and minerals were formulated (SARC) and fed to juvenile meagre (FCPCT). Growth, survival, histopathological evaluation, biochemical and gene expression of oxidative enzymes were determined.

MATERIAL & METHODS

Diets

The formulation, proximate composition and fatty acid content of the experimental diets are shown in **Table 24.1.1.5.1. & 24.1.1.5.2** respectively. Five isolipidic (16.7% lipid) and isoproteic (49.6% protein) fish meal and fish oil based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se. Diet C (100 $mg \cdot kg^{-1}$ C), Diet C+Mn/Zn/Se (100 $mg \cdot kg^{-1}$ C, 40 $mg \cdot kg^{-1}$ Mn, 200 $mg \cdot kg^{-1}$ Zn, 1.5 $mg \cdot kg^{-1}$ Se), Diet CC (600 $mg \cdot kg^{-1}$ C), Diet CCC (1200 $mg \cdot kg^{-1}$ C), Diet CCCC (3200 $mg \cdot kg^{-1}$ C). The analysed dietary content of vitamin E, C and K and minerals Mn, Zn and Se for each treatment is showed in **Table 24.1.1.5.2.**

Table 24.1.1.5.1. Table Raw material composition and analysis of the experimental diets

	Diet				
Raw Material (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
Wheat	17,39	17,37	17,22	17,03	16,38
Corn gluten	5,00	5,00	5,00	5,00	5,00
Wheat gluten	6,78	6,79	6,86	6,95	7,25
Soya concentrate	25,08	25,07	25,02	24,95	24,73
Fish meal	35,00	35,00	35,00	35,00	35,00
Fish oil	10,41	10,41	10,41	10,41	10,41
Phospahte	0,14	0,14	0,14	0,14	0,14
Vitamin E	0,03	0,03	0,03	0,03	0,03
Vitamin C	0,01	0,01	0,16	0,33	0,90
Premix vit min	0,10	0,10	0,10	0,10	0,10
Vitamin K	0,00698	0,00698	0,00698	0,00698	0,00698
Astaxanthin	0,05	0,05	0,05	0,05	0,05
Zinc sulphate	0,00	0,01111	0,00	0,00	0,00
Selenium Sodium selenite	0,00	0,00115	0,00	0,00	0,00
Manganese Manganese sulphate	0,00	0,00261	0,00	0,00	0,00
[VOLUME]	100,00	100,00	100,00	100,00	100,00
DRY_MAT	91,78	91,78	91,79	91,82	91,89
V MOIST	8,23	8,23	8,21	8,19	8,12
C PROT	50,00	50,00	50,00	50,00	50,00
C FAT	16,00	16,00	16,00	16,00	16,00
ASH	7,44	7,46	7,54	7,66	8,07
Zinc (mg/kg)	161,11	200,00	160,97	160,80	160,24
Manganese (mg/kg)	31,65	40,00	31,61	31,56	31,39
Selenium (mg/kg)	0,98	1,50	0,98	0,98	0,98
VIT E (mg/kg)	300,00	300,00	300,00	300,00	300,00
VIT K (mg/kg)	35,00	35,00	35,00	35,00	35,00
VIT C (mg/kg)	100,00	100,00	600,00	1200,00	3200,00
ASTA (mg/kg)	50,00	50,00	50,00	50,00	50,00

Table 24.1.1.5.2. Diets fatty acid composition (percentage of fatty acids) in diets of meagre. Data expressed as means of three technical replicates per batch of diet.[1]Includes 15:0 and 17:0.[2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5.[3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	**C**	**C+Mn/Se/Zn**	**CC**	**CCC**	**CCCC**
14:00	7.97	7.52	7.64	7.55	8.12
16:00	22.49	21.49	20.88	20.28	22.20
18:00	4.32	4.14	4.06	3.98	4.30
20:00	0.30	0.38	0.33	0.32	0.35
Σ Saturated[1]	36.60	35.00	34.47	33.71	36.61
16:1n-7	7.76	7.44	7.60	7.64	8.11
18:1n-9	12.07	12.89	11.18	10.91	11.62
18:1n-7	3.14	3.06	3.01	3.00	3.19
20:1n-7	3.21	3.11	2.86	2.75	2.98
22:1n-11	4.59	4.38	4.02	3.84	4.15
Σ Monosaturated[2]	32.92	32.98	30.73	30.15	32.19
18:2n-6	6.14	6.72	5.57	5.14	5.28
18:3n-6	0.28	0.27	0.29	0.29	0.30
20:2n-6	0.23	0.23	0.23	0.23	0.25
20:3n-6	0.10	0.10	0.11	0.11	0.12
20:4n-6	0.73	0.74	0.81	0.85	0.87
Σ n-6PUFA[3]	7.64	8.21	7.18	6.81	1.71
18:3n-3	1.09	1.26	1.09	1.08	1.13
18:4n-3	1.77	1.82	2.08	2.19	2.19
20:3n-3	0.09	0.09	0.09	0.09	0.09
20:4n-3	0.49	0.49	0.55	0.58	0.59
20:5n-3	7.87	8.14	9.60	10.26	10.24
22:5n-3	0.91	0.94	1.11	1.19	1.19
22:6n-3	7.84	8.32	10.07	10.72	10.73
Σ n-3PUFA[4]	20.06	21.07	24.59	26.10	26.15
(n-3+n-6) PUFA	27.70	29.27	31.77	32.91	27.86
Total n-3 LC-PUFA[5]	1711	1789	2133	2275	2275
Proximate composition					
Proteins (%)	49.6	49.3	49.6	48.9	49.5
Lipids (%)	16.8	16.8	16.4	16.8	16.5
Moisture (%)	7.2	8.0	7.9	8.2	7.6
Ash (%)	7.1	6.9	7.0	7.0	7.4
Vitamin E (mg/kg)	228	242	243	241	255
Vitamin C (mg/kg)	98	96	586	1180	2835
Vitamin K (mg/kg)	23	23	23	22	23
Mn (mg/kg)	37	49	34	34	35
Zn (mg/kg)	130	180	130	140	140
Se (mg/kg)	1.1	1.6	1.1	1.1	1.2

Experimental fish

The experiment was carried out at the FCPCT facilities (Las Palmas, Canary Islands, Spain). The juvenile meagre were obtained from induced spawns at the FCPCT facilities. Juveniles were acclimated to the experimental condition and the basal diet for 2 weeks. The experiment was carried out in 21 fibre glass tanks of 500 L with 100 fish/tank (3.20 kg/m^3) for all the diets. The initial mean weight was 15.75 ± 0.56g. Fish were reared under the natural light conditions throughout the feeding trail. The juvenile meagre were fed 3 times per day (8:00, 11:30, 15:00), 6 days per week during 90 days with the different experimental diets. All the uneaten feed was collected daily from each tank and dried in order to calculate the daily feed intake. Dead fish were recorded daily and survival was determined. After 90 days of feeding samples were collected for histology, biochemistry analysis and gene expression analysis.

Growth performance

Growth data were analysed according the following equations: Survival (%) = 100*(final number fish – initial number fish)/ initial number fish; Growth (%) = ((final mean weight – initial mean weight)/initial mean weight)*100; Weight gain = (final mean weight- initial mean weight); SGR (specific growth rate) = 100 x (ln final mean weight – ln initial mean weight)/ number of days; FI = feed intake (g)/fish per day; FCR (feed conversion ratio) = feed intake (g)/ weight gain (g); K (condition factor (%)) = 100*(fish weight/ (fish length)3); HSI (hepatosomatic index (%)) = 100*(liver weight / fish weight); VSI (viscerosomatic index (%)) = 100* (fish weight - eviscerated fish weight)/fish weight.

Histopathology

At the beginning (n = 50) and at the end of the trial (n = 30 fish per diet) fish were sacrificed and samples of liver, kidney and heart were collected and fixed in 4 % buffered formalin for histological analysis. The samples were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm, fixed to the microscope slide, heated and finally stained with haematoxylin and eosin (H&E), Ziel-Neelsen (ZN), Fite-Faraco method and Gram stain. Then, the samples were used for histopathological evaluation.

Histopathology scoring

The severity of granulomatosis was individually scored in each organ. The severity of the granulomas was classified in each organ depending on the number of granulomas observed during the microscopy evaluation. The average severity was classified in liver, kidney and heart according to the criteria shown in Table **Table 24.1.1.5.3**.

Table 24.1.1.5.3. Severity score of granulomas in liver, kidney and heart.

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Biochemical analysis

Feed and fish biochemical composition analysis were conducted following standard procedures. Lipids in liver, heart, kidney and feeds were extracted with a chloroform-methanol (2:1 v/v) mixture following the Folch method. Protein content (Kjeldahl method), dry matter and ash were also determined. Fatty acids from total lipids were prepared by transmethylation. Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following the standardized conditions of FCPCT facilities. The concentration of vitamins in diets was analysed by HPLC. TBARs were measured in triplicate from extracted total fatty acids (10mg/ml) of liver, kidney and heart.

Gene expression

Kidney, liver and heart were aseptically collected from 4 fish per tank at the final sampling and stored at -80°C until further analysis. Total RNA was extracted from, approximately, 100 mg of sample using TRI Reagent® (Sigma). Purity was assessed by spectrophotometry (A260/A280), followed by a visual quality assessment via agarose gel electrophoresis on 2% agarose gel stained with GelRed ™ Nucleic Acid Gel Stain (Biotium). The cDNA was synthesized from 1 µg of total RNA using the iScript cDNA Synthesis Kit (BIORAD) in 20 µl reactions, which included 4 µl 5× iScript Reaction Mix, 1 µl iScript Reverse Transcriptase (BIORAD), 13 µl Milli-Q sterile water and 2 µl RNA (1 µg) of the sample. The reverse transcription was done in a thermal cycler (iCycler) at 25 C for 5 min, 60 min at 42°C and finally heating samples for 5 min at 85°C. PCR primers sequences used for the PCR amplification of the cDNAs of the target genes were CAT, SOD and GPX. The relative transcript abundance of glutathione peroxidase, superoxide dismutase and catalase was determined by quantitative real time PCR (qPCR). Primer efficiency for each gene was previously evaluated to ensure that it was close to 100%. All PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate using 10 µl Thermo Scientific Luminaris Color Higreen qPCR Master Mix (Bio-Rad Hercules, California), 1 µl of forward and reverse primers (100 pmol·µl^{-1}), 6 µl water nuclease-free and 5 µl of a 1:10 dilution of the cDNA, with the exception of the reference genes, which were determined using 2 µL of cDNA, in a final volume of 20 µl. In addition, amplifications were carried out with a systematic negative control (NTC non-template control) containing no cDNA. The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50°C for 2 min, a denaturation at 95°C for 10 min, followed by 35 cycles: 15 s at 95°C, 30 s at the annealing Tm and 30 s at 72°C. Expression level of each gene was normalized by the corresponding expression of β-Actin, Elongation factor 1α and Tubulin, which were chosen as the most stable according to GeNorm.

Statistical analysis

All statistical analyses were done with Statgraphics. The normality was checked with the test Kolmogorov Smirnov test. The homogeneity of variance was performed with the Levene test. With the variables that satisfied the normality and homogeneity was carried out a parametric one-way (ANOVA) and Tukey test. For non-parametric variables, data which did not display a normal distribution and homogeneity of variance, a Kruskal-Wallis test was used. To compare two variables, a t-student test was used for the variables with normality and a Mann-Whitney test for the non-parametric. A significance level of 0.05 was used. The number of fish scored in each level of severity was evaluated in a frequency distribution manner.

RESULTS

Growth performance

Inclusion of different levels of dietary C and minerals Mn, Se and Zn did not affect meagre final weight, length and growth parameters **(Table 24.1.1.5.4).** Growth, SGR, FCR, and K factor were significantly higher in the treatments with low density (100 fish/tank). Juvenile meagre grew from ~ 15.75 g to ~ 94 g in 90 days. A good food conversion ratio (FCR) was obtained among all the dietary treatments (0.75~0.80) but without significant differences.

Table 24.1.1.5.4. Meagre (*Argyrosomus regius*) growth performance after being fed for 90 days with diets containing different levels of vitamin C and Mn, Zn and Se. Data are means ± SD. Values in each row with a different superscript are significantly different (P<0.05). FCR, food conversion ratio; SGR, specific growth rate.

Diet	Final weight (g)	Length (cm)	FCR	SGR	survival (%)
C	94.05 ± 21.05^{ab}	18.12 ± 1.63	0.75 ± 0.01^{a}	2.02 ± 0.02b	98.3 ± 3.6
C+ Mn/Se/Zn	94.94 ± 18.96^{b}	18.07 ± 1.25	0.76 ± 0.02^{a}	1.99 ± 0.05ab	97.8 ± 2.0
CC	94.66 ± 23.16^{ab}	18.07 ± 1.50	0.76 ± 0.01^{a}	2.00 ± 0.03ab	98.0 ± 2.0
CCC	96.83 ± 22.08^{b}	18.15 ± 1.40	0.75 ± 0.01^{a}	2.02 ± 0.06b	98.7 ± 3.1
CCCC	96.03 ± 23.75^{b}	18.24 ± 1.51	0.76 ± 0.01^{a}	2.00 ± 0.02ab	97.8 ± 3.2

Tissue lipid content and fatty acid profiles

There were no differences in the tissue lipid content among the fish fed the different experimental diets, being for kidney around 4.5%, liver 16 % and heart 4.4%. The fatty acid profile of the tissues of fish reflected the dietary fatty acid content. The highest levels of total monounsaturated fatty acids were observed in liver, followed by kidney and heart, however the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) was higher in the heart, followed by kidney and liver. All the other fatty acids were similarly distributed in the three tissues (**Table 24.1.1.5.5, Table 24.1.1.5.6. & Table 24.1.1.5.7**).

Table 24.1.1.5.5. Hepatic fatty acid composition (percentage of fatty acids) of meagre fed different experimental diets. Data are expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	C	C+Mn/Se/Zn	CC	CCC	CCCC
14:00	2.69 ± 0.19	2.70 ± 0.21	2.55 ± 0.15	2.58 ± 0.04	2.74 ± 0.14
16:00	23.66 ± 1.79	24.66 ± 2.59	24.93 ± 1.15	24.27 ± 0.66	23.33 ± 0.38
18:00	7.87 ± 1.21	7.71 ± 0.86	8.21 ± 0.33	7.54 ± 0.38	7.11 ± 0.23
20:00	0.25 ± 0.03	0.26 ± 0.02	0.25 ± 0.03	0.24 ± 0.02	0.23 ± 0.02
Σ Saturated[1]	35.11 ± 2.77	35.97 ± 3.41	36.55 ± 1.60	35.26 ± 0.92	34.11 ± 0.65
16:1n-7	9.44 ± 0.16	8.91 ± 0.39	9.30 ± 0.12	9.22 ± 0.38	9.46 ± 0.09
18:1n-9	21.92 ± 0.78	22.22 ± 1.38	22.33 ± 0.69	21.78 ± 0.89	21.45 ± 0.55
18:1n-7	3.24 ± 0.29	3.42 ± 0.19	3.36 ± 0.09	3.40 ± 0.12	3.49 ± 0.16
20:1n-7	2.93 ± 0.07	2.85 ± 0.26	2.74 ± 0.13	2.78 ± 0.02	2.83 ± 0.16
22:1n-11	2.09 ± 0.07	1.97 ± 0.23	1.98 ± 0.12	1.95 ± 0.05	2.01 ± 0.18
Σ Monosaturated[2]	41.75 ± 1.11	41.53 ± 1.19	41.79 ± 0.82	41.24 ± 1.33	41.44 ± 0.92
18:2n-6	5.54 ± 0.49	6.11 ± 0.66	5.36 ± 0.20	5.60 ± 0.37	6.12 ± 0.29
18:3n-6	0.15 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.03	0.18 ± 0.00
20:2n-6	0.24 ± 0.02	0.25 ± 0.03	0.24 ± 0.00	0.24 ± 0.01	0.26 ± 0.01
20:3n-6	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.11 ± 0.00
20:4n-6	0.50 ± 0.13	0.53 ± 0.12	0.48 ± 0.09	0.51 ± 0.11	0.58 ± 0.02
Σ n-6PUFA[3]	6.77 ± 0.78	7.40 ± 0.90	6.56 ± 0.35	6.86 ± 0.57	7.53 ± 0.29
18:3n-3	0.68 ± 0.13	0.79 ± 0.11	0.65 ± 0.06	0.68 ± 0.08	0.73 ± 0.00
18:4n-3	0.71 ± 0.26	0.71 ± 0.20	0.69 ± 0.14	0.73 ± 0.18	0.82 ± 0.04
20:3n-3	0.08 ± 0.03	0.08 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
20:4n-3	0.52 ± 0.18	0.56 ± 0.22	0.53 ± 0.10	0.56 ± 0.10	0.62 ± 0.02
20:5n-3	4.35 ± 0.74	3.83 ± 1.41	3.63 ± 0.89	3.88 ± 1.12	4.37 ± 0.32
22:5n-3	1.41 ± 0.24	1.26 ± 0.38	1.31 ± 0.11	1.29 ± 0.35	1.49 ± 0.08
22:6n-3	6.74 ± 1.45	6.00 ± 1.21	6.36 ± 0.79	7.53 ± 0.61	6.81 ± 0.56
Σ n-3PUFA[4]	14.49 ± 2.93	13.24 ± 3.52	13.25 ± 1.96	14.74 ± 1.53	14.92 ± 0.95
(n-3+n-6) PUFA	21.25 ± 3.69	20.64 ± 4.36	19.81 ± 2.28	21.61 ± 2.07	22.45 ± 0.67
Total n-3 LC-PUFA[5]	13.02 ± 0.45	11.65 ± 1.12	11.83 ± 1.03	13.26 ± 0.72	13.29 ± 0.53
Lipids (%)	17.39 ± 1.05	16.21 ± 1.72	15.88 ± 1.09	17.44 ± 1.80	14.07 ± 3.28
Proteins (%)	7.98 ± 0.31	8.29 ± 0.22	8.34 ± 0.37	8.17 ± 0.40	8.32 ± 0.30
Moisture (%)	62.89 ± 0.77	64.21 ± 1.82	63.61 ± 1.24	62.30 ± 2.36	64.03 ± 2.60
Ash (%)	0.67 ± 0.06	0.70 ± 0.23	0.65 ± 0.18	0.52 ± 0.07	0.71 ± 0.20

Table 24.1.1.5.6. Fatty acid composition (percentage of fatty acids) of kidney from meagre fed different experimental diets. Data are expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	**C**	**C+Mn/Se/Zn**	**CC**	**CCC**	**CCCC**
14:00	3.82 ± 0.38	3.80 ± 0.66	3.27 ± 0.65	3.89 ± 0.53	3.73 ± 0.42
16:00	20.07 ± 0.20	19.78 ± 0.94	20.90 ± 0.86	20.10 ± 0.77	20.26 ± 0.27
18:00	5.97 ± 0.32	5.66 ± 0.90	6.85 ± 0.78	5.72 ± 0.84	6.11 ± 0.74
20:00	0.26 ± 0.01	0.25 ± 0.00	0.26 ± 0.01	0.25 ± 0.01	0.26 ± 0.02
Σ Saturated[1]	31.07 ± 0.20	30.38 ± 1.31	32.13 ± 0.97	30.89 ± 0.96	31.27 ± 0.57
16:1n-7	5.17 ± 0.73	5.18 ± 0.92	4.36 ± 0.98	5.30 ± 0.68	5.07 ± 0.67
18:1n-9	11.48 ± 0.48	12.10 ± 0.96	10.51 ± 0.89	11.17 ± 0.38	11.06 ± 0.26
18:1n-7	2.99 ± 0.10	3.02 ± 0.10	3.16 ± 0.20	2.97 ± 0.03	2.99 ± 0.06
20:1n-7	2.37 ± 0.23	2.41 ± 0.27	2.14 ± 0.22	2.35 ± 0.24	2.26 ± 0.13
22:1n-11	2.55 ± 0.38	2.57 ± 0.61	2.01 ± 0.55	2.58 ± 0.52	2.37 ± 0.33
Σ Monosaturated[2]	26.30 ± 1.99	27.01 ± 2.78	23.72 ± 2.57	26.05 ± 1.94	25.38 ± 1.35
18:2n-6	6.32 ± 0.25	6.64 ± 0.35	6.02 ± 0.33	6.34 ± 0.53	6.20 ± 0.18
18:3n-6	0.14 ± 0.01	0.14 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
20:2n-6	0.27 ± 0.02	0.28 ± 0.03	0.31 ± 0.05	0.26 ± 0.02	0.27 ± 0.03
20:3n-6	0.13 ± 0.00	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.00	0.13 ± 0.00
20:4n-6	1.93 ± 0.34	1.83 ± 0.58	2.63 ± 0.80	1.92 ± 0.41	2.01 ± 0.25
Σ n-6PUFA[3]	9.30 ± 0.25	9.50 ± 0.32	9.81 ± 0.71	9.30 ± 0.20	9.27 ± 0.11
18:3n-3	0.85 ± 0.06	0.94 ± 0.16	0.69 ± 0.14	0.82 ± 0.12	0.79 ± 0.08
18:4n-3	1.19 ± 0.15	1.20 ± 0.28	0.93 ± 0.27	1.24 ± 0.28	1.15 ± 0.23
20:3n-3	0.10 ± 0.00	0.09 ± 0.00	0.10 ± 0.02	0.09 ± 0.02	0.08 ± 0.01
20:4n-3	0.52 ± 0.06	0.50 ± 0.08	0.47 ± 0.07	0.52 ± 0.08	0.51 ± 0.04
20:5n-3	9.56 ± 0.24	9.24 ± 0.76	10.26 ± 0.96	9.86 ± 0.13	9.78 ± 0.31
22:5n-3	1.67 ± 0.04	1.60 ± 0.09	1.74 ± 0.13	1.71 ± 0.10	1.69 ± 0.03
22:6n-3	16.70 ± 1.89	16.88 ± 1.72	17.11 ± 0.40	16.86 ± 1.63	17.33 ± 1.12
Σ n-3PUFA[4]	30.60 ± 1.90	30.44 ± 2.25	31.31 ± 1.04	31.11 ± 1.24	31.33 ± 0.64
(n-3+n-6) PUFA	39.90 ± 2.02	39.94 ± 2.47	41.12 ± 1.74	40.42 ± 1.27	40.60 ± 0.74
Total n-3 LC-PUFA[5]	28.45 ± 2.10	28.22 ± 1.21	29.58 ± 1.05	28.95 ± 2.30	29.31 ± 0.95
Lipids (%)	4.69 ± 0.23	4.18 ± 0.11	4.10 ± 0.29	4.53 ± 0.33	4.47 ± 0.45
Proteins (%)	14.43 ± 0.23	15.30 ± 0.23	15.14 ± 0.49	15.30 ± 0.30	14.61 ± 0.54
Moisture (%)	80.01 ± 0.32	80.12 ± 0.68	80.47 ± 0.24	80.27 ± 0.51	79.81 ± 0.24
Ash (%)	0.45 ± 0.03	0.47 ± 0.04	0.45 ± 0.05	0.45 ± 0.03	0.46 ± 0.03

Table 24.1.1.5.7. Cardiac fatty acid composition (percentage of fatty acids) of meagre fed different experimental diets. Data are expressed as means of three technical replicates per batch of tissue. [1]Includes 15:0 and 17:0. [2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Fatty acids (%)	**C**	**C+Mn/Se/Zn**	**CC**	**CCC**	**CCCC**
14:00	1.36 ± 0.14	1.14 ± 0.22	1.36 ± 0.20	1.25 ± 0.08	1.48 ± 0.24
16:00	19.07 ± 0.25	18.62 ± 0.80	18.90 ± 0.20	18.75 ± 0.55	18.66 ± 0.30
18:00	9.54 ± 0.48	10.94 ± 1.58	9.47 ± 0.52	9.69 ± 0.33	9.08 ± 0.37
20:00	0.26 ± 0.01	0.24 ± 0.03	0.24 ± 0.00	0.25 ± 0.01	0.25 ± 0.01
Σ Saturated[1]	30.73 ± 0.36	31.63 ± 1.04	30.42 ± 0.47	30.39 ± 0.77	29.97 ± 0.44
16:1n-7	1.71 ± 0.19	1.35 ± 0.30	1.75 ± 0.38	1.47 ± 0.17	2.12 ± 0.39
18:1n-9	7.92 ± 0.33	7.33 ± 0.38	7.81 ± 0.64	7.33 ± 0.24	8.36 ± 0.60
18:1n-7	3.47 ± 0.14	3.48 ± 0.19	3.51 ± 0.09	3.52 ± 0.14	3.45 ± 0.09
20:1n-7	1.72 ± 0.04	1.62 ± 0.14	1.69 ± 0.10	1.61 ± 0.02	1.75 ± 0.11
22:1n-11	0.98 ± 0.08	0.86 ± 0.16	0.96 ± 0.12	0.90 ± 0.05	1.02 ± 0.16
Σ Monosaturated[2]	16.74 ± 0.54	15.59 ± 0.89	16.66 ± 1.29	15.76 ± 0.43	17.67 ± 1.28
18:2n-6	4.96 ± 0.20	5.01 ± 0.20	4.96 ± 0.21	4.82 ± 0.12	5.02 ± 0.29
18:3n-6	0.15 ± 0.08	0.17 ± 0.11	0.19 ± 0.01	0.15 ± 0.09	0.19 ± 0.01
20:2n-6	0.36 ± 0.00	0.37 ± 0.04	0.36 ± 0.02	0.36 ± 0.02	0.35 ± 0.01
20:3n-6	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00
20:4n-6	3.29 ± 0.19	3.52 ± 0.16	3.26 ± 0.17	3.44 ± 0.07	3.15 ± 0.19
Σ n-6PUFA[3]	9.90 ± 0.28	10.26 ± 0.26	9.89 ± 0.11	9.96 ± 0.18	9.82 ± 0.12
18:3n-3	0.49 ± 0.06	0.45 ± 0.06	0.47 ± 0.06	0.45 ± 0.01	0.50 ± 0.06
18:4n-3	0.26 ± 0.05	0.20 ± 0.08	0.28 ± 0.07	0.24 ± 0.06	0.32 ± 0.07
20:3n-3	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.00
20:4n-3	0.30 ± 0.01	0.27 ± 0.02	0.31 ± 0.03	0.36 ± 0.14	0.32 ± 0.02
20:5n-3	8.96 ± 0.04	8.59 ± 0.34	9.09 ± 0.15	8.87 ± 0.31	8.72 ± 0.47
22:5n-3	2.08 ± 0.07	1.99 ± 0.09	2.07 ± 0.06	2.05 ± 0.02	2.06 ± 0.05
22:6n-3	27.30 ± 0.56	27.84 ± 0.44	27.66 ± 1.28	28.67 ± 0.63	27.44 ± 1.99
Σ n-3PUFA[4]	39.49 ± 0.58	39.44 ± 0.34	39.95 ± 1.05	40.72 ± 0.54	39.45 ± 1.88
(n-3+n-6) PUFA	49.39 ± 0.36	49.70 ± 0.33	49.84 ± 1.07	50.68 ± 0.40	49.28 ± 1.85
Total n-3 LC-PUFA[5]	38.64 ± 2.10	38.69 ± 2.03	39.13 ± 1.52	39.95 ± 0.87	38.54 ± 1.32
Lipids (%)	4.47 ± 0.92	3.91 ± 0.78	4.41 ± 0.76	4.81 ± 0.89	4.04 ± 0.37
Proteins (%)	15.82 ± 1.47	15.58 ± 0.15	15.16 ± 0.50	15.48 ± 0.74	15.55 ± 0.25
Moisture (%)	82.70 ± 0.21	82.46 ± 0.30	82.53 ± 0.18	81.84 ± 0.30	82.26 ± 0.41
Ash (%)	0.35 ± 0.04	0.40 ± 0.03	0.33 ± 0.04	0.35 ± 0.05	0.40 ± 0.03

The level of lipid peroxides, as indicated by TBARS content (µmol g-1 larval tissues), was not affected in liver by diet, but was significantly lower in heart and kidney of fish fed with the diet CCC (**Figure 24.1.1.5.1., Figure 24.1.1.5.2 & 24.1.1.5.3, respectively**).

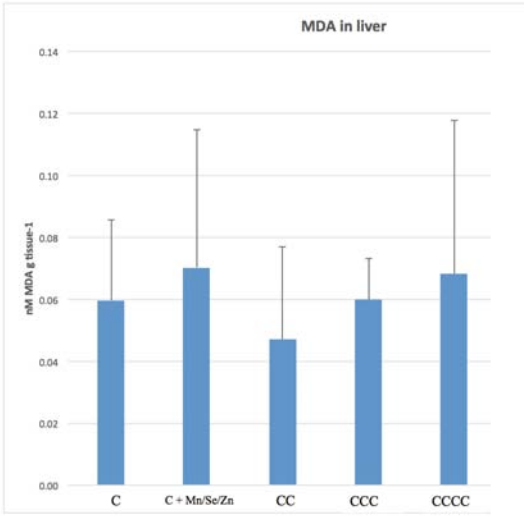


Figure 24.1.1.5.1. TBARS content in liver of juvenile meagre after 90 days of feeding with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences (P<0.05).

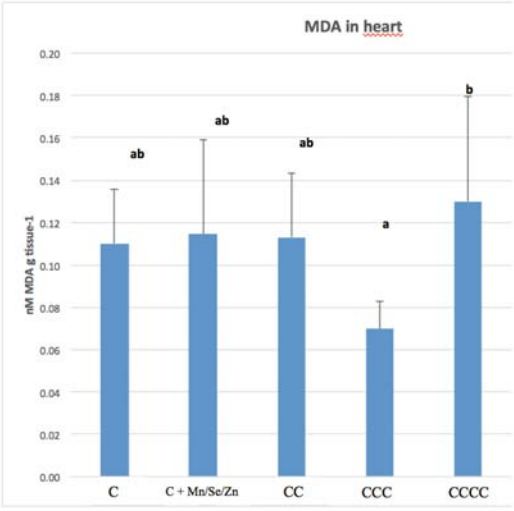


Figure 24.1.1.5.2. TBARS content in heart of juvenile meagre after 90 days of feeding with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences (P<0.05).

Histopathology

Gross granulomas in tissues (liver, kidney and heart) were only observed in 2 fish, and were not related to any dietary treatment. The histopathological evaluation revealed different stages of granuloma development (**Figure 24.1.1.5.4**). At initial stages, granulomas were observed as isolated and irregular aggregates of macrophages and some lymphocytes (**Figure 24.1.1.5.4a**) that later were forming concentric layers (**Figure 24.1.1.5.4b**). These aggregates progressively lead to a necrotic centre with an external layer of fibrocytes (**Figure 24.1.1.5.4c**). In the final stages, the granuloma was completely composed of laminar material, especially observed in heart and kidney (**Figure 24.1.1.5.4d**).

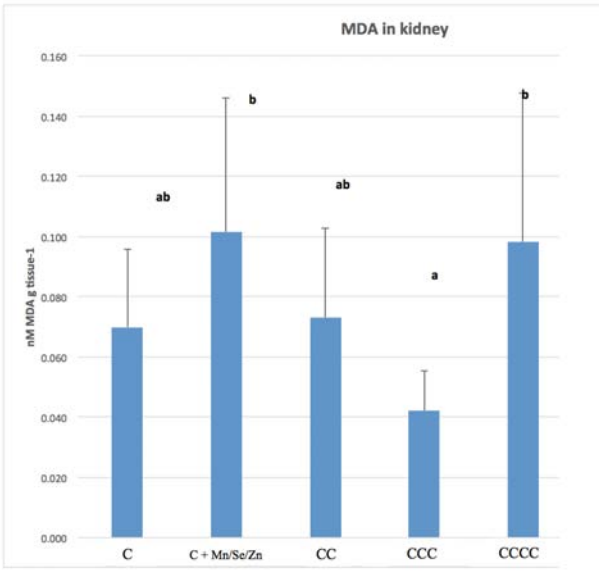


Figure 24.1.1.5.3. TBARS content in kidney of juvenile meagre after 90 days of feeding with the experimental diets. Each value represents mean ± SD (n=21). Different letters denote significant differences (P<0.05).

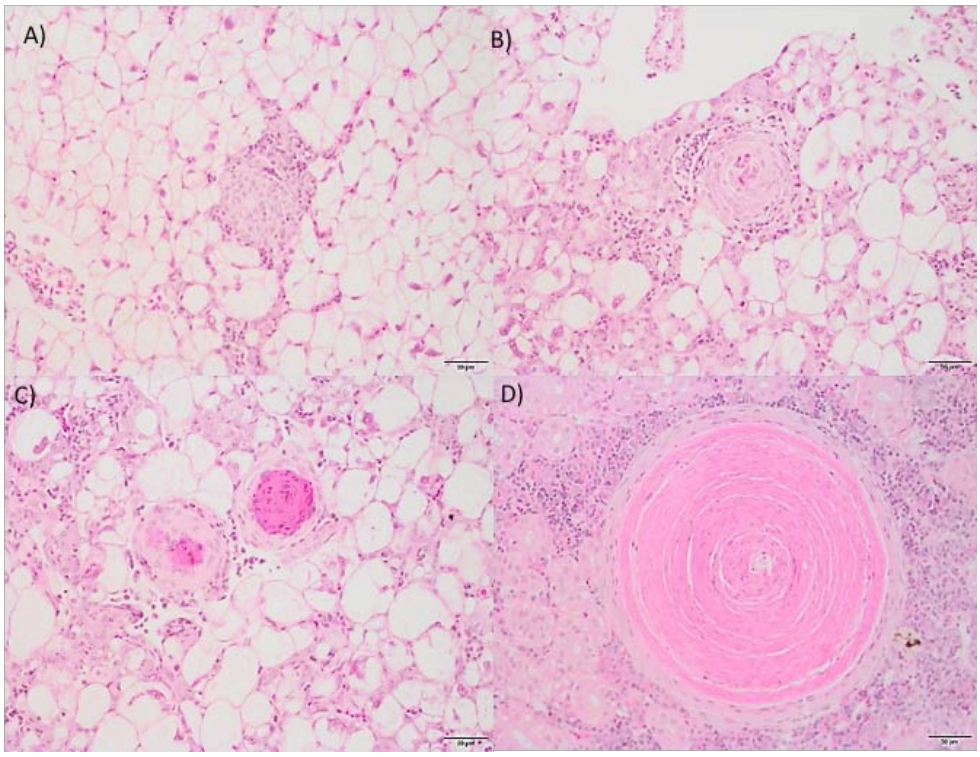


Figure 24.1.1.5.4. Different stages of granuloma development. **A)** Irregular aggregations of macrophages and inflammatory cells. **B)** Concentric layers of macrophages and inflammatory cells**. C)** Necrotic centre with an external layer of fibrocytes. **D)** Granuloma composed completely of laminar material in kidney.

The most affected organ was the liver followed by the kidney and heart. A significantly lower number of fish with hepatic granulomas was observed when a high level of vitamin C was added to the feeds (CCC-CCCC diet) (**Table 24.1.1.5.8**).

Table 24.1.1.5.8. Percentage of affected liver, kidney and heart with granulomas, of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se after 90 feeding with experimental diets. Data are means ± SD. Values in each row with a different superscript are significantly different (P<0.05).

Diets	Liver	Kidney	Heart
C	1.37 ± 1.13	0.73 ± 0.78	0.17 ± 0.38
C+Mn/Se/Zn	1.40 ± 1.07	0.77 ± 0.97	0.07 ± 0.25
CC	1.33 ± 1.09	1.00 ± 0.87	0.10 ± 0.31
CCC	0.97 ± 1.03	1.03 ± 0.93	0.03 ± 0.18
CCCC	1.23 ± 1.28	0.87 ± 0.97	0.03 ± 0.18

The severity score did not show significant differences among fish fed the different dietary treatments in any tissue after 90 days of feeding, however there was a tendency to a decrease in the severity of granulomatosis in liver, for instance in liver 1.37 in diet KEC vs 0.97 in diet KECCC (**Table 24.1.1.5.9).**

Table 24.1.1.5.9. Average granuloma severity score in liver, kidney and heart of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se after 90 feeding with experimental diets. Data are means ± SD. Values in each row with a different superscript are significantly different (P<0.05).

Diets	Liver	Kidney	Heart
C	76.7 ± 3.51^{cb}	60.0 ± 6.30	16.7 ± 20.82
C+Mn/Se/Zn	76.7 ± 2.89^{cb}	60.0 ± 0.00	6.7 ± 11.50
CC	83.3 ± 5.77^{c}	70.0 ± 5.00	10.0 ± 17.32
CCC	63.3 ± 1.53^{a}	66.7 ± 4.58	3.33 ± 0.58
CCCC	63.3 ± 1.15^{a}	56.7 ± 5.77	3.33 ± 0.58

No calcification was observed at any stage of the analyzed tissue. The specific staining (Ziel-Neelsen, Fite-Faraco and Gram stain), were negative, discarding a possible infectious origin (**Figure 24.1.1.5.5)**

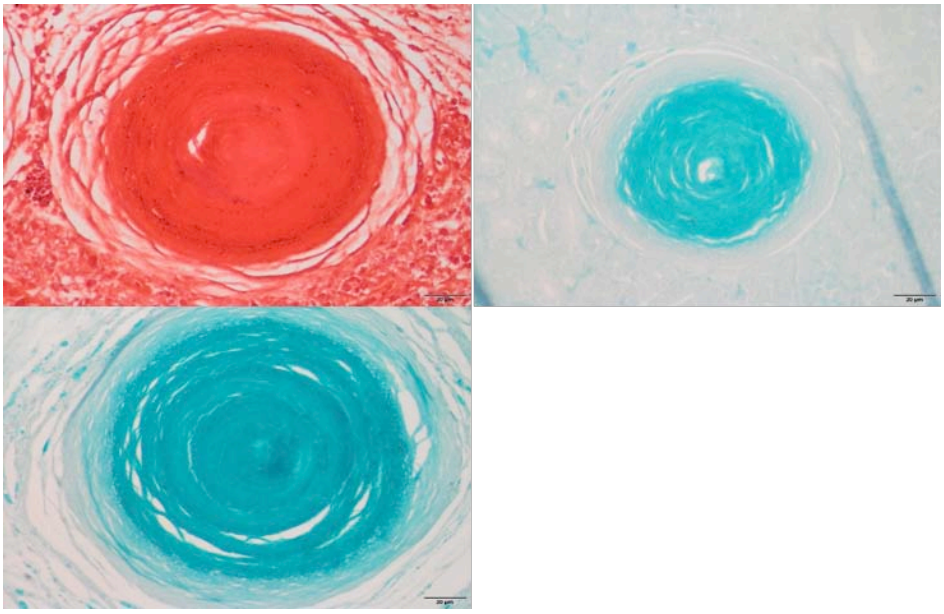


Figure 24.1.1.5.5. A) Gram stain of granuloma in liver, B) Ziel-Neelsen stain of granuloma in kidney and C) Fite-Faraco stain in liver.

Gene expression analysis

There were significant differences in *cat*, *gpx* and *sod* expression in the liver, the expression of this enzyme was higher in fish fed with high levels of vitamin C (CCCC diet) compared to fish fed diet C (**Figure 24.1.1.5.6, Figure 24.1.1.5.7. & Figure 24.1.1.5.8**).

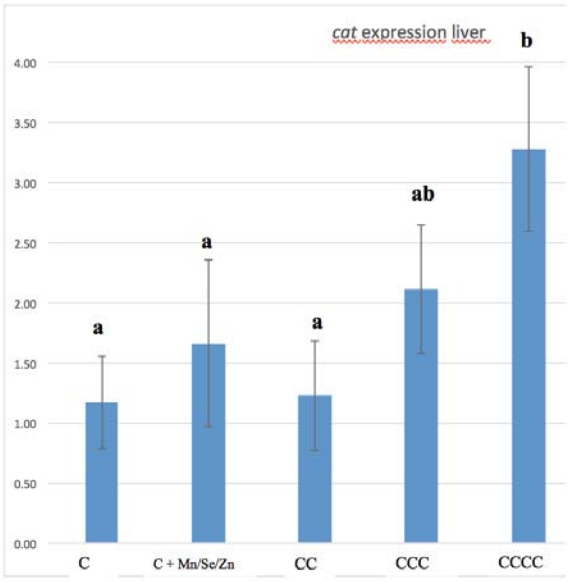


Figure 24.1.1.5.6. *cat* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

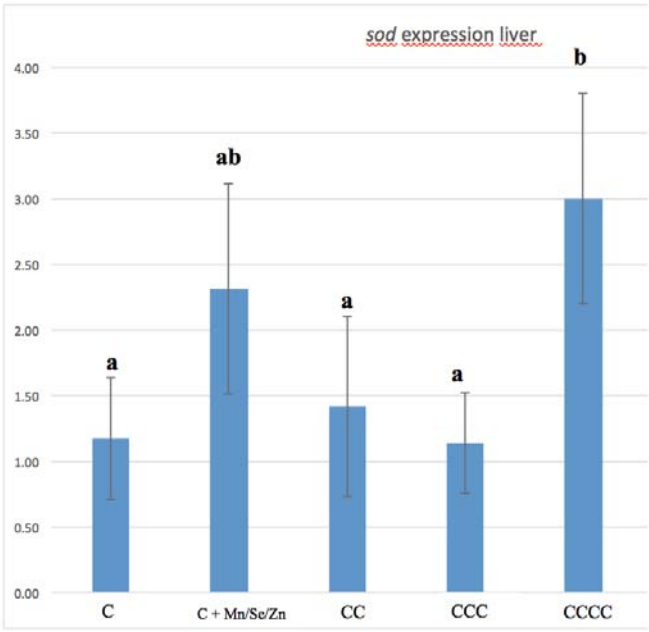


Figure 24.1.1.5.7. *sod* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

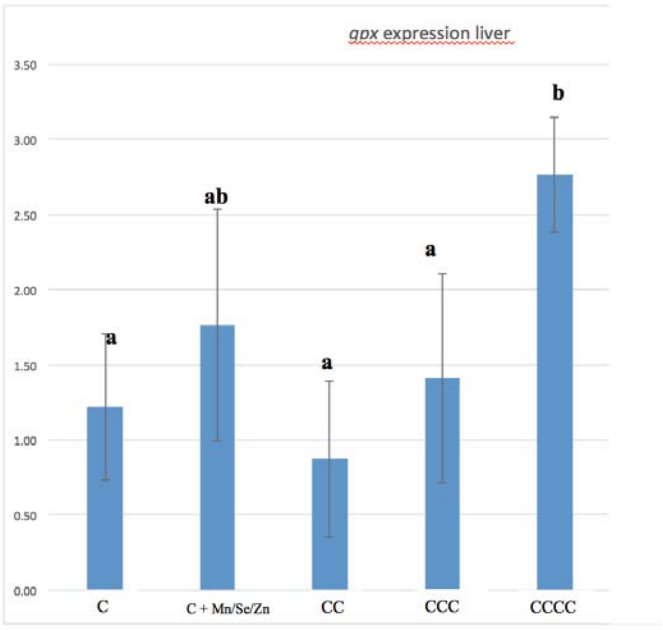


Figure 24.1.1.5.8. *gpx* expression levels measured by real-time PCR in liver of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

Significant differences were also observed in the gene expression of *cat* in kidney in fish fed diet CCC (**Figure 24.1.1.5.9**). No differences were observed in the expression of *sod* and *gpx* (**Figure 24.1.1.5.10 & Figure 24.1.1.5.11).**

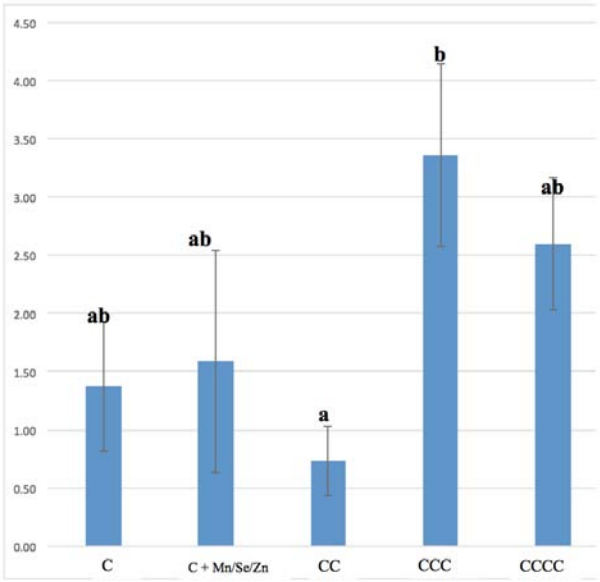


Figure 24.1.1.5.9. *cad* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

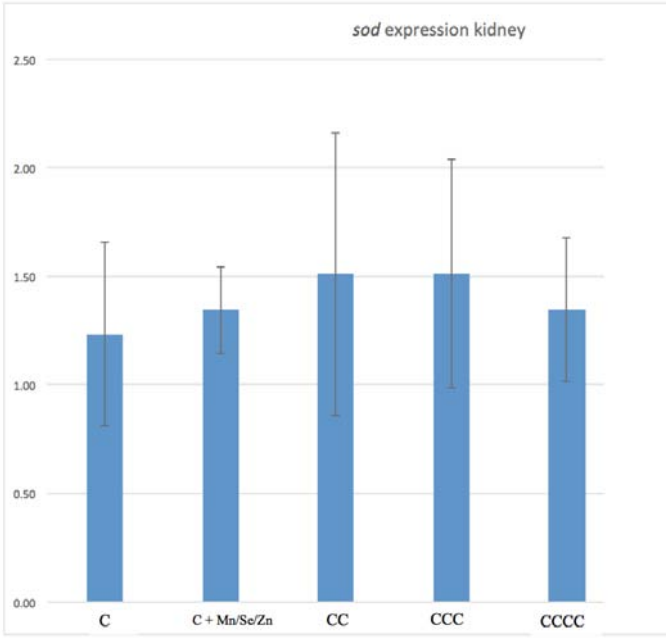


Figure 24.1.1.5.10. *sod* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

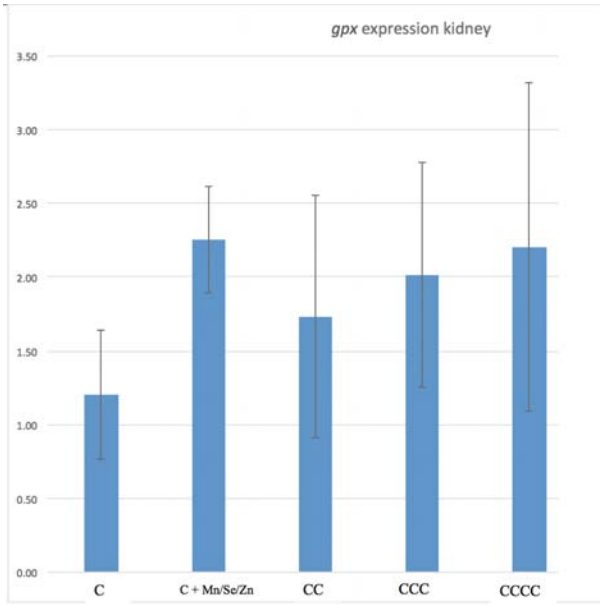


Figure 24.1.1.5.11. *gpx* expression levels measured by real-time PCR in kidney of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

The expression of *cat* in heart was not affected by the inclusion of different levels of vitamin C or Mn, Zn and Se (**Figure 24.1.1.5.12**). However, significant differences (P<0.05) were obtained in the gene expression of *sod* and *gpx* **(Figure 24.1.1.5.13 & Figure 24.1.1.5.14)**. The expression was increased in fish fed with high levels of vitamin C (CCCC).

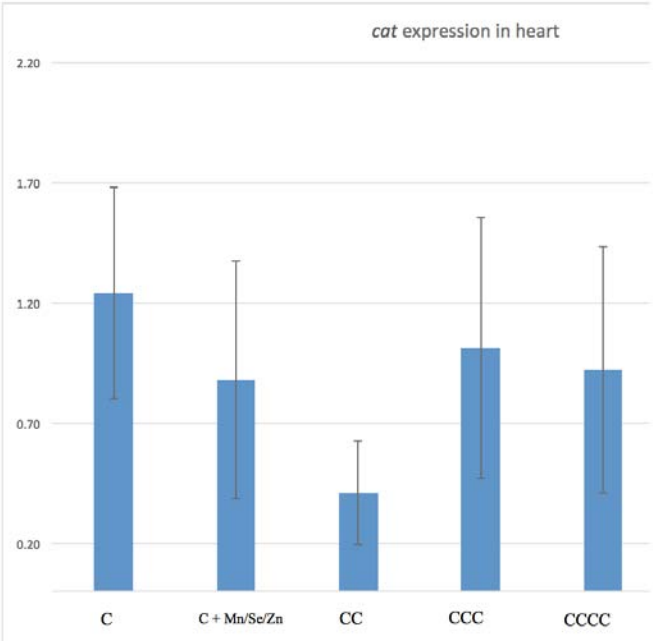


Figure 24.1.1.5.12. *cad* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

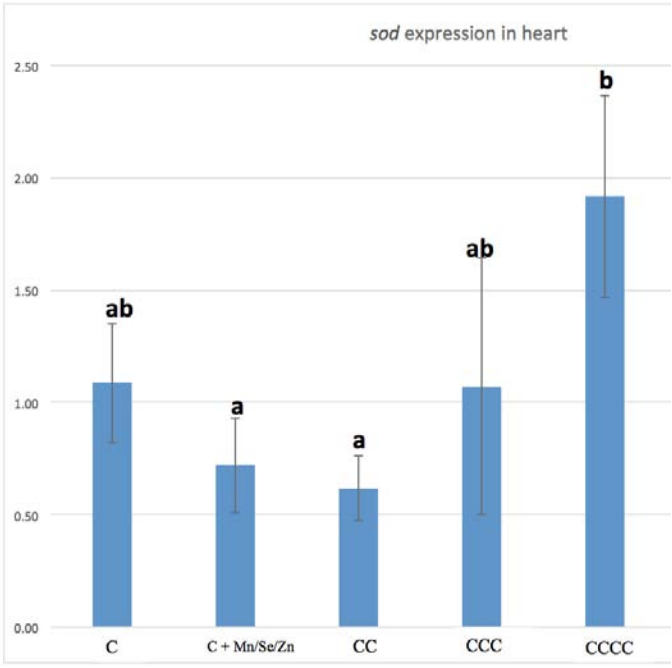


Figure 24.1.1.5.13. *sod* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

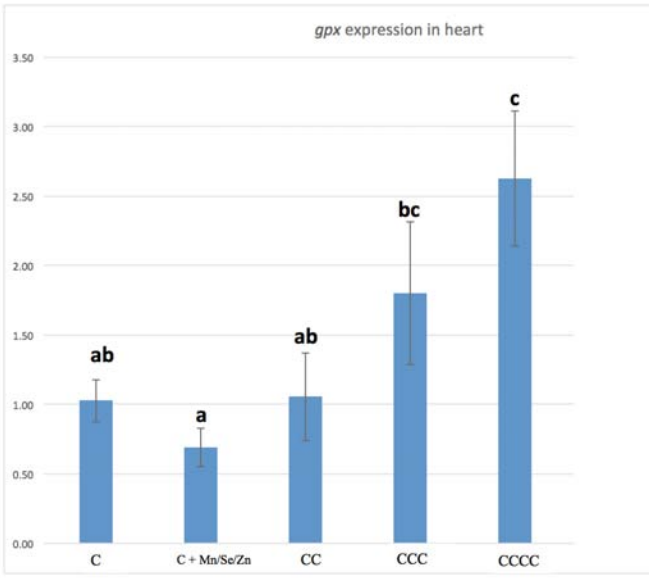


Figure 24.1.1.5.14. *gpx* expression levels measured by real-time PCR in heart of *Argyrosomus regius* after 90 days of feeding the experimental diets. Different letters denote significant differences (P<0.05).

Sub-task 24.1.2. Health and pathological assessment (HCMR, Pantelis Katharios).

This work is still in progress and is scheduled to finish in Month 54. Description of the work and the results have been submitted with the previous report.

Task 24.2. Chronic Ulcerative Dermatopathy (led by HCMR, Pantelis Katharios).

Two parallel rearing trials of meagre in borehole and natural seawater were conducted in order to study the development of Chronic Ulcerative Dermatopathy (CUD). Eggs produced in May 2015 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the rearing trial which was performed in duplicate 40m^3 tanks (HCMR). The rearing trial lasted from 1-56 days post hatching (dph). Every day measurements of pH, CO_2, O_2 and T were made in two water sources in order to identify the aetiological agent of the condition. Fish were sampled according the protocol in **Table 24.2.1.**

Table 24.2.1. Sampling protocol for the CUD experiment

	Days post hatching (dph)																			
	1	2	3	4	5	6	7	9	11	13	15	17	19	21	26	31	36	41	46	51	56
SEM																					
Histology																					
qPCR																					

For qPCR: 10 fish from each tank were frozen in liquid nitrogen and stored at -80ºC until analysis. For qPCR, total RNA was isolated from the head of meagre by using the Nucleospin RNA plus Kit (Macherey-Nagel) according to the manufacturer's instructions, and cDNAs were synthesized from 1 μg RNA by using a QuantiTect Reverse transcription kit (Qiagen). The sets of degenerate oligonucleotide primers used for cathepsin K, TRAP and vATPase were the following and were determined by Prof. Secombes' team (P5) in Aberdeen:

CathK	F	ACGCTCACTCCAAATCCAACTG
	R	CCGTGCCGCTACAATTCATCA
TRAP	F	CGTAATTGCTGCCATCTCTGT
	R	CTGTTCTCCTGTGCTTAGCCTAC
vATPase	F	TGTATGCCTGTTATGCCATTG
	R	TCCTGAGCGATGAAGTTCTT

The mRNA expression of genes encoding for CathK, TRAP and vATPase was determined with quantitative polymerase chain reaction (qPCR) assays using the KAPA SYBRH FAST qPCR Kit (Kapa Biosystems). Reactions were cycled and the resulting fluorescence was detected with Mini Thermal Cycler (Bio-Rad) under the following cycling parameters: 95oC for 3 min (DNA Polymerase activation step), 95oC for 15s (denaturation step), 60ºC for 30 s (annealing step), 72ºC for 20 s (extension step), 36 cycles (step 2–step 4). Levels of cathK, TRAP and vATPase mRNA were normalized based on the reference gene β-actin. A relative standard curve was constructed for each gene, using 4 serial dilutions (1:5) of a pool of all cDNA samples.

For scanning electron microscopy (SEM): 3 fish from each tank were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 1 or 2 days (depending on the size of the fish) and then stored in sodium cacodylate buffer at 4ºC.

For histology: 3 fish from each tank were fixed in 4% formaldehyde: 1% glutaraldehyde at 4°C. Subsequently they were dehydrated in gradually increased ethanol solutions (70-96%) and then embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections of 4 μm were obtained with a microtome (RM 2035, Leica, Germany). After drying, slides were stained with methylene blue/azure II/basic fuchsin according to Bennett et al. (1976) and examined under a light microscope.

Results

At the end of the rearing trial all the fish reared in borehole water had visible lesions associated with CUD in comparison with the fish reared in natural seawater (**Figure 24.2.1**).

Figure 24.2.1. Meagre reared in natural seawater (left) and borehole water (right). All fish reared in borehole water had visible lesions on the head associated with CUD.

Growth performance

The average length and weight of the fish of the different water sources at the end of the rearing trial (56 dph) are presented in Figure 24.2.2. The growth performance of the fish was not affected by the different source of water ($p>0.05$)

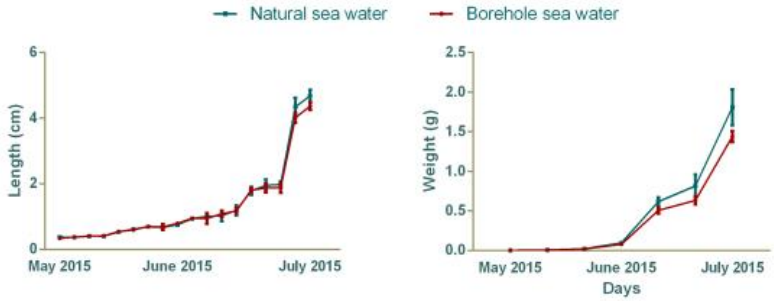


Figure 24.2.2. Average length and weight of meagre reared in borehole and natural seawater. The values are mean±SD.

Physicochemical analysis of water

The physicochemical analysis of the two water sources is presented in **Figure 24.2.3**. The pH was lower and CO_2 higher in borehole water in comparison to natural sea water while T was higher in seawater from June, while O_2 levels did not different between the two sources.

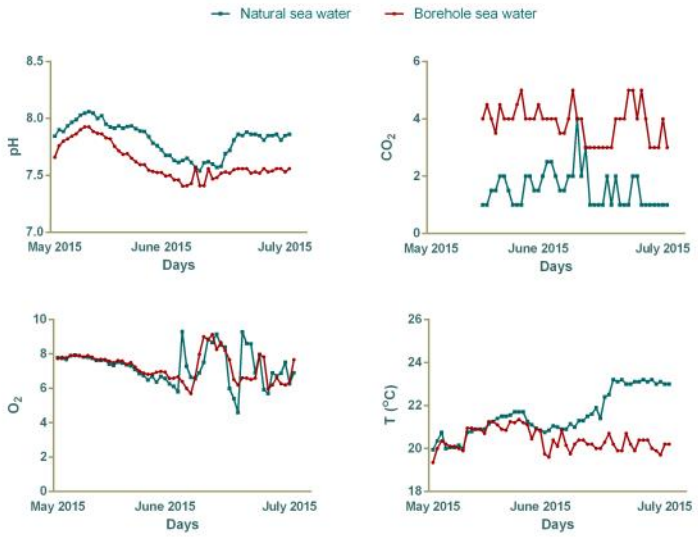


Figure 24.2.3. Physicochemical analysis of two different sources of water.

Expression of CathK, TRAp and vATPase

Expression profile of CathK, TRAP and vATPase of the different water sources was significant different at the end of the rearing trial (56dph). In particular, cathepsin K and TRAP expression was 2.7 and 2.1 times higher, respectively, in the fish of the borehole water group compared to the seawater group ($t(17)=2.26$, $p=0.037$ for cathepsin K and $t(17)=2.41$, $p=0.028$ for TRAP). The expression of vATPase did not exhibit significant differences between the two water sources ($t(17)= -.219$, $p=0.830$) (**Figure 24.2.4**).

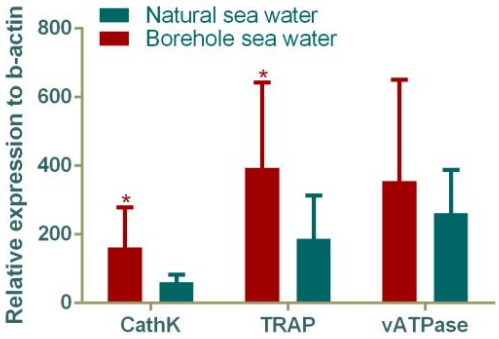


Figure 24.2.4. Relative expression of CathK, TRAP and vATPase of meagre's head reared in borehole and natural sea water at the end of the rearing trial (56dph). Values are means±SD. * = statistically significant differences between the two water sources ($p<0.05$).

Scanning Electron Microscopy (SEM)

From the SEM observations it was found that the main affected areas were the supraorbital commissure (SOCom) which joins the left and the right supraorbital canals (SO), the infraorbital canal (IO), the mandibular canal (MD) and the area of the nostril (R). The supraorbital canal (SO) of both healthy and CUD-affected fish was outlined by a distinct series of pores **(Figure 24.2.5 (A),(B)).** Instead, both the IO and the MD canals of CUD-affected fish were open with no pore-bearing canal roof **(Figure 24.2.5 (B),(D), (F))**. The canals had a groove-like appearance where damaged canalized neuromasts were exposed **(Figure 24.2.6)**. The nares of CUD-affected fish often had wider openings than healthy individuals however, the olfactory rosette was not damaged (**Figure 24.2.5. (E),(F)**).

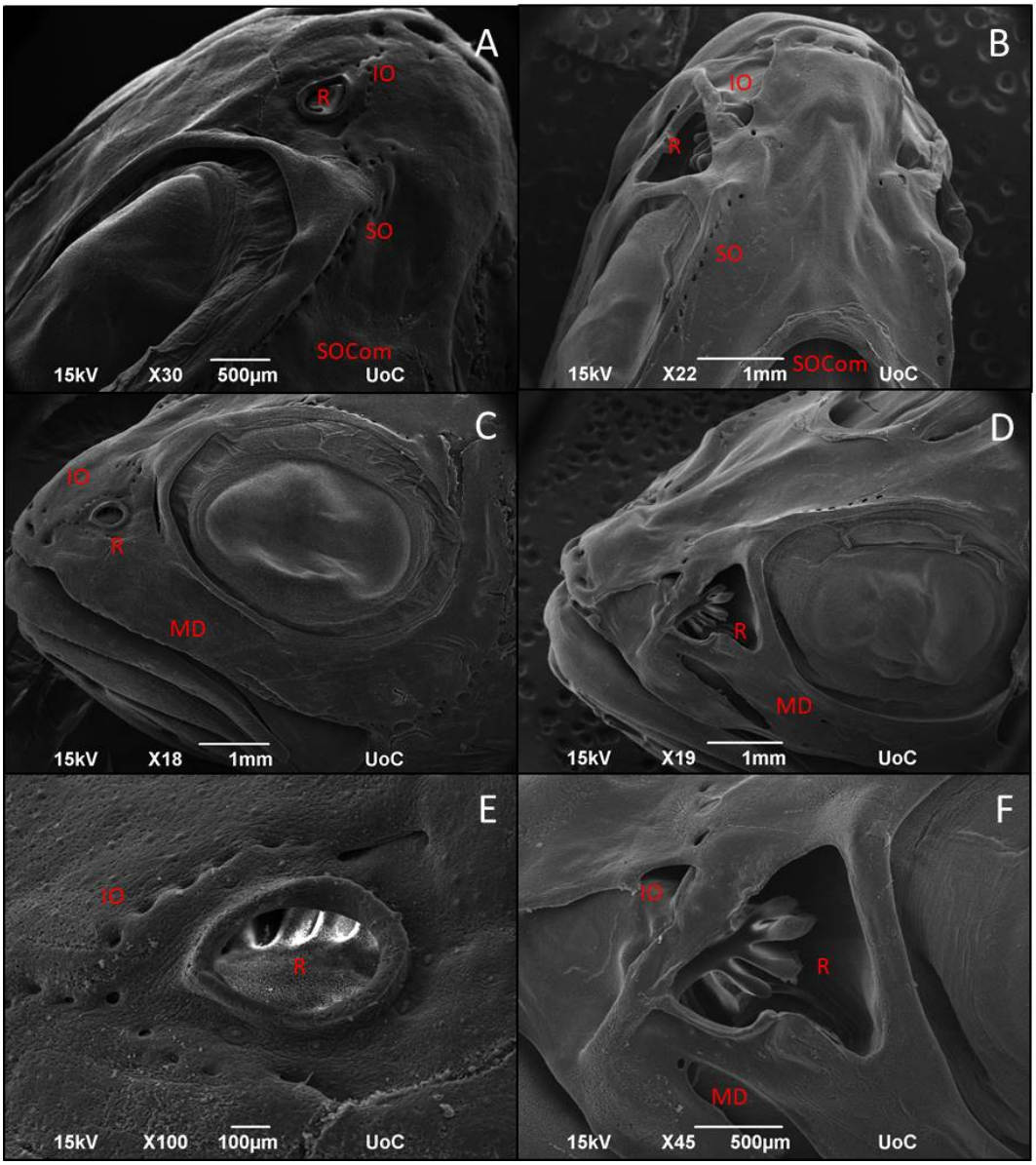


Figure 24.2.5. SEM micrographs of healthy and CUD-affected juvenile meagre (56dph). Dorsal view showing the supraorbital canal (SO), the infraorbital canal (IO) and supraorbital commissure (SOCom) of healthy **(A)** and CUD-affected meagre **(B)**. Lateral view of healthy (**C**) and CUD-affected meagre (**D**) showing the infraorbital canal, the nostril (R) and the mandibular canal (MD). Higher magnification of the nostril (R) with the infraorbital canal (IO) and the mandibular canal (MD) of healthy (**E**) and CUD-affected meagre (**F**).

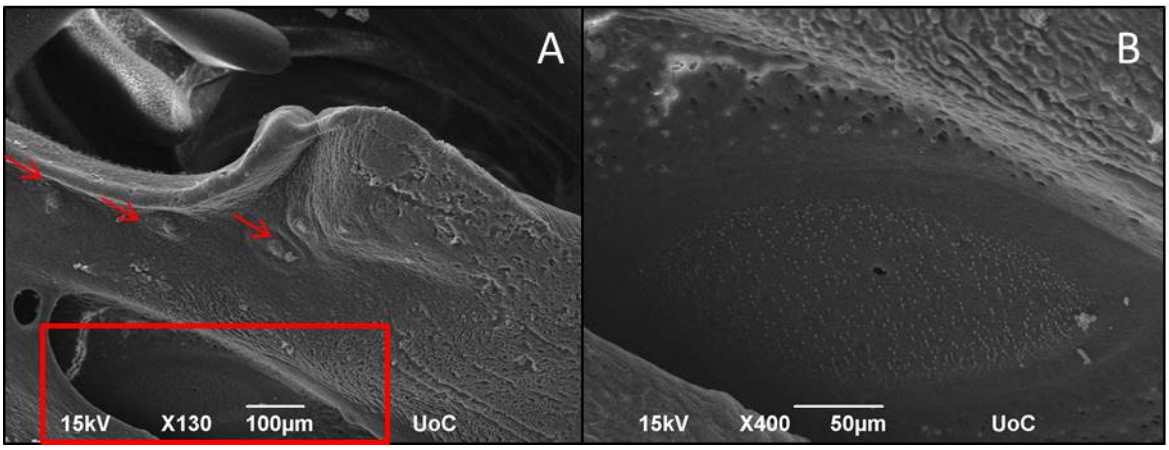


Figure 24.2.6. SEM micrographs of CUD-affected juvenile meagre (56dph). **A.** Lateral view of the nostril with the opened mandibular canal (framed area). Arrows indicate normal superficial neuromasts around the nostril. **B.** Higher magnification of the framed area showing an exposed damaged neuromast.

Histology

From the comparative histological analysis of meagre reared in borehole and natural seawater no differences were observed until 41 dph. F**igure 24.2.7** shows an infraorbital canal **(A,B)** and a mandibular canal **(C, D)** of meagre reared in natural seawater and in borehole water on 56dph. In meagre of natural seawater the canals were completely developed. Instead in meagre from borehole water we observed erosion, ulceration and loss of the basal membrane while the neuromasts were exposed to the external environment.

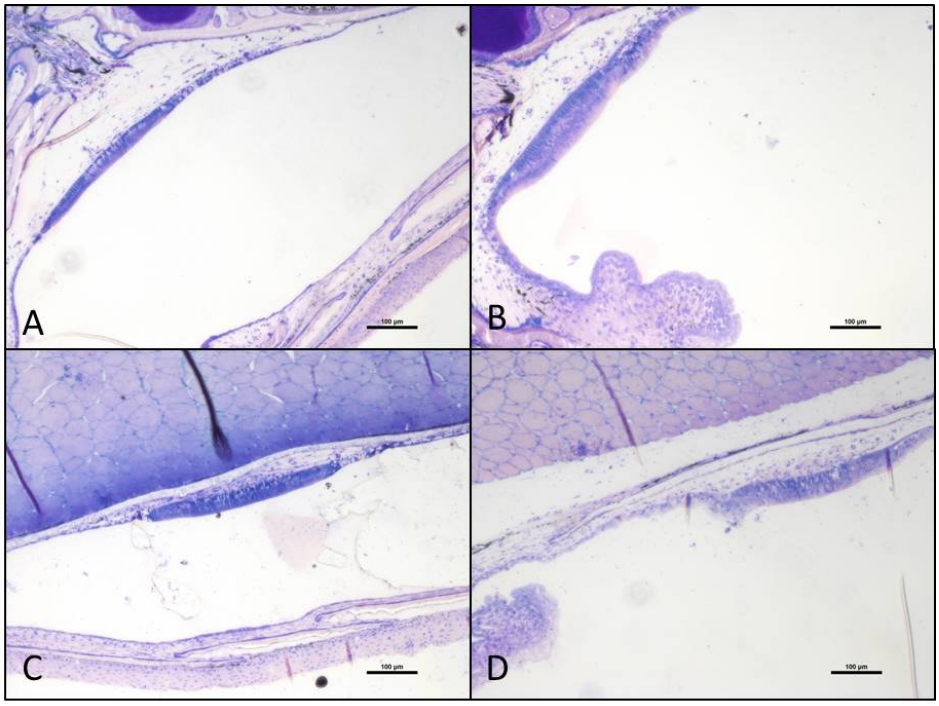


Figure 24.2.7. Cross sections of an infraorbital canal of healthy **(A)** and CUD-affected meagre **(B)** and a mandibular canal of healthy **(C)** and CUD-affected meagre **(D)**.

Further experiments and analysis

A second rearing trial was performed in order to investigate whether CO_2 in borehole water is the cause of the development of CUD lesions. In this trial, we used 2 parallel rearing tanks supplied with natural sea water. In one of this tank we adjusted the pH to 7.4 by providing CO_2. We cultured meagre from eggs to 50 dph. Analysis of the results of this trial is still ongoing, however first indications suggest that neither pH nor CO_2 are the factors affecting the development of CUD lesions. Furthermore, analysis of the heavy metal content of the fish is ongoing. All the results will be presented in the respective deliverable

Conclusions

The results indicate that the disease can be induced with the use of borehole water. The hypothesis tested is that borehole water which is rich in CO_2 as indicated also by the lower pH compared to the pH of natural seawater increases the enzymatic activity of the osteoclasts. The CO_2 activates the osteoclasts which are in close proximity with the environment like the osteoclasts of the lateral line canals. In such a case there would be an environmentally induced imbalance between osteoclasts (bone resorbing cells) and osteoblasts (bone depositing cells) that would cause the lesions seen in the fish located exclusively in the lateral line canals. The qPCR results are in agreement with this hypothesis since there is a big overexpression of the genes that are related to the osteoclast activity in the fish grown in borehole water.

Task 24.3. Anti-parasitic treatments (led by IRTA, Ana Roque).

Development of a challenge method for *Scianocotyle pancerii*

Transporting parasitized fish long distances is probably not feasible since fish may not survive, therefore an alternative source of parasites was needed.

Two alternative infestation models were devised.

1) Place live adult parasites directly on the gills of naive anaesthetised juvenile fish.
2) Place eggs in the tank water and wait to see whether infection of naive juvenile fish would occur.

For this, a trip was made to a fish farm in the Valencian coast, 5h away from IRTA and several gills of freshly dead fish were collected after they were confirmed to be infected with *Scianocotyle*. Gills were then brought to IRTA in damp conditions by placing them in sterile Petri dishes filled with paper wet with sterile seawater and sealed with parafilm. Petri dishes were then placed in cold boxes and brought back to IRTA.

Upon arrival, gills containing eggs were placed inside a tank containing 15 fish around 100g ww. Two lots of 20 parasites were also collected to place directly on the gills of two fish. A fortnight later the two juveniles exposed directly to adult parasites and another two fish from the tank exposed to parasite eggs, were sacrificed and gills searched for parasites. The two juveniles exposed to the parasite were infected, however the ones from the egg tank were not. Unfortunately, no published information was found on the length of the lifecycle of this species of Monogean. Another week later, two more fish were sacrificed, which again did not have any parasites. A week later (4 weeks), two fish were sacrificed and one of them did have some parasites on the gills, therefore the leftover 9 fish were all sacrificed to estimate the prevalence which was 2 out of 11 (around 20%). From this result, it was decided that it was worth using this challenge model for future studies.

A final experiment was set up to test the efficiency of cinnamon as anti-parasiticide for meagre infested with *S. panceri*. Once more eggs from infested meagre were collected from a farm in the Mediterranean coast. For 5 weeks, 180 juveniles were exposed in four groups of 20 to infected gills with eggs attached. Throughout this process some fish were lost. In the end of the 5 weeks, fish were confirmed to have parasites and the experiment started. To do this, 10 fish from each tank were anesthetized and under and gills would be observed under a strong light with the help of a magnifying glass. For the experiment, three diets were

prepared as described before, untreated diet, diet with EO of cinnamon and a new diet prepared with an aqueous extract of Equinaecea.

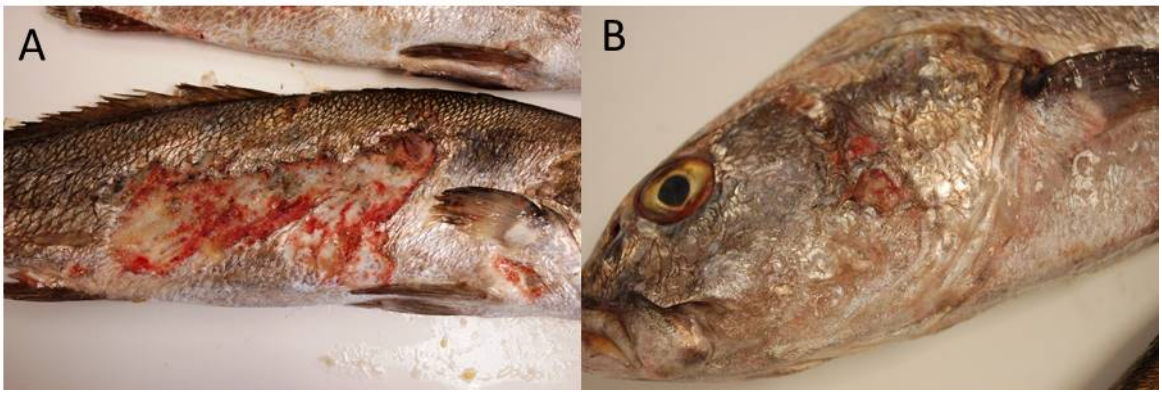
Three replicate tanks were used per treatment each with 17 fish per tank. Fish were fed 2 x 7.5g of the corresponding diet a day at 8 am and 3 pm during three weeks. Two days before offering starting the experiment, an initial sampling was performed using nine fish which were weighed, blood sampled and samples were taken for histology from liver, intestine, stomach, gill and anterior kidney. From the fresh blood sample, a haematocrit was performed and then plasma was separated to be frozen for further analysis. Prevalence was estimated from these nine fish and another 45 fish were anesthetized to get a better idea of the initial prevalence. On days 8 and 15, nine fish (one from each tank) were sacrificed, prevalence was estimated, and blood and organs sampled for histology. On the last day of the experiment, day 22, all fish were sacrificed to estimate the prevalence per treatment and nine fish were blood and organ sampled.

Results are currently under analysis.

Task 24.4. *Nocardia* infection in meagre (led by HCMR, Pantelis Katharios).

Sub-task 24.4.1 Isolation and characterization of the pathogen (HCMR, Pantelis Katharios).

The aim of this task was to monitor meagre from various locations in Greece and try to identify and isolate *Nocardia* spp., or other granuloma-associated pathogens and to assess whether these bacteria and fungi represent an actual hazard for the species. During the first years of the DIVERSIFY project we have examined a large number of apparently healthy and fish exhibiting disease signs of varying sizes from various localities using microbiological, histological and molecular techniques. In most of the cases examined, no bacterial growth was observed on the solid media used while none of the isolated bacteria had phenotypes consistent to *Nocardia* spp. In addition to the bacteria isolated in solid media, PCR analysis was performed directly on SG-affected tissues and organs using specific primers against the suspected pathogens, *Nocardia* spp., *Mycobacterium* spp., and *Ichthyophonus hoferi*. All samples examined with this method were negative for all 3 pathogens surveyed, except 2 fish that we received in June 2016 from a commercial fish farm located in Astakos, West Greece. These fish had severe dermal lesions and ulceration of the skin and considered suspicious for *Nocardia* spp. infection due to the distinct morphology of these lesions (Figures 24.4.1).



Figures 24.4.1. A. Severe ulceration of the skin of cultured meagre. B. Nodular morphology of the dermal lesions, appearance alarming for *Nocardia* spp. infection in meagre.

PCR for *Nocardia* spp. was positive in 4 out of the 6 different organs examined, including skin, heart, kidney and liver from both individuals. Positive PCR samples from both species were sequenced and compared against GenBank sequences using BLAST algorithms. The analysis showed 100% identity with *Nocardia*

seriolae. Histological analysis of the *Nocardia*-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* spp. in meagre.

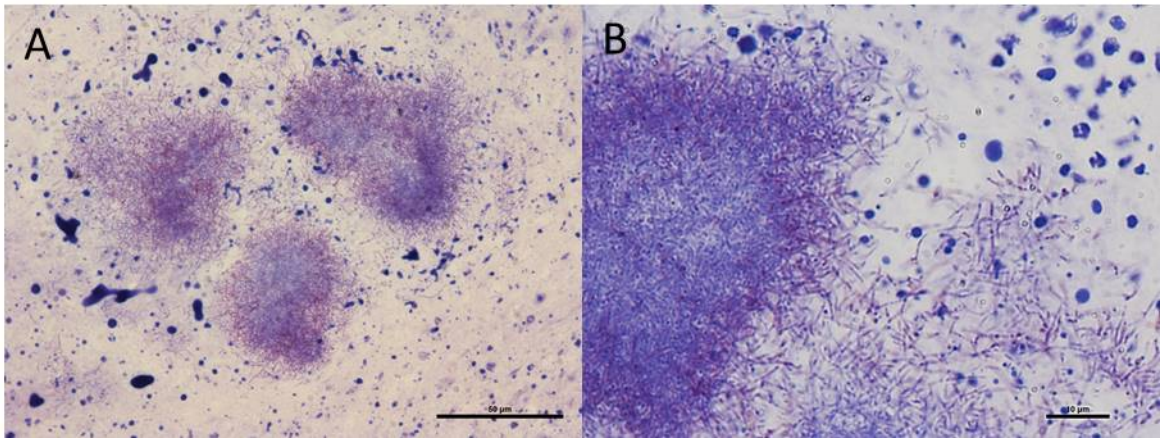


Figure 24.4.2. Section of dermal lesion of meagre showing acid-fast elongated and branching bacteria, consistent with the typical morphology of *Nocardia* spp.

The conclusions of this task are that nocardiosis is present in Greece, most probably in a confined geographical region, however it is not the cause of SG. Generally, the species does not seem to be very susceptible to common bacterial infections, however there are sporadic reports suggesting that several pathogens may become problematic in the future.

The full details concerning this task have been submitted in ***Deliverable 24.4 Isolation and characterization of Nocardia from infected meagre.***

Sub-task 24.4.2. Preparation of an autogenous vaccine (HCMR, Pantelis Katharios).

This task has changed to include a vaccine trial using an established commercial vaccine against a bacterial fish pathogen that is widely transmitted in aquaculture settings, *Vibrio anguillarum*. The reason for the change are three-fold. First, the original supposition was that unknown species of Nocardia may be causative in some pathologies seen in meagre in culture (see Elkesh et al. 2013), but attempts at isolation and other data on symptomology of granulomas seem not to support this. Second, *V. anguillarum* has a wide host range, and as meagre culture expands and intensifies it is likely that this pathogen may prove to be problematic. Third, since commercial vaccines against *V. anguillarum* already exist for bath and intraperitoneal immunization use, but have not been evaluated in meagre in a precise quantitative manner using gene expression analysis, it would be useful to understand more fully the utility of these vaccines in this new aquaculture species. In the previous attempts to carry out this task using meagre of ~10 gm weight, there was unforeseen problems with a ciliate parasite infestation that killed all the fish in the experiment four weeks after i.p. vaccination against *V. anguillarum*. Upon necropsy, many of the animals displayed adhesions of the intestine with the mesentery membranes causing constrictions of the intestine. This likely impaired digestion and enhanced stress of the fish and thus impairing their ability to combat the ciliate parasite that ultimately killed them by an inflammatory response of the branchial tissue leading to suffocation. This prevented us from completing this task. The following year (2017) we have had difficulties in obtaining non-vaccinated fish for using in a trial. It is for these reasons that we will be preforming this task in the coming year using somewhat younger fish that can be acquired earlier in the production cycle prior to any vaccination and use a bath exposure vaccination protocol to try to achieve completion of this task and

evaluate the effectiveness of vaccination against *V. anguillarum* using a live bacterial challenge and measure gene expression of selected immune genes.

In the first trial gill, gut, head kidney and spleen samples were taken 1, 3 and 5 days post vaccination to determine the early vaccination response / adjuvant effect by way of QPCR. These samples are currently stored in RNAlater at -80^{o}C to be analysed in the near future.

Task 24.5. First characterisation of the immune system (led by UNIABDN, Chris Secombes).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.3 Cloning of key marker genes of innate and adaptive immune responses in meagre.*** Additionally, manuscripts have been published in peer-reviewed journals covering description of gene expression analysis of several of the identified immune transcripts from meagre (see Campoverde et al., 2017; Milne et al., 2017).

Task 24.6. Monitor specific immune responses (led by UNIABDN, Chris Secombes).

The results of the analysis of antibody and cytokine kinetics post stimulation with PAMPs, has been reported in DL24.10. Tissue samples were collected from fish following exposure to poly I:C, LPS and β-glucan, with qPCR assays optimized to detect expression of adaptive immune genes (eg IgT/ IgM, TCR, Th cytokines) and key antimicrobial, antiviral and pro-inflammatory genes, based on the results of D24.3. Similarly, primary cell cultures from different tissues (gills, gut, head kidney, spleen) were stimulated *in vitro* with these PAMPS, and the kinetics of immune gene induction followed. The data shows that the meagre immune response to PAMPs is robust and can be tailored to the potential type of pathogen that is encountered. The responses vary by tissue, likely linked to the cell composition present at different immune sites, and the need for immediate or more prolonged responses. These data show the potential to modulate immune responses in meagre in culture, such as by delivery of immunostimulants, to enhance particular immune pathways at a time of disease risk.

Following on from the 2nd report, we have now also generated monoclonal antibodies (Mabs) against meagre IgM (Z69) and IgT (Z55). These were initially tested by Western blotting, using serum from healthy meagre and run on a NuPAGE gel at 100v for 1 h, then transferred to a nitrocellulose membrane and exposed to the Mabs and developed. As seen in **Figure 24.6.1**, the Z69 and Z55 Mabs each detect a single protein of the correct size expected for their respective immunoglobulin, with meagre IgM and IgT having expected weights of 66.09kDa and 58.93kDa.

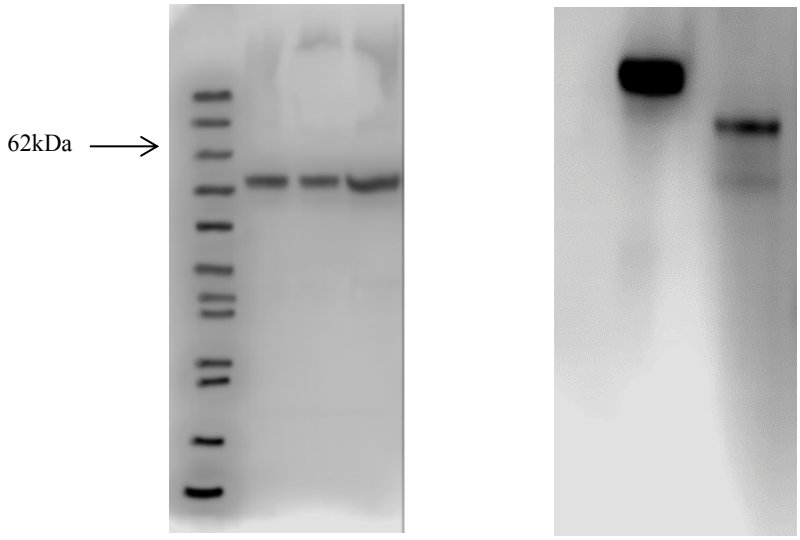


Figure 24.6.1. Western blots of meagre serum exposed to Z69 and Z55. A) Shows meagre serum diluted 1:30 in triplicate incubated with Z69 anti-meagre IgM. B) Shows meagre serum diluted 1:15 exposed to Z69 anti-meagre IgM (left) and to Z55 anti-meagre IgT (right).

The specificity of anti-meagre Mabs was next tested in immunohistochemistry (IHC). Gills, gut, head kidney and spleen were taken from healthy meagre, fixed and processed for use in IHC. Sections were then exposed to anti-meagre Mabs, which were then visualised by the addition of goat anti-mouse Ig labelled with HRP and then DAB substrate. Z69 and Z55 showed a specific binding activity, as seen in **Figure 24.6.2** for head kidney (a major site of B cells in fish), where cytoplasmic/membranous staining was observed for both Mabs, although relatively fewer IgT+ cells were apparent. To be sure that Mab staining was specific for IgM and IgT protein expression in situ hybridisation was also performed using probes specific for IgM and IgT, as seen in **Figure 24.6.2**.

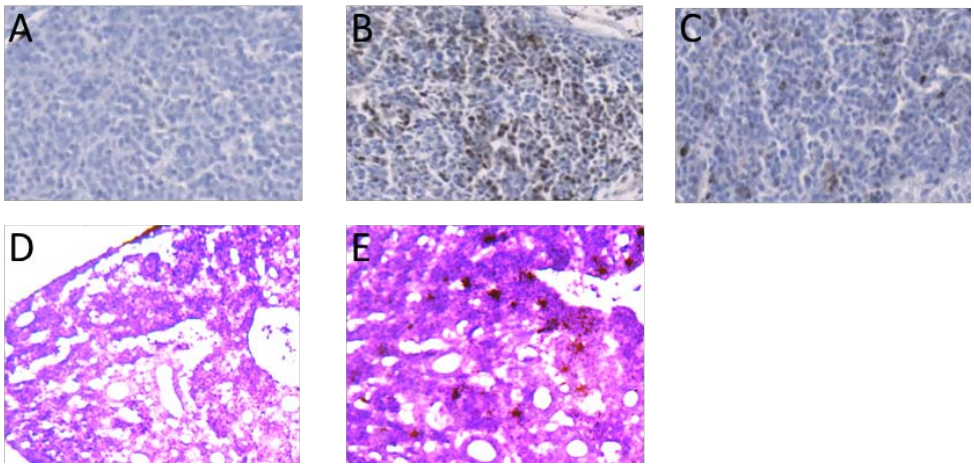


Figure 24.6.2. The expression of IgM and IgT in meagre head kidney. A) Control – shows tissue that underwent IHC with an anti-rainbow trout IgM Mab. B) Z69 staining (IgM). C) Z55 staining (IgT). D) In situ hybridisation using meagre IgM sense probe. E) In situ hybridisation using meagre IgM anti-sense probe.

The meagre Mabs were next tested in an ELISA assay to confirm they were able to detect serum immunoglobulins in their native state. Serum from healthy meagre was taken from 8 fish, diluted serially and used to coat the wells of a 96 well plate. A standard indirect ELISA assay was then performed. As seen in **Figure 24.6.3**, Z69 could detect total IgM in serum up to a dilution of ~1:65,536, whereas Z55 is capable of detecting IgT in serum diluted to ~1:16,384. Assays to detect specific antibody responses are therefore available for future use, such as to detect specific IgM and IgT against *V. anguillarum* post-vaccination. In preliminary analysis of serum from vaccinated fish in task 24.4.2, titres of 1:16 – 1:32 were detectable for IgM in serially diluted serum and titres of 1:4 – 1:8 were detectable for IgT.

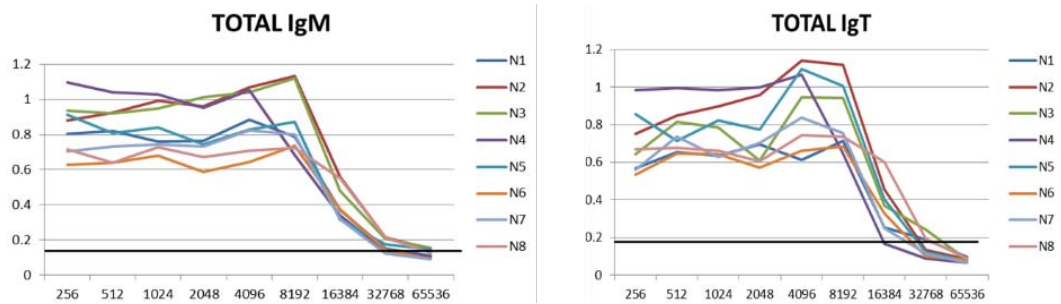


Figure 24.6.3. Z69 and Z55 detection of total serum Ig. Z69 (left) and Z55 (right) were used in ELISA to determine the detection limit using serially diluted meagre serum. Double the optical density of the negative control is represented by the horizontal line and anything above this is considered positive.

Task 24.7. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in meagre (led by FCPCT, Daniel Montero).

During the reporting period, two diseases outbreaks were recorded.

- Disease outbreaks occurred in October 2016. Fish size: 5g coming from weaning facilities. Outbreak: continuous mortality. Results: *Vibrio alginolyticus*, *Vibrio harveyi*. Positive Nested-PCR Nodavirus

- Disease outbreak occurred in November 2016. Incidence of mortality during an experiment from WP 8 (nutrition of meagre). Fish size: 23g. nº of fish: 3 samples per diet. Cultivation in YEME (to rule out the presence of *Nocardia* spp.) And AS + salt and BHI + salt. Two bacterial strains were isolated (**Table 24.7.1.**): strain CE1 from CCCP3 and DDDP1 diets, strain CE2 from diets BBBP3 and DDDP1.

Table 24.7.1. Biochemical test of bacteria isolated from liver of experimental fish from WP8.

Bacteria	Strain EDC1	Strain EDC2
Morfology	bacilus	bacilus
Gram	-	+
Oxidase	+	-
Catalase	+	+
Motility	+	-
O/F glucose	F	
TSA	+	+
Salt requirement	+	-
Swarming	+	-
DIAGNOSTIC	Vibrio spp.	*Bacillus sp*

A challenge test was also conducted with the isolated *Nocardia* reported in previous reports of activity. A total of 180 fish with an average initial weight of 69.50 g were randomly into nine groups, so that 20 fish per group were housed in each 500 mL-tank. They were inoculated by intraperitoneal injection with 1 mL of bacterial suspension with different concentrations of bacteria, from 10^1 to 10^8 CFU/mL, except the control group which was inoculated with sterile phosphate buffered saline (PBS). Inocula were prepared from a culture in YEME medium (broth) supplemented with 0.1% Tween 80, in order to reduce the typical formation of clumps by *Nocardia* spp. After incubating and centrifuging the culture, bacteria were suspended in PBS until an optical density of approximately 0.5 at 600 nm to finally prepare serial dilutions.

Due to the chronic nature of this disease, three samplings for microbiological and histological analysis were carried out after 30, 37 and 51 days post infection (dpi), so 4 fish per tank and time point were randomly selected to be sacrificed (**Figure 24.7.1.).** Also, spontaneously dead fish were recorded daily and then, analyzed as described below for all sampled fish.

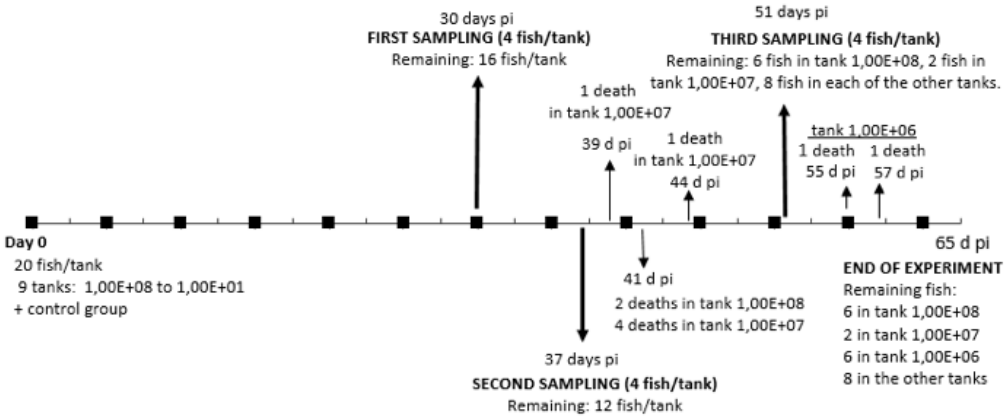


Figure 24.7.1. Timeline of an experimental infection in meagre by *Nocardia* spp., with the number of fish sacrificed per timepoint and spontaneous deaths observed.

Bacteriology

Bacterial recovery was from liver, spleen and kidney sections onto blood agar (5% defibrinated sheep blood (v/v)) supplemented with 1.5% sodium chloride (DSBA+1.5%NaCl), brain-heart agar with 1.5% sodium chloride (BHIA+1.5%NaCl) and YEME medium (agar). Cultures were incubated at 25ºC for 3 weeks, but checked daily; then, positive ones were confirmed by nested-PCR.

Histopathology

Samples of the liver, spleen and kidney were fixed in 10% buffered formalin and processed for paraffin sectioning to evaluate possible histological findings or lesions due to infection, and stained with haematoxylin and eosin (H&E) and other special stains: Gram stain and modified Ziehl-Neelsen´s staining method (ZN).

Results

Clinical signs

Unexpectedly, any typical gross signs, such as necrosis and ulceration on skin or nodules in internal organs were visible. Lethargy was observed in the second half of the challenge as also as weight discrepancies between animals inoculated with 10^8-10^6 CFU/mL and the remaining groups, inoculated with lower doses. Nonetheless, microscopic granulomas were found during histological evaluation, as shown further below (See Histopathology).

Mortality rate

A total of 10 dead fish were found only in groups with the higher doses (10^8-10^6 CFU/mL). The first case was recorded at 39 days post infection and deaths peaked at 41 dpi and stopped at 57 dpi. When the inoculated dose was 10^6 CFU/mL, dead fish appeared at the end of the experimental period (**Figure 24.7.2**).

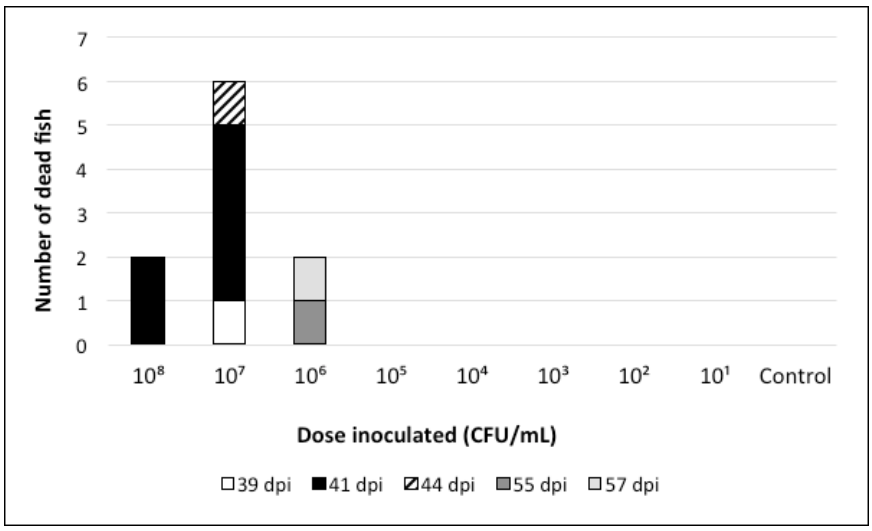


Figure 24.7.2. Deaths registered throughout an experimental infection in meagre with *Nocardia brasiliensis.* Inoculated doses from 10^1 to 10^8 CFU/mL.

The cumulative mortality was 17% for doses above 10^6 and 0% at concentrations below 10^6 CFU/mL (**Figure 24.7.3**).

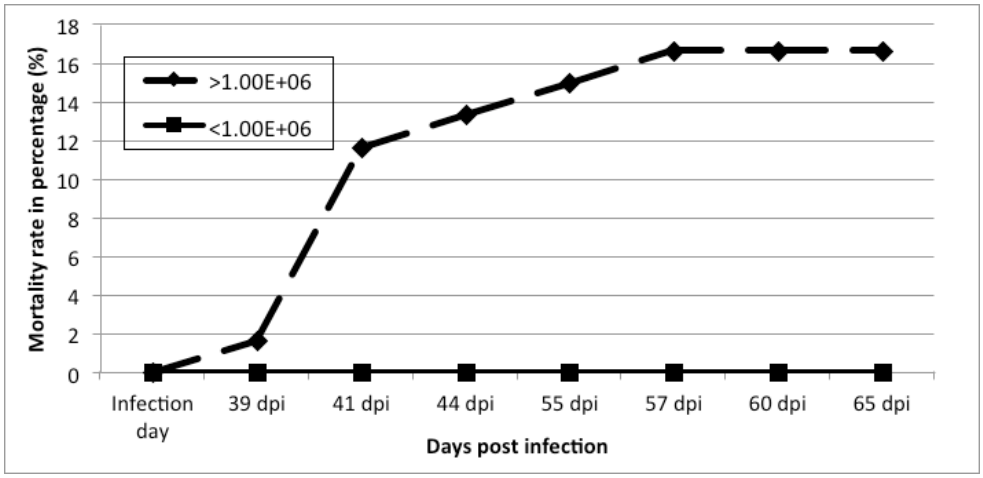


Figure 24.7.3. Cumulative mortality of meagre infected with *Nocardia brasiliensis* at concentrations above and below 10^6 CFU/mL.

Bacteriology

N. brasiliensis was isolated at the first sampling point (30 dpi) from 50% fish inoculated with a dose of 10^8 CFU/mL as also as 10^7 CFU/mL and from 25% of fish injected with 10^4, 10^3 and 10^2 CFU/mL dose. At the second sampling point (37 dpi), the bacterium was only recovered from nearly 50% and 100% of fish inoculated with doses 10^8 and 10^7 CFU/mL, respectively (**Figure 24.7.4**). It was possible to isolate the bacteria from the organs of dead fish up to 41 days post infection. From that moment on, *Nocardia* spp. was not detected. Bacteria recovery was higher from liver (11%) than from spleen (4%) and kidney (4%), the first month than the last period, and in groups "10^8" and "10^7" than in the others (**Figure 24.7.4**).

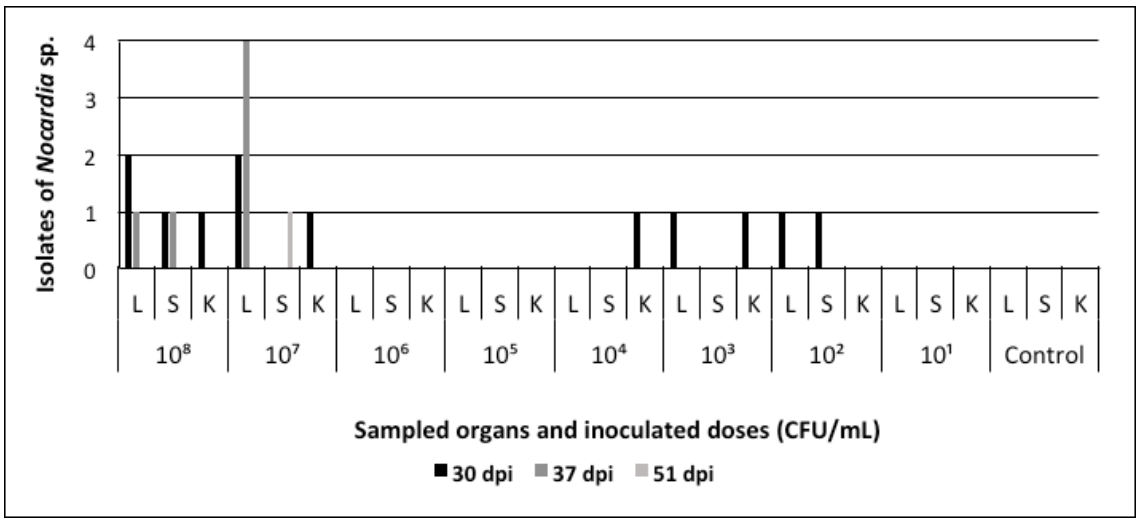


Figure 24.7.4. Number of experimentally infected meagres from which *Nocardia brasiliensis* was isolated. Isolates from collected fish organs per experimental group at each sampling point. Abbreviations: L, liver; S, spleen; K, kidney.

Histopathology

Microscopic granulomas were observed from 30 days post infection in internal organs of 25% of fish injected with 10^8, 10^6, 10^5 and 10^4 CFU/mL, reaching 50% of fish with granulomas in the groups inoculated with bacteria concentrations of 10^3 and 10^2 CFU/mL. Then, the incidence of granulomas increased irregularly throughout the experiment and between doses. (**Figure 24.7.5**). It was observed that cases where only one granuloma per organ was visible represented 88%, while only 11% showed to have more than one granuloma. 89% of the animals with more than one granuloma per organ were inoculated with 10^8 and 10^7 CFU/mL. In dead fish, microscopic granulomas were observed in all organs sampled at 41 days post infection –all fish from tanks with 10^8 and 10^7 CFU/mL-, in the case found two days before, at 39 dpi, no granuloma was observed. Kidney was the tissue most affected with the development of granulomas (26%), followed by liver (12%). Some were found in the spleen (3%) (**Figure 24.7.5**).

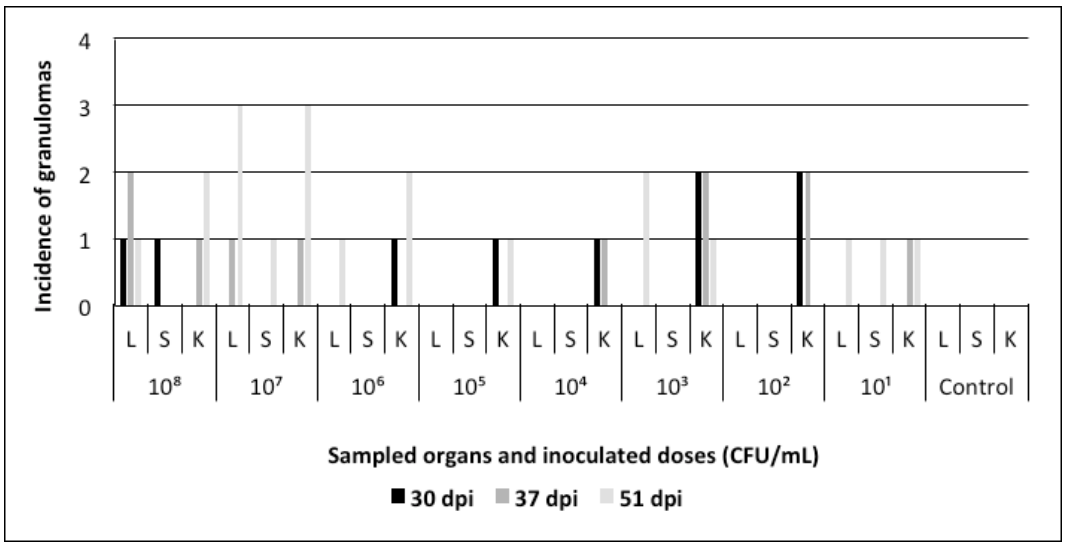


Figure 24.7.5 Fish with microscopic granulomas in collected organs at the sampling points of a challenge in meagre with *N. brasiliensis.* Abbreviations: L, liver; S, spleen; K, kidney.

Histologically, there were several types of granulomas: composed by aggregated macrophages (**Figure 24.7.6a**), with macrophages arranged in concentric layers (**Figure 24.7.6b**), with a necrotic center and layers of macrophages (**Figure 24.7.6c**), a larger necrotic center with an external layer of fibrocytes (**Figure 24.7.6d** and **Figure 24.7.6e**) and, the least expected type, completely composed of laminar material without necrotic centre (**Figure 24.7.6f**).

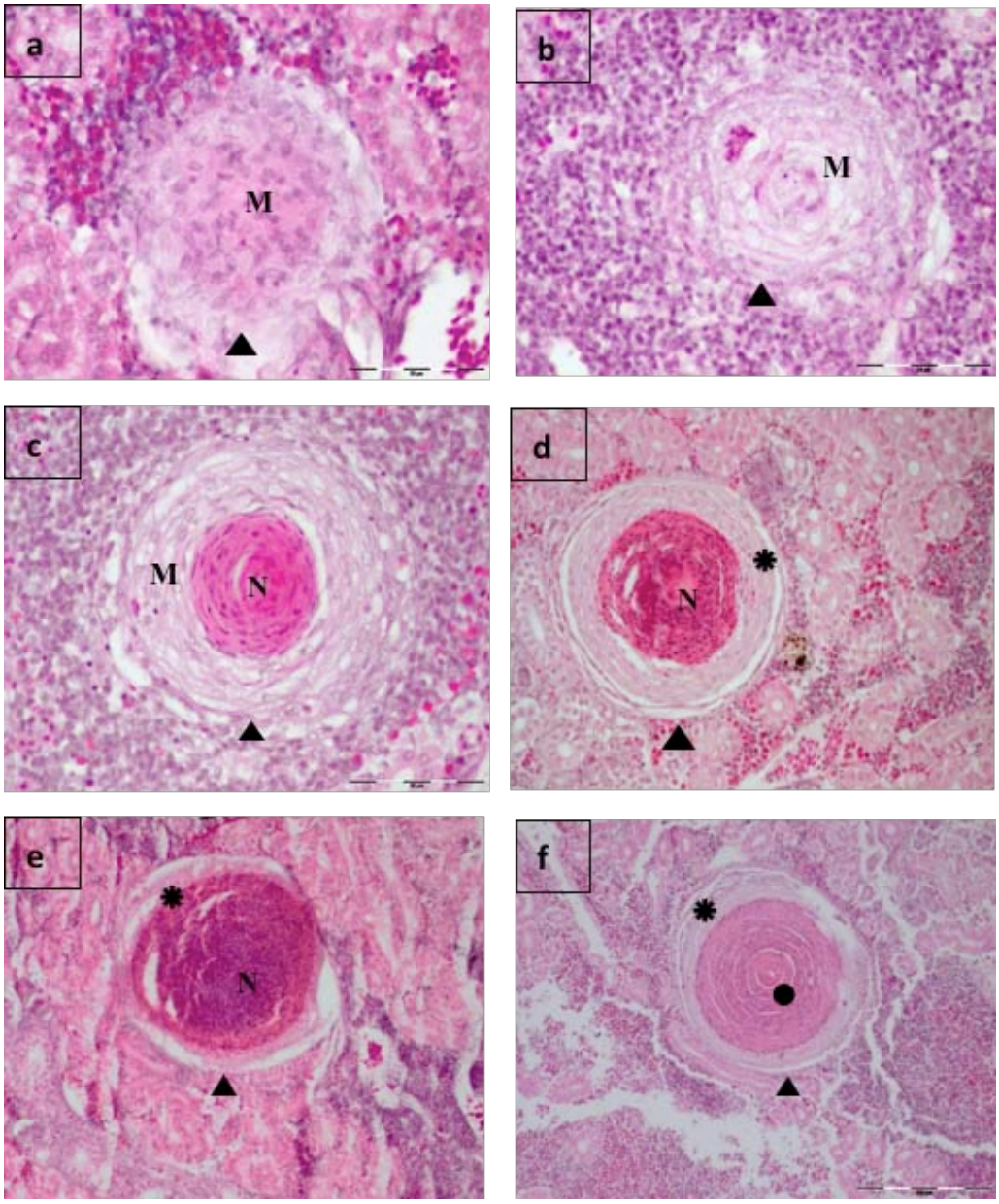


Figure 24.7.6 Histopathology of nocardiosis in meagre. Different types of granulomas: (a) aggregates of macrophages form the granuloma (b) similar to the first but the cells are arranged in concentric layers (c) with necrotic center (d) composed by necrotic center and surrounded by layers of macrophages and an outer layer of fibrocytes (e) like the previous granuloma without the layers of macrophages (f) a different pattern of granuloma development, composed completely of laminar material. Symbols: ▲, granuloma; **M,** macrophages**; N,** necrotic center; *, layer of fibrocytes; •, laminar material. Magnifications: a-d: x40; e-f: x20. Scale bars: a-c: 50 µm; f: 200 µm.

All granulomas were found in kidney, liver and spleen (**Figure 24.7.7a,b,c**) and most of them were understood to represent different stages of development, despite all types being observed at any dose and at any time.

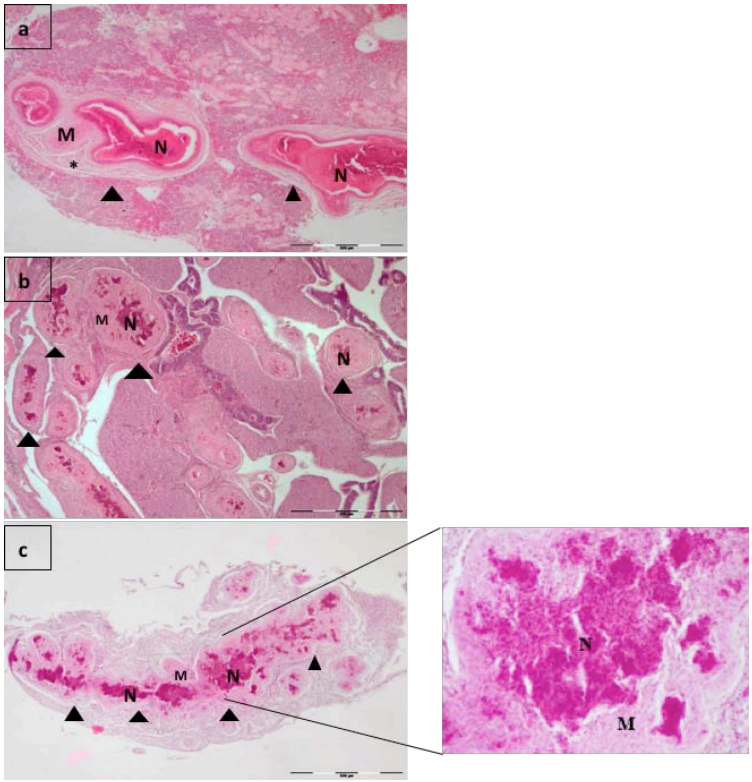


Figure 24.7.7 Histopathology of nocardiosis in meagre. Presence of granulomatous foci (▲) in (a) kidney, (b) liver and (c) spleen (with a magnification from the marked area), varied in size with marked necrotic centers (**N**), surrounded by macrophages (**M**) and connective tissue (*). Magnification: x4. Scale bar: 500 µm.

Moreover, we could not find any bacteria in histological sections, even with special stains (**Figure 24.7.8**).

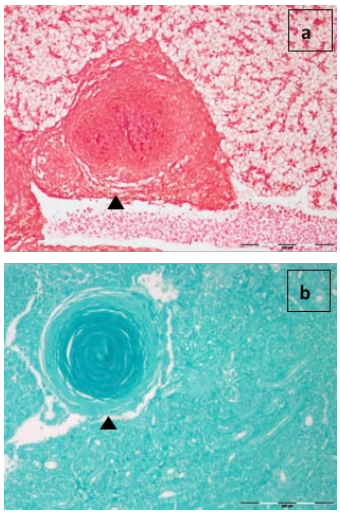


Figure 24.7.8 Special stains, (a) Gram and (b) Ziehl-Neelsen, did not reveal the presence of *Nocardia spp*. in histological sections. ▲, granuloma. Magnification: x10. Scale bar: 200 µm.

Task 24.8 Diagnostic-recommendation manual for meager health (led by HCMR, Pantelis Katharios).

In this task we foresee to develop a diagnostic manual for meagre disease. This will be a practical diagnostic manual and recommendation guide for meagre health issues targeted to fish health specialists and aquaculture scientists and producers. The manual will be the synopsis of major findings of WP24. It will be published in electronic format (pdf file) and uploaded in the project website, and will be freely available for the public. The manual will be organized in chapters describing the major diseases of the species with original photographic material, epidemiological and pathological data. Responsible for the compilation and organization of the manual will be P1. HCMR, and all partners involved (P2. FCPCT, P3. IRTA, P5. UNIABDN) will contribute according to their participation with chapters, photographic material, diagnostic keys etc. The task is still in progress and runs throughout the lifespan of the WP. Several bacterial strains have been isolated, however none of these can be considered a primary pathogen and are probably environmental opportunists.

Deviations from Annex I and their impact:

There were no deviations from the DOW.

WP 25 Fish health – greater amberjack

WP No:	25	**WP Lead beneficiary:**		P5. UNIABDN
WP Title (from DOW):	Fish health – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P2. FCPCT	P8. IEO	P15. ULL
Lead Scientist preparing the Report (WP leader):	Chris Secombes			
Other Scientists participating:	Pantelis Katharios (P1), Daniel Montero (P2), Felix Acosta (P2), Chris Secombes (P5), Douglas Milne (P5), Virginia Martín (P8), Salvador Jerez (P8), Juana Cejas (P8), Covadonga Rodríguez (P15), Jose Pérez (P15), Pilar Foronda (P15).			

Objectives

6. Provide early diagnosis tools for Epitheliocystis,
7. Develop "antiparasite diets" to be used prior to sea cage culture,
8. Begin characterisation of the immune system, with a focus on mucosal (skin/gill) defences,
9. Develop anti-monogenean parasites infection rearing protocols.
10. Develop diagnostic-prevention-treatment methods for diseases in greater amberjack.

Summary of work reported in the previous Reporting Period (1-12 Mo):

A number of studies were undertaken to study the disease issues affecting greater amberjack culture:

- Task 25.1 – Establishment of a mesocosm for amberjack culture, with sampling undertaken to determine bacterial presence with a focus on species previously associated with epitheliocytsis occurrence in Greece.
- Task 25.2 – Mass production of amberjack juveniles for subsequent studies aimed at promoting parasite resistance. Tissue samples were collected and sent to P5. UNIABDN to begin Task 25.3.
- Task 25.3 – Initial design of primers for cloning and sequencing of amberjack immune genes, with a focus on mucosal defences. Samples from P2 were used for PCR, with several products obtained that were in the process of being cloned for sequence confirmation.
- Task 25.4 – A collector device was piloted to detect and quantify the level of infestation of amberjack with monogenean parasites, without the need to handle the fish. The method was based on egg counts that were done periodically. The collector was optimised in terms of mesh size to use, position of the collector in the tank and duration in the tank. Studies of the viability of the collected eggs was also reported.
- Task 25.5 – Studies of the seasonality of potential diseases of amberjacks was started. Several strains of bacteria were isolated from skin ulcers, including *Vibrios* of the *harveyi* clade, and *Staphylococcus epidermidis*. A challenge test with *Photobacterium* subsp. *piscicida* was performed by ip injection but the fish were not susceptible to this species at the dose used (10^3cfu/fish).
- Task 25.6 – Various Greek fish farms were visited for a health status survey. Monogenean and digenean gill parasites were found and analysis of the associated pathology was begun. The anthelmintic praziquantel appeared an effective treatment.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In the 2nd Periodic Report we made progress against all of the tasks, as outlined below. This included further mesocosm trials in **Task 25.1** for development of rapid detection methods for epitheliocystis, and screening of gill samples from different Greek fish farms. In **Task 25.2** four subtasks were undertaken including; A) Morphological study on the incidence of monogenean parasite in greater amberjack skin, B) Determination

of environmental conditions that can modulate greater amberjack resistance to parasitic infection, C) Formulation of a diet supplemented with mucus stimulation products, and D) Standardization of monogenean cultures. In **Task 25.3** primers for detection of 11 immune genes were optimized for qPCR, ready for studies of mucosal defences, with initial PAMP stimulation in vivo revealing good induction at mucosal sites such as gills. Further grow out trials were undertaken in **Task 25.4**, to assess the relationship between monogenean parasite egg number and fish mortality and the impact of several potential anti-monogenean treatments, with mannose looking promising. Diagnosis of bacterial and viral infections was undertaken with juveniles in **Task 25.5**, with *Bacillus oceanisediminis* and *Aeromonas* spp. being detected. Challenge trial were also performed to assess relative disease susceptibility to two bacterial species, namely *Listonella anguillarum* and *Photobacterium damselae* subsp. *piscicida*. The fish were found to be refractory to the former. Lastly in **Task 25.6** a broodstock fish was diagnosed with a neoplastic lesion in the kidney, identified as a renal cystic adenocarcinoma, with associated *Vibrio* sp. detectable but unrelated to the tumor.

Summary of progress towards objectives (31-48 Mo):

In the current reporting period progress has been made against all tasks and is outlined in detail below. This included progress in identifying the causative agent of epitheliocystis. Whilst the mesocosm studies gave no clear results, samples collected from collaborating fish farms in Greece revealed that, in contrast to the prevalent belief that Epitheliocystis is caused by Chlamydia, at least in Greece the main pathogens causing Epitheliocystis disease are bacteria that belong in the β- or γ-proteobacteria. These bacteria have a mainly intracellular life cycle and their cultivation in vitro has not yet been accomplished. Studies to promote resistance to parasitic incidence on greater amberjack trialled two different prebiotics, namely MOS and cMOS. Positive effects were found for the cMOS and prebiotic combination (MOS + cMOS) following challenge with *N. girellae*, in terms of lower infestation levels and lower numbers of parasites per cm^2 of skin. Immune gene expression analysis of skin and gills also showed positive effects with cMOS. Whether cMOS could impact on bacterial load was also studied. After feeding for 90 days the fish were stressed by crowding and prevalence of opportunistic bacteria detected in samples from liver and spleen. Whilst crowding resulted in 100% prevalence for opportunistic bacteria in both tissues, twice the number of bacterial species (Vibrio's) were present in the control diet fed fish vs cMOS fed fish. Further immune gene analysis was undertaken using cell suspensions from kidney and spleen, and showed these cells are highly responsive to PAMPS, with differences in kinetics and magnitude of increases seen dependent upon the stimulant. Studies of one of the antimicrobial molecules in greater amberjack, piscidin, showed good bacterial growth inhibition against two fish pathogens, *Vibrio anguillarum* and *Yersinia ruckeri*. The effect of stocking density on parasite (*Neobenedenia melleni*) egg production was studied and revealed that egg number tended to decrease (P=0.08) with increased culture density. Several anti-attachment factors were also trialed and two treatments (cumin and mannose) showed a reduction in egg number over the following 2-8 days. These effects were compared to traditional anti-parasite chemical treatments, including copper sulphate, formaldehyde and hydrogen peroxide. Copper sulphate and hydrogen peroxide had no effect on egg number bur formaldehyde was effective, especially when used on several occasions. Nevertheless the potential of mannose in particular was apparent and might be improved by further optimisation of treatment dose and frequency of application. These practical applications will ultimately be incorporated into a diagnostic recommendation manual, to be published at the end of the DIVERSIFY programme.

Details for each Task

Task 25.1. Study of Epitheliocystis during larval rearing (led by HCMR, Pantelis Katharios).

The objective of the study was to develop tools for the rapid identification of epitheliocystis in larval cultures of greater amberjack. The main aetiological agents that are being monitored are members of the *Chlamydiacae* and the newly described bacteria *Ca* Ichthyocystis spp and *Ca* Endozoicomonas cretensis.

The tools have been already developed and their validation required the natural infection of greater amberjack larvae since the Epitheliocystis agents are unculturable. To this direction we have foreseen the use of mesocosm culture with the use of untreated seawater as the most appropriate system to study the disease

and validate the molecular tools. In the first reporting period, we presented the results obtained from the first rearing trial using a mesocosm system. Unfortunately, that trial ended very soon at 12 dph due to a massive mortality caused by unidentified reasons. Nevertheless, no epitheliocystis-causative agent could be traced.

In 2015 we performed a second trial. This time, for safety reasons, especially taking into account the limited availability of greater amberjack eggs, we used two parallel mesocosm cultures with greater amberjack and meagre (*Argyrosomus regius*). In addition, we started a survey in the Greek fish farms to investigate the spread of the newly identified Epitheliocystis causative agents, *Candidatus* Ichthyocystis spp. and *Candidatus* Endozoicomonas cretensis. It should be noted that this study was not foreseen in the DoW but has been included in this task without any additional cost for the project.

Rearing trials and detection of the pathogens

2015 experiments

Two parallel larval rearing trials were made in HCMR using the mesocosm technology described earlier. **Table 25.1.1** contains the information concerning these trials and the samples obtained.

Table 25.1.1. Data of the rearing trials and the samples obtained

Date	Water source	Argyrosomus regius		Seriola dumerili		Samples
		sampling	age (dph)	sampling	age (dph)	
2/7/2015	Borehole	1	hatching			filtrate + eggs + larvae
3/7/2015	Borehole		1			
4/7/2015	Borehole		2			
5/7/2015	Borehole		3			
6/7/2015	Borehole		4			
7/7/2015	Borehole	2	5			filtrate + larvae
8/7/2015	Sea	3	6	1	Hatching	filtrate + larvae
9/7/2015	Sea		7		1	
10/7/2015	Sea	4	8	2	2	filtrate + larvae
11/7/2015	Sea		9		3	
12/7/2015	Sea		10		4	
13/7/2015	Sea	5	11	3	5	filtrate + larvae
14/7/2015	Sea		12		6	Larvae
15/7/2015	Sea	6	13	4	7	filtrate + larvae
16/7/2015	Sea		14		8	Larvae
17/7/2015	Sea	7	15	5	9	filtrate + larvae
18/7/2015	Sea		16		10	
19/7/2015	Sea		17		11	
20/7/2015	Sea	8	18	6	12	filtrate + larvae
21/7/2015	Sea		19		13	Larvae

22/7/2015	Sea	9	20	7	14	filtrate + larvae
23/7/2015	Sea		21		15	
24/7/2015	Sea	10	22	8	16	Larvae
25/7/2015	Sea		23		17	
26/7/2015	Sea		24		18	
27/7/2015	Sea		25	9	19	Larvae
28/7/2015	Sea		26		20	
29/7/2015	Sea		27	10	21	Larvae
30/7/2015	Sea		28	11	22	Larvae
31/7/2015	Sea		29	12	23	Larvae
1/8/2015	Sea		30		24	
2/8/2015	Sea		31		25	
3/8/2015	Sea		32	13	26	Larvae

Ten L of water were taken from the water column of the tanks at each sampling in triplicates. Samplings were made using a special sampler.

Samples were fractionated. Particularly, they were consecutively filtered through decreasing pore diameter filters: 250 μm, 120 μm, 53 μm and 25 μm. The samples obtained (filtrates & larvae) were used for **1)** histology **2)** molecular analysis and **3)** preserved and stored for future use. In addition, 1-2 L of flow-through water from the abovementioned fractionation passed through a 0.22 μm filter. This filter was divided in three equal pieces and used for **1)** molecular analysis **2)** isolation of bacteria in general nutrient media and **3)** preservation and storage for future need.

In total, 10 samplings were made for *Argyrosomus regius* and 13 for *Seriola dumerili* in 2015. The filtrates were placed in 50 ml falcons and centrifuged immediately at 5,000 rpm for 15 min. The supernatant was discarded and the pellet was transferred into a 1.5 ml Eppendorf, re-centrifuged at 20,000 rpm and the resulting pellet was stored until further analysis. The filtrates for histology and molecular analysis were preserved in preservative, PBF 10% and RNA later respectively while the filtrates for future use were stored in -80^{o}C. Filtrates from the 0.22 μm filters were preserved in glycerol 25% at -80^{o}C.

Total DNA extraction was performed from the filtrates, the individual larvae samples and the bacteria isolated in bacterial cultures. Filtrate and larvae samples were digested using Proteinase K (1-3 h at 56^{o}C) and DNeasy Blood & Tissue Kit was used for the DNA extraction. DNA of the microorganism from the filter of 0.22 μm diameter was similarly extracted whereas DNA from the cultivated bacteria was extracted via boiling process. Specific pairs of primers were used for each pathogen. The data of primers and PCR condition are presented in detail on **Table 25.1.2.**

Table 25.1.2. Data of the pairs of primers and the PCR conditions for the detection of pathogens, the microorganism targets.

Pathogen	Primer	Primer sequence (5'-3')	Annealing temperature	Extension duration	Product's length
Endozoicomonas spp.	Endo sp F	AGTAGGGAGGAAAGGTTGAAGG	60ºC	30 sec	400 bp
	Endo sp R	CCCAGAATACAAGACTCCGGAC			
Ichthyocystis spp.	Ichthyo sp F	AACTARGATGGTGGCGAGTG	60-62ºC	1 min	900 bp
	Ichthyo sp R	CGCACATGTCAAGGGTAGG			
Chlamydiaceae	IGF	GACTAGGTTGGGCAAG	55ºC	30 sec	300 bp
	IGR	AGCTCTTA(T/G/A)(C/T)AACTTGGTCTGTA			

One third of the filter with diameter 0.22 μm was used for the isolation of microorganisms of each sampling day. In order to achieve the isolation of the etiological agents, pieces of the filter were cultivated in Marine agar and Marine broth.

There was no evidence of the disease in any of the greater amberjack or the meagre larvicultures conducted. The survival of fish and the duration (12 days) of the rearing process was remarkably low of the experimental larvae rearing in 2014 (**reported in previous reporting period**). Possibly, this problem was related to bad quality of the eggs and some technical issues that appeared as well (e.g regulation of temperature of the tanks, shading of the tanks etc.), since there were no isolations of pathogens from the fish. On the experiment of 2015 the technical issues were solved resulting to a 33-day rearing duration with the survival and development of the larvae being similar to that referred in bibliography.

Briefly, in the two parallel cultures none of the microorganism-targets were detected on the larvae. This was in accordance with the fact that Epitheliocystis disease clinical signs (eg inclusions in gills and skin) were not found in either fresh preparation or in histology. Positive signal for *Chlamydiaceae* and *Endozoicomonas* spp. appeared for some of the filtrates. Positive signal for *Chlamydiaceae* was observed in different filtrates of both experiment and also in algae sample from the microalgae cultures that was used for the rearing process during the early developmental stages of the larvae. Positive signal for *Endozoicomonas* spp. was observed in different filtrates of the same sampling days during the first days of the experiment (2015) but it was not detected in any of the following samplings. Finally, there was no positive signal for *Ca Ichthyocystis* spp of the samples analyzed.

Fish farm survey

Survey 1

Apart from the experimental larvae rearing that were performed for the isolation, the observation and the understanding of the biology and the life cycle of Epitheliocystis agents, at the HCMR facilities in Crete, gill samples of cultured gilthead seabream with Epitheliocystis were collected from collaborating fish farms of Greece. The samples collected were from sea bream farming in cages at the regions of Arkadia, Argolida, Galaxidi, Astakos, and Euvoia. The samples were undergone PCR and histology analysis in HCMR while analysis was completed at the Veterinary Department of Zurich University (prof. Lloyd Vaughan) within the framework of cooperation of other research programs (Aquaexcel, KRIPIS). qPCR, Fluorescent in situ

hybridization (FISH), TEM και genomic analysis were performed in Zurich. The typical morphology of the cysts of Epitheliocystis in gilthead seabream gills is shown in **Figure 25.1.1**.

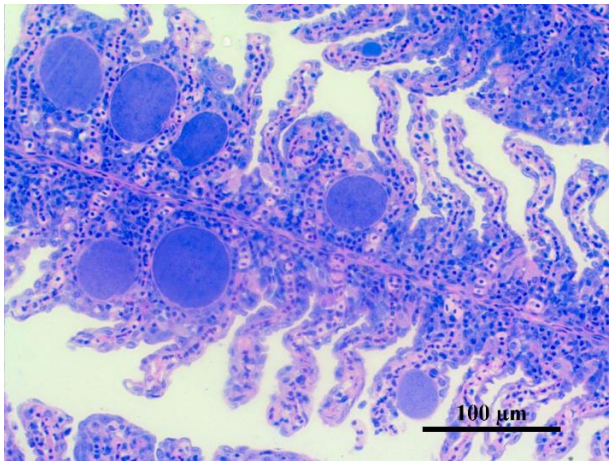


Figure 25.1.1. Typical appearance of Epitheliocystis. Intracellular inclusions in the gills epithelium of the secondary lamellae can easily be observed. The sample is from a sea bream farmed in Argolida.

Two different morphological types of cysts were observed in gill histology. The majority of the cysts were big with diameter 80-100 μm circumscribed in a fine, thin membrane whereas there were few others of smaller size with the outer membrane slightly thicker and the content of the cyst being intensely granular.

Gill samples with Epitheliocystis from the different fish farms of Greece were screened using PCR with specific primers (**Table 25.1.2**) for the particular species in order to identify the presence of the pathogen in other regions of Greece as well.

More than 60 samples were screened in total covering the time period 2012-2015 and the regions of: Argolida, Arkadia, Astakos, Saronikos, Galaxidi and Euvoia. All samples were positive for Ichthyocystis and Chlamydia of the Piscichlamydia genus. qPCR analysis showed that the majority of the load belonged to Ichthyocystis identifying it as the main and dominant pathogen in Greece with wide geographical distribution.

The results of the experiments, the samplings and all the analysis process revealed that, in contrast to the prevalent belief that Epitheliocystis is attributed to Chlamydia, at least in Greece the main pathogens causing Epitheliocystis disease are bacteria that belong in the β- or γ-proteobacteria. These bacteria have a mainly intracellular life cycle and their cultivation in vitro has not yet been accomplished.

Survey 2

Since the study of Epitheliocystis is still in progress for our group, trying to acquire comprehensive knowledge of the disease, the life cycle of the pathogens and its effect on the host, more samples of sea bream gills are being collected from the collaborating fish farms. In combination with the molecular, histology and electron microscopy analysis that are being conducted, we will try to observe if there is a correlation between:

- Season (collecting samples whenever an incident of Epitheliocystis emerges in a fish farm)
- the water temperature of the farm cages sampled region
- the fish age
- the time period fish were put out in the sea cages before they got infected
- the prevalence (how many fish were infected out of the total number screened during the sampling from the cages)

- and the intensity (an estimation of how severe the infection is related to the number and size of the cysts observed on the gills).

The new samples covered the period March 2015 – September 2016. They were originated from 3 different hosts, gilthead seabream, European seabass and greater amberjack cultured in 8 different geographic localities in Greece (**Figure 25.1.2**).

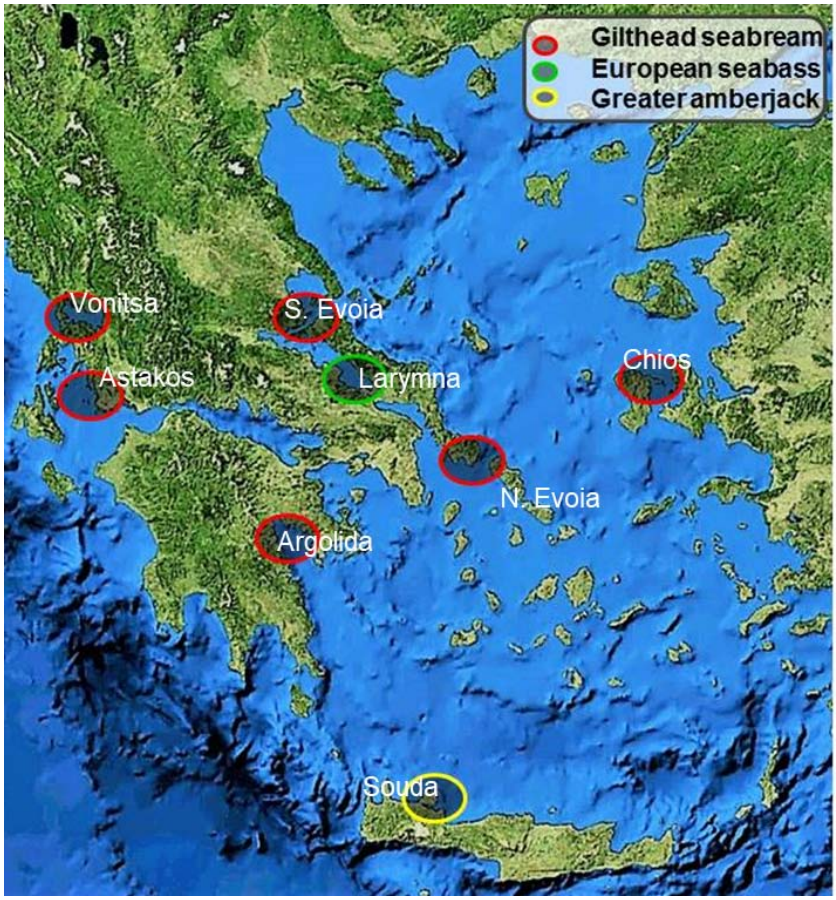


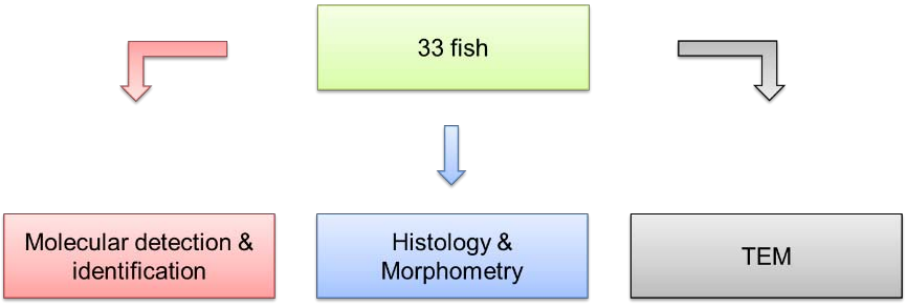
Figure 25.1.2. Sampling stations. Different color code indicates the fish species sampled.

The number of fish sampled per species together with all information regarding culture conditions are shown in **Table 25.1.3.**

Table 25.1.3. Data on the samples processed for the detection of Epitheliocystis.

Fish species	n	Age (months)	Time in the sea (months)	Weight (g)	Temperature (˚C)
Gilthead seabream	21	4 - 9	1.5 - 6	10-70	13.8-23
European seabass	3	10.5	6	15	12
Greater amberjack	9	4	2	60 - 139	26

The pipeline for the analysis of the samples is shown in **Figure 25.1.3.**



Amplification of 16S rRNA gene fragments with specific primers for
- *Ca.* Ichthyocystis spp.
- *Endozoicomonas spp.*
- Chlamydiae

Visualization of the results
Agarose-gel with Et-Br

Identification of pathogens
- Sequencing of the PCR products (Sanger)
- Comparison of the sequences with other sequences of NCBI GenBank (BLAST)

Phylogenetic analysis
- Alignment of sequences with ClustalW
- Phylogenetic trees were made using Tamura-Nei model with Neighbor joining analysis at 1000 bootstrap.

Histology
Formalin fixation – resin embedment – routine polychromic staining

Light microscopy examination of gills' sections.
Nikon microscope

Morphometrics
- Photos' acquisition **:** NIS elements (Nikon)
- Morphometric measurements **:** Image J software

Statistics
- Normalization of data measurements (ln(x)= x′)
- Levene's test: Homogeneity of variances
- t-test or one-way ANOVA: detection of significant differences among the groups
- Tuckey HSD Post-Hoc tests: multiple comparisons
- Significance: p-value<0.05

Figure 25.1.3. The identification pipeline for the processed samples.

In addition, selected samples were also processed for Electron Microscopy at the EM Lab of the University of Crete.

Of the 33 samples analyzed,

- All (33) samples were positive for *Ca.* Ichthyocystis spp.
- 15 samples were positive for Chlamydiae
- 2 samples were positive for *Endozoicomonas spp.*

Chlamydiae were found in gilthead seabream from Astakos, S. Evoia, N. Evoia, Argolida, Vonitsa and in European seabass from Larymna. *Endozoicomonas* was found in gilthead seabream from Chios.

The phylogeny of the 3 different aetiological agents of Epitheliocystis was analyzed separately for each bacterial species.

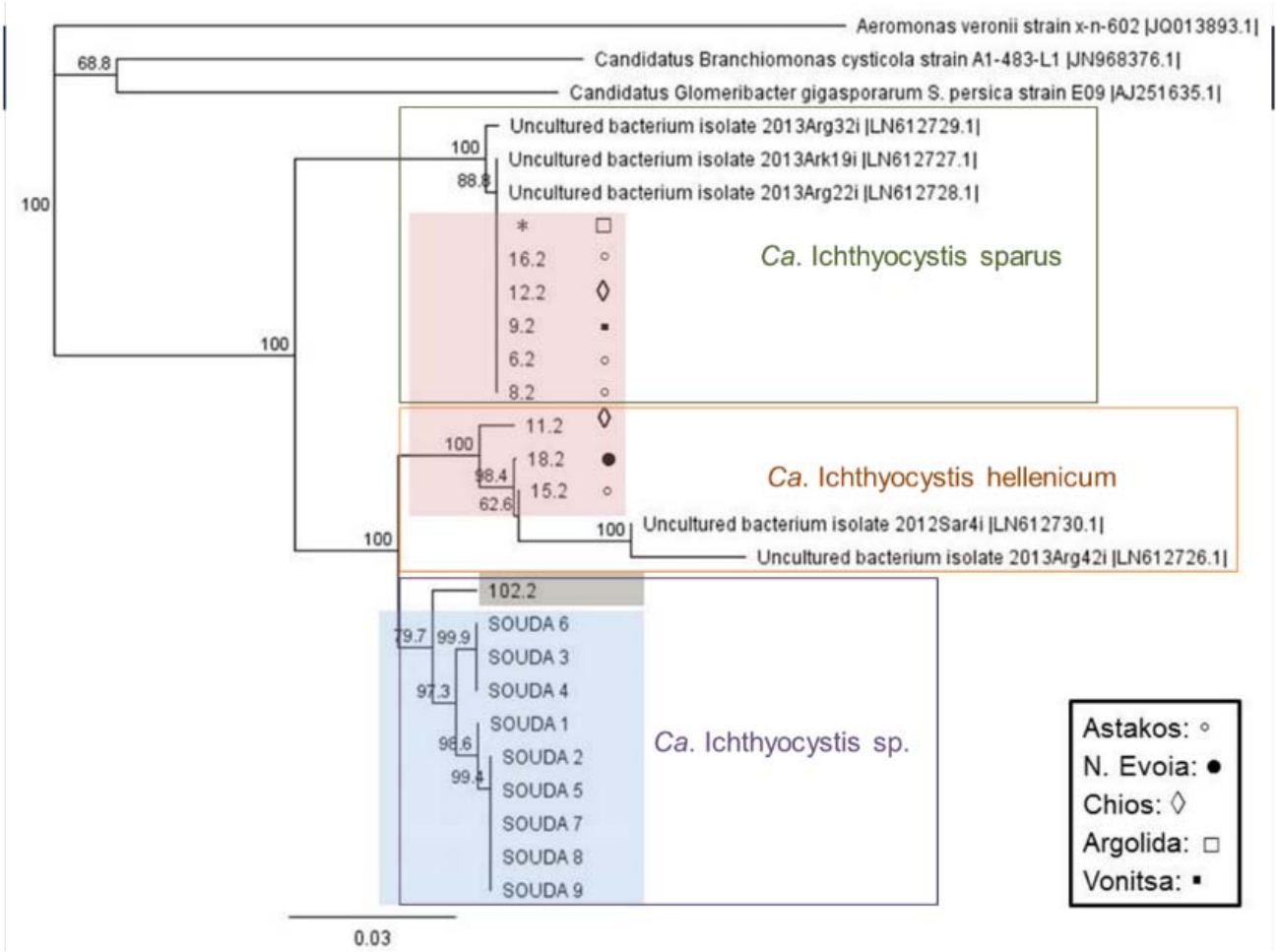


Figure 25.1.4. Phylogenetic analysis of the *Ca. Ichthyocystis* spp.-positive samples. The majority of the samples from mainland Greece are clustered together with the two species of the genus already described previously; *Ca. Ichthyocystis sparus* and *Ca. Ichthyocystis hellenicum*. However, there is a third cluster (blue color) containing the samples from on-growing adult greater amberjack from Crete (Souda) and a sample from seabass from Larymna (102.2). This cluster may represent a novel species of the genus.

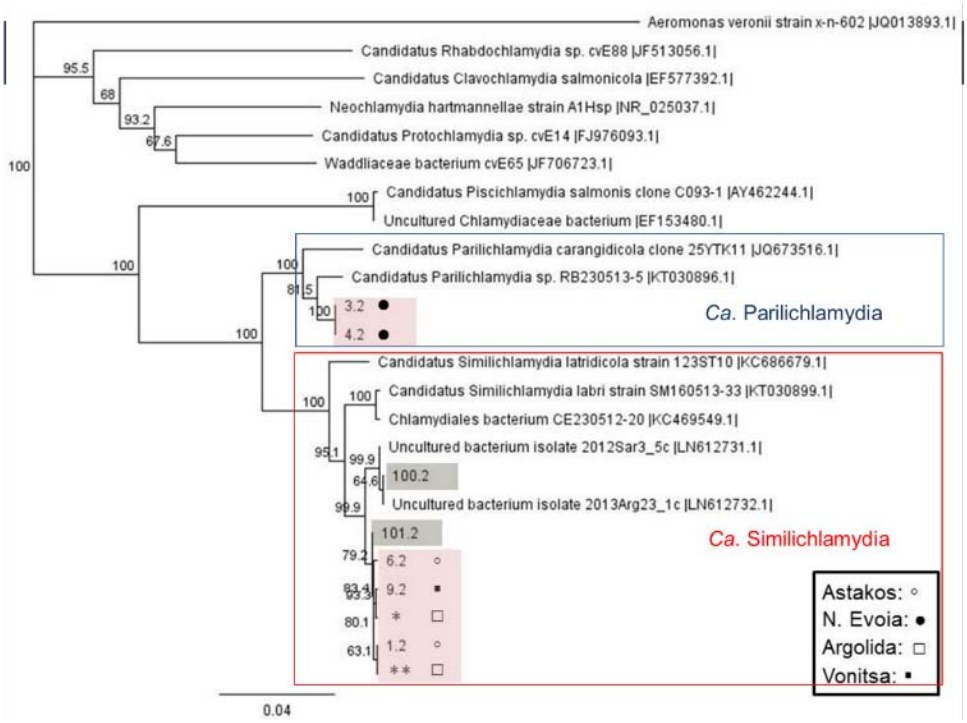


Figure 25.1.5. Phylogenetic analysis of the *Chlamydiae*-positive samples. The samples obtained from this survey cluster with *Ca. Similichlamydia* and *Ca. Parilichlamydia*.

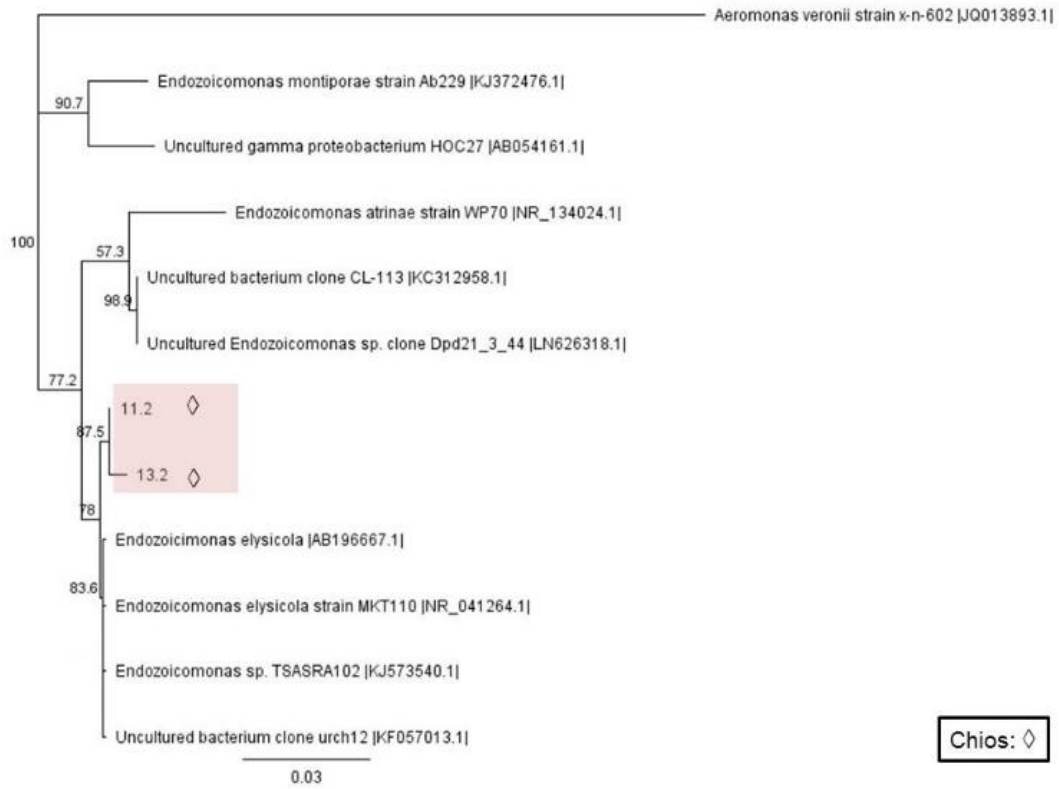


Figure 25.1.6. Phylogenetic analysis of the *Endozoicomonas*-positive sample. This sample was obtained from seabreams cultured in Chios.

Histologically, the cysts of epitheliocystis can be divided in 4 morphologically distinct groups. Type a: big cysts containing finely granular material, type b: big cysts with coarse granular material, type c: cysts with coarse granular material surrounded by a "ring" formation and type d: small cysts with ring surrounded by a capsule **(Figure 25.1.7)**.

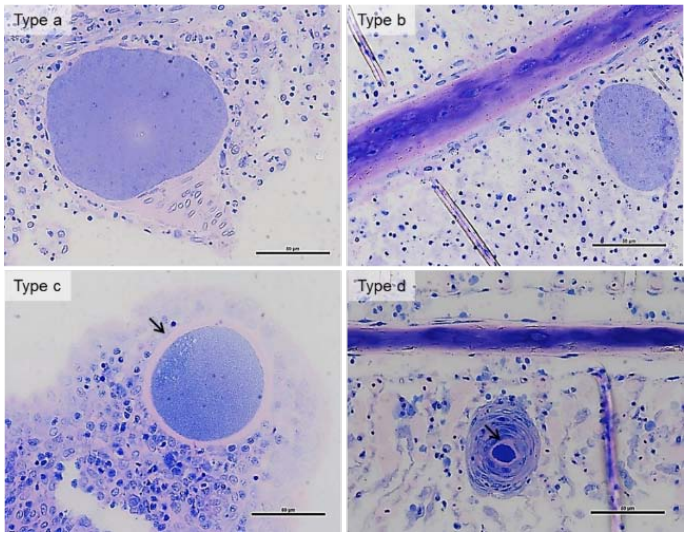


Figure 25.1.7. The four different types of cysts identified in the survey.

The morphometric characteristics of the cysts are shown compared based on the cyst type (**Figure 25.1.8**) the fish species (**Figure 25.1.9**). Morphometry of the cysts obtained from greater amberjack are shown in **Figure 25.1.10**.

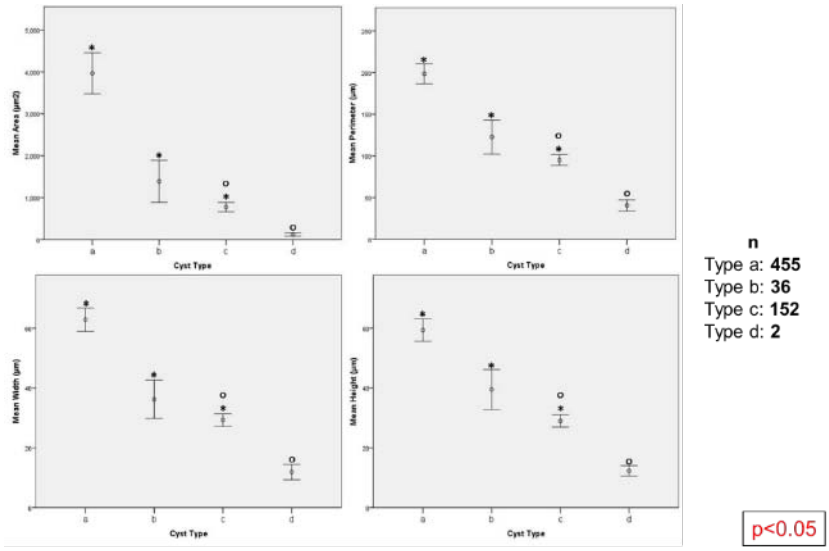


Figure 25.1.8. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the 4 cyst types.

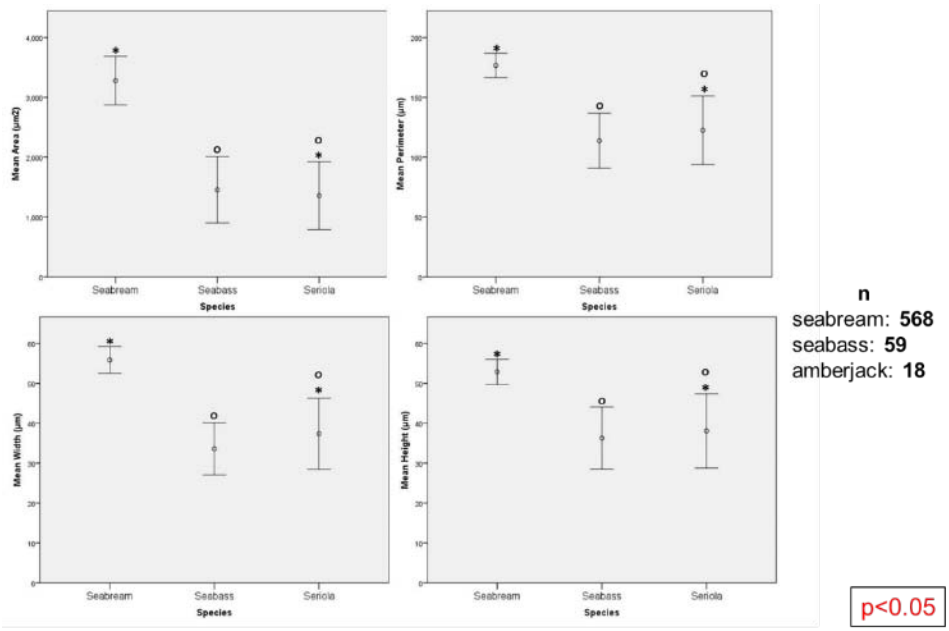


Figure 25.1.9. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the cysts in the three species studied.

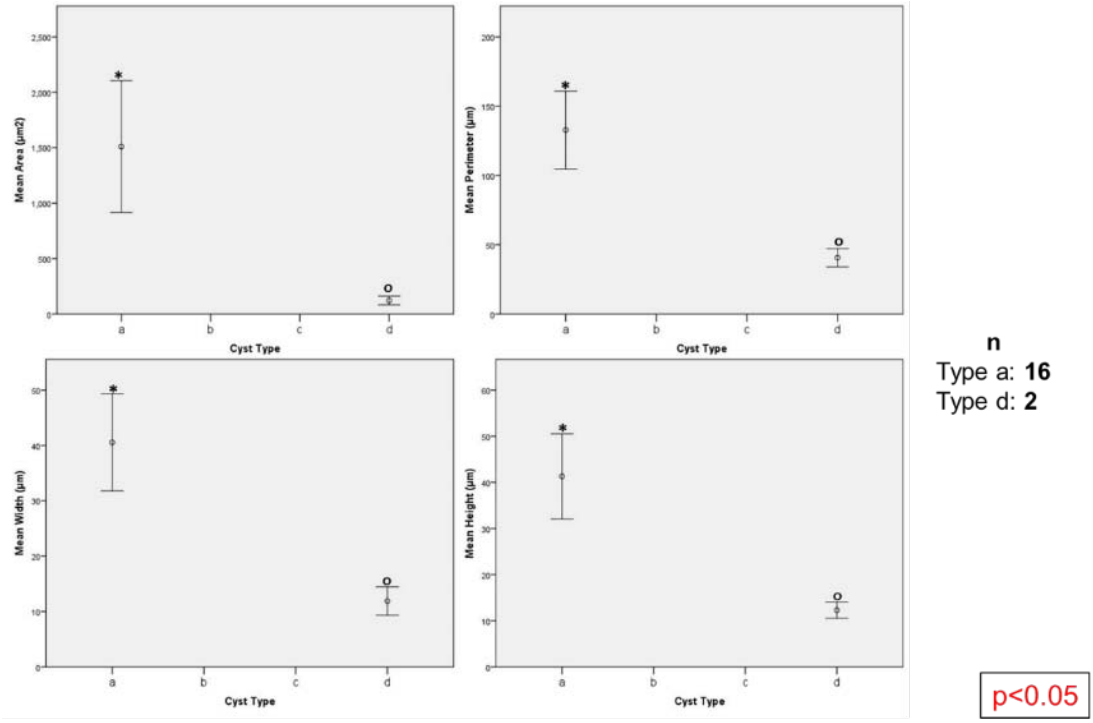


Figure 25.1.10. Mean±SD of the area (upper left), perimeter (upper right), width (lower left) and height (lower right) of the cysts in the samples of greater amberjack.

Transmission Electron Microscopy (TEM)

Two samples were processed for TEM; both were gills from seabream, one from Astakos (8.3) and one from Chios (14.3). The morphology of the bacteria in both cases are consistent with *Ca.* I*chthyocystis* spp. (**Figure 25.1.11**).

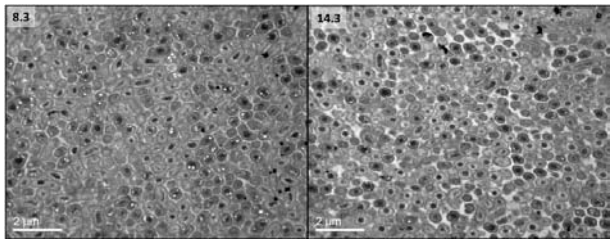


Figure 25.1.11. TEM micrograph of the bacteria inside the cysts. In both cases the bacteria are densely packed and their morphology is consistent with that of *Ca. Ichthyocystis* spp.

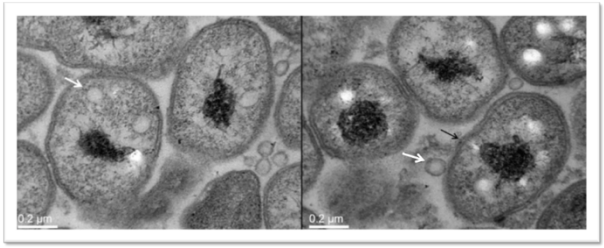


Figure 25.1.12. TEM micrograph of the bacteria inside the cysts. The bacteria have a visible double membrane (black arrow) and contain spherical bodies (white arrows). Compact nucleoids appear dark in the centre of the bacteria.

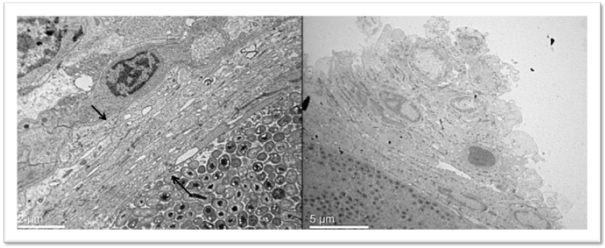


Figure 25.1.13. Bacteria are clearly separated from the fish tissue by interconnected epithelial cells which form a thick layer.

In HCMR facilities, Epitheliocystis has affected larviculture using 'Mesocosm' technique many times. The disease has emerged twice in sharpsnout seabream (*Diplodus puntazzo*) larviculture (Katharios et al. 2008, 2015) and in greater amberjack and common dentex (*Dentex dentex*) culture. From the incidents referred above, molecular identification was succeeded for the second incident of Epitheliocystis outbreak in *Diplodus puntazzo* (Katharios et al. 2008, 2015) when the main pathogen related to the disease was *Candidatus* Endozoicomonas cretensis n. sp. One of the main problems is that most of the times, in the same

incident, more than one microbial agents related to the disease coexist. As a result, the identification of the pathogen responsible for Epitheliocystis demands the use of techniques such as qPCR or FISH.

Despite that the disease did not occur on the experimental larviculture performed within the framework of the DIVERSIFY project, there was molecular identification of bacteria that can be potential pathogens responsible for Epitheliocystis outbreaks. Concerning Chlamydia, the main signal was detected in algae samples leading to the conclusion that they were environmental Chlamydia, irrelevant with the disease, since the primers used for Chlamydia detection were universal primers for Chlamydia signature primers.

From the survey we conducted in the Greek fish farms we confirmed our hypothesis that Epitheliocystis can be caused by various unrelated pathogens which in many cases infect simultaneously the same host. We also confirmed that the most significant pathogen is *Ca.* Ichthyocystis spp. and we have possibly found a novel species of the genus, which infects adult greater amberjacks.

Analysis is still ongoing and all the results of this task will be included in the Deliverable D25.6.

Part of these results were presented at the European Association of Fish Pathologists Conference held in Belfast 4-8 September 2017: Antonakaki Angeliki, Vaughan Lloyd, Maja Ruetten, Seth-Smith Helena, Petropoulos Ioannis, Dourala Nancy, Kantham K. Papanna, Katharios Pantelis. *Epitheliocystis disease in Greece is caused by a wide variety of unrelated bacteria*

Task 25.2. Promoting resistance to parasitic incidence on greater amberjack (led by FCPCT, Daniel Montero).

Trial Design

Two hundred and sixteen fish (331.4 ± 30 g) were separated in twelve cylindroconical 1,000 L tanks (eighteen each) in open circuit. Fish were fed by hand 3 times per day to apparent satiety, and the uneaten pellets recovered.

Diets used combined a *Seriola* base diet designed by SKRETTING® (Stavanger, Norway) with two different prebiotics, namely MOS and cMOS (BIOMOS® and ACTIGEN® developed by ALLTECH®). Diet C (control) was composed exclusively by *Seriola* base diet and without prebiotic. Each diet was randomly assigned to triplicate groups of fish (n=3x3).

Sampling procedures

A sampling point was conducted at time 0, 30 days and 60 days, where growth and feed data were collected. At 90 days fish growth and feed data were also recorded, and head kidney, spleen, gills and skin of 3 fish per tank were sampled for gene expression. Additionally, skin mucus and blood for serum extraction were collected. After that, a final parasite challenge with *N. girellae* was performed.

Results

Growth performance

No significant differences were observed in final weight or SGR among groups ($p>0.05$), although cMOS diet obtained the highest values (**Table 25.2.1**). Similarly, feed efficiency and feed intake showed better indexes were recorded in cMOS diet despite no significant differences among groups were observed.

Serum and skin mucus immunological parameters

No differences were obtained for serum and skin mucus lysozyme activity ($p<0.05$) among groups. Nevertheless, significant differences were observed for serum bactericidal activity ($p<0.05$) also showing by two-way ANOVA that no interaction was obtained among prebiotics. Skin mucus bactericidal activity did not show significant differences ($p>0.05$) despite being cMOS diet the one with the highest values (**Table 25.2.1**).

Parasite challenge

Parasite challenge showed significant differences among groups ($p<0.05$) for all the parameters measured. On one hand, infestation level showed the highest values in fish fed control (C) and MOS diet, while cMOS and prebiotic combination (MOS + cMOS) diet presented the lower infestation levels (**Table 25.2.1**). Number of parasites per cm^2 of fish showed equivalent results, being control and MOS diets the ones with higher number of parasites per cm^2, while cMOS and prebiotic combination the lowest values. Regarding parasite size, control diet showed the highest value and cMOS as well as prebiotic combination presented the lowest ($p<0.05$).

Table 25.2.1. Results obtained at the end of the trial (90 days). Different letters denote significant differences. Interaction among MOS and CMOS is compared with Two-way ANOVA. SGR: Specific growth rate; FCR: Feed conversion ratio; S.M: Skin mucus; Lyso. Act.: Lysozyme activity; Bact. Act.: Bactericidal activity; Nº para. /surface: Nº of parasites per cm^2 of skin surface area.

	Diet C	MOS	cMOS	MOS + cMOS	Interaction MOS-CMOS
Final Weight (g)	1046.75 ±129.61	1024 ±161.17	1090.37 ±135.49	1036.55 ±126.88	P>0.05
SGR (%)	1.096 ±0.04	1.095 ±0.06	1.139 ±0.09	1.081 ±0.07	P>0.05
FCR	1.537 ±0.14	1.523 ±0.04	1.44 ±0.10	1.44 ±0.16	P>0.05
Intake (g)/100g fish	43.75 ±1.45	44.29 ±3.39	43.32 ±3.31	43.99 ±2.65	P>0.05
S.M. Lyso. Act. (µg/ml)	15.45 ±1.71	14.45 ±4.74	13.45 ±0.94	13.7 ±5.98	P>0.05
Serum lyso. Act. (µg/ml)	37.81 ±4.08	42.4 ±2.5	34 ±3.50	36.45 ±3.79	P>0.05
S.M. Bact. Act. (%)	3.72 ±1.86	5.03 ±1.21	6.54 ±0.89	5.22 ±2.61	P>0.05
Serum Bact. Act. (%)	4.89 ±1.06 ^{a}	5.91 ±1.70 ^{ab}	8.27 ±1.05 ^{b}	9.51 ±1.27 ^{b}	P>0.05
Parasit. Degree level	2-3 ^{a}	2 ^{a}	1-2 ^{b}	1-2 ^{b}	
Parasite total length (mm)	4.44 ±0.31 ^{a}	3.9 ±0.43 ^{ab}	3.32 ±0.40 ^{b}	3.56 ±0.43 ^{b}	P>0.05
Nº para. / Surface (cm^2)	0.101 ±0.01 ^{a}	0.087 ±0.02 ^{a}	0.015 ±0.01 ^{b}	0.042 ±0.01 ^{b}	P>0.05

Gene expression

MOS and cMOS diets produced an effect in the immune genes selected for all the tissues. Skin gene expression data showed significant differences for TNFα, iNOS, IFNd, IFNγ, MUC-2, IL-17D and IgT, being cMOS treatment the one that produced the higher response (**Figure 25.2.1**). Gill expression of Hepcidin, Defensin, Mx protein, IFNd and IL-17D produced significant different among groups, also being cMOS the prebiotic with higher effect (**Figure 25.2.2**). Despite that, gill was the tissue with less differences recorded among groups. Head kidney gene expression of iNOS, IFNd, IL-10, IL-17D, IL-22, Mx protein and IgM showed differences among groups, being higher MOS response except in adaptive immune system parameters (**Figure 25.2.3**). Hepcidin, defensin, TNFα, IFNγ, IL-1β and IgT gene expression in spleen were significantly different among groups, being MOS the prebiotic related with the higher response (**Figure 25.2.4**).

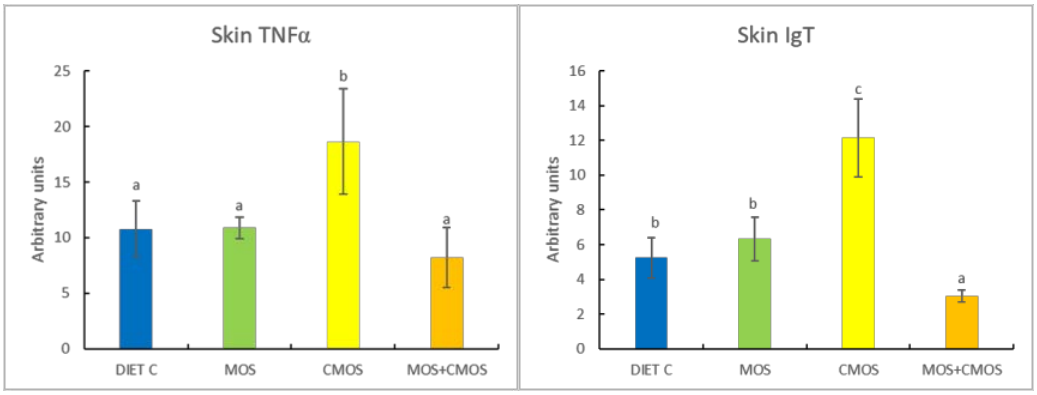


Figure 25.2.1. Skin TNFα and IgT expression. Different letters denote significant differences ($p < 0.05$).

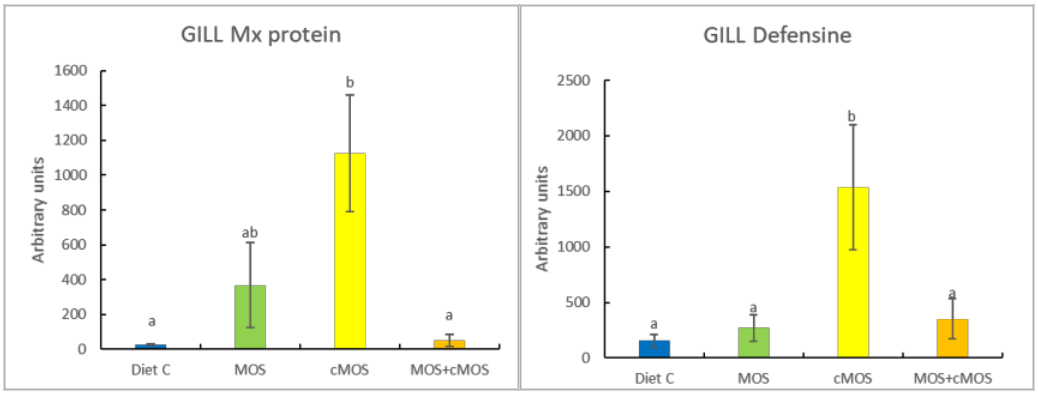


Figure 25.2.2. Gill Mx and defensin expression. Different letters denote significant differences ($p<0.05$).

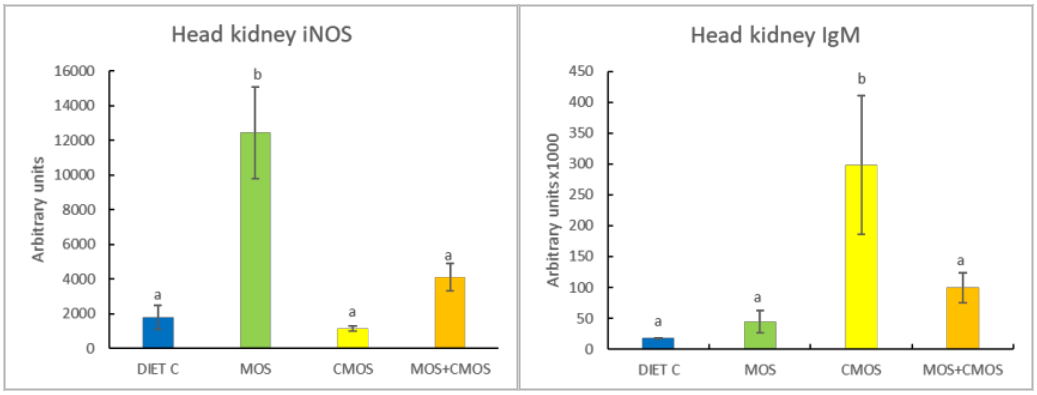


Figure 25.2.3. Head kidney iNOS and IgM expression. Different letters denote significant differences ($p<0.05$).

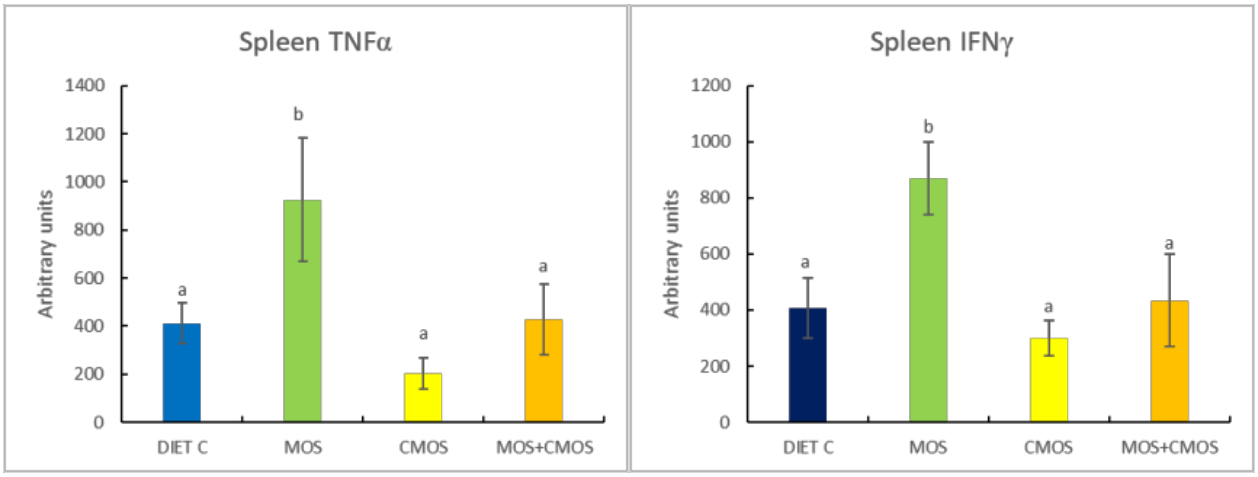


Figure 25.2.4. Spleen TNFα and IFNγ expression. Different letters denote significant differences ($p<0.05$).

Task 25.3. Identification of immune markers (led by UNIABDN, Chris Secombes).

To better understand how amberjack immune molecules are induced following infection/ vaccination, fish were stimulated *in vivo* and *in vitro* with three selected Pathogen Associated Molecular Patterns (PAMPs), with PBS exposed fish/ cells acting as controls.

As reported previously an in vivo study was performed where the immune stimulants poly I:C, LPS and flagellin were injected intraperitoneally into greater amberjack and led to the modulation of key immune genes in the gills, gut, head kidney and spleen, as assessed by QPCR assays.

In tandem, an in vitro stimulation of cell suspensions derived from the head kidney and spleen was performed, by exposing these cells to the same three PAMPs for 4, 12 or 24 h. QPCR assays were then performed using the produced CDNA and the results analysed. **Figure 25.3.1** shows representative results from this large data set and demonstrates how cytokine expression is modulated in the cell suspensions following PAMP stimulation. IL-17D is highly upregulated following PAMP stimulation after 24 h in head kidney cells, but in spleen cells modulation occurs earlier, after 4 h following flagellin exposure and after 12 h following poly I:C exposure. IL-22 upregulation can be observed 24 h after LPS and flagellin exposure in head kidney cells, while in spleen cells earlier upregulation is again apparent, after just 4 h in spleen cells, but with a lower fold increase. IL-22 was also upregulated in spleen cells in response to poly I:C, but only 12 h after stimulation. TNFα appears to be highly upregulated (50-430 fold) in head kidney cells 24 h after PAMP exposure, while a lower (~5 fold increase) but significant increase is seen in spleen cells only after 4 h with LPS and flagellin but persists to 12 h with poly I:C stimulation. When looking at the expression of these 3 cytokines a clear difference in the profile of each gene in response to PAMPs is apparent, with cell type (head kidney and spleen cells) influencing the magnitude and kinetics of the response seen.

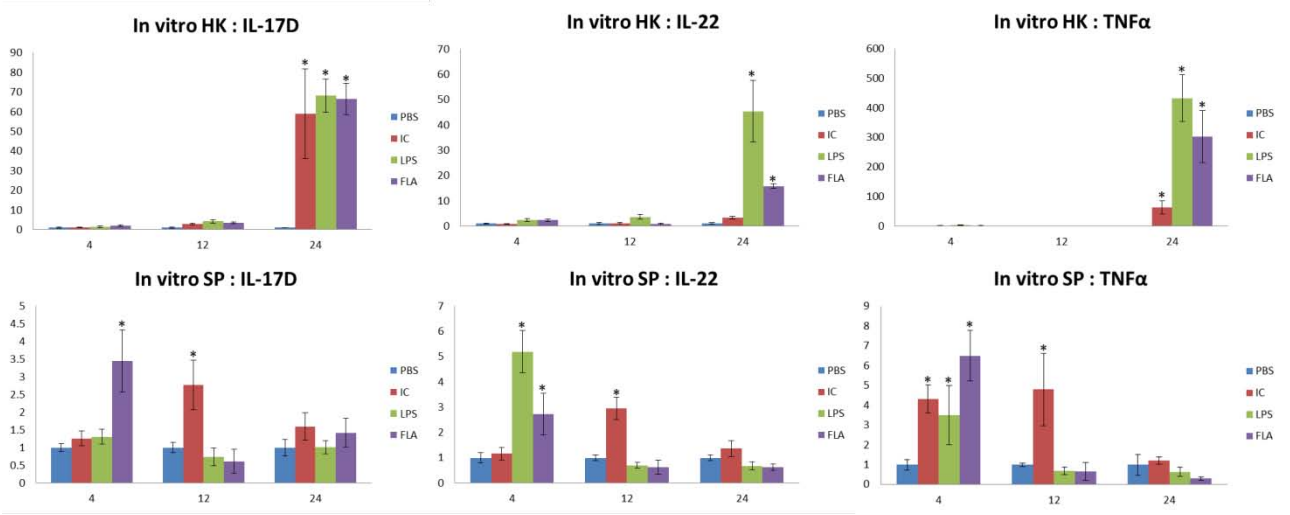


Figure 25.3.1. Fold change in gene expression following in vitro PAMP stimulation of head kidney (HK) and spleen (SP) cells. The graphs show the fold change in expression of three key immune genes following stimulation with poly I:C (red), LPS (green) and flagellin (purple) after 4, 12 and 24 h. Target genes were normalized against EF-1α expression and the data presented as the mean of 6 samples ± SEM. Groups statistically differing from the PBS control cells (One-way ANOVA, P≤0.05) are indicated by an asterisk (*).

Amberjack mucosal defences

As reported previously the amino acid sequence for the greater amberjack piscidin antibacterial peptide has been identified and the peptides properties and structure predicted. Since the last report the piscidin active peptide has been synthesised using a solid phase multiple peptide system and then purified to >85% purity using HPLC by BIOMATIK. The peptide was then incubated with several different bacterial strains at a range of concentrations and the growth of the bacteria recorded by monitoring the OD_{650} using a spectrophotometer. Measurements were taken before the addition of piscidin, after the addition of piscidin and then every 30 min for 3 h. A representative example of the results can be seen in **Figure 25.3.2**. Exposure to piscidin has a profound effect on the growth of both *Vibrio anguillarum* and *Yersinia ruckeri*, which was notably reduced when incubated with piscidin at a concentration of 400 ng/ml. Growth was completely arrested when both bacteria were incubated with a concentration of 800 ng/ml of piscidin.

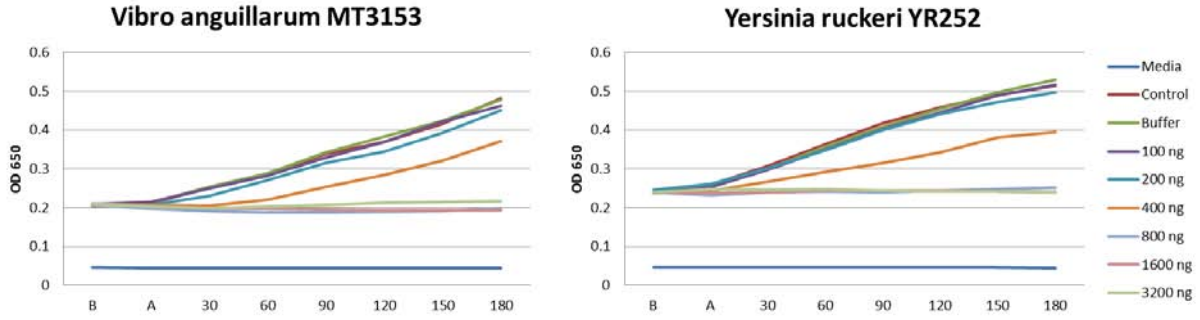


Figure 25.3.2. Growth of bacterial strains incubated with piscidin. These graphs record the OD_{650} of bacterial strains suspended in TSA media. The bacteria were incubated with piscidin ranging from 100 ng/ml to 1,600 ng/ml. Controls of TSA containing no bacteria (Media), bacteria growing in media unhindered (Control) and bacteria incubated with the buffer used for piscidin reconstitution (Buffer) were also performed. Readings were taken before the addition of piscidin (B), after the addition of piscidin (A) and then every 30 min (30, 60, 90, 120, 150, 180) for 3 h.

Task 25.4. Effectiveness of stocking density and anti-oncomiracidia attaching substances in the control of monogenean parasites (led by IEO, Salvador Jerez, Juana Cejas, Virginia Martín; ULL, Covadonga Rodríguez, Jose Pérez, Pilar Foronda).

Some preliminary experiments have been performed and useful information for the working plan initially established in the DOW concerning the efficacy of baths with anti-oncomiracidia substances and the stocking density of greater amberjack juveniles, has been obtained.

Stocking density assay

A collector device, previously designed at IEO facilities, has been used to weekly monitor the infestation level by monogenean parasites of 480 *Seriola dumerili* juveniles (average weight 175.7 ± 56.4 g, average total length 20.2 ± 2.3 cm) distributed in 12 indoor 4 m^3 tanks during 120 days (**Sub-task 21.3.2 Definition of optimal stocking density)**. The biometric and culture parameters during the trial are shown in **Table 25.4.1.**

Fish were infested by the skin fluke *Neobenedenia melleni,* and the number of eggs registered weekly. Eggs from other monogenean parasites were not collected.

Table 25.4.1. Biometric and culture parameters during the trial at different densities (T1, Low Density; T2, Medium Low Density; T3, Medium High Density; T4, High Density).

Treatment	**T1**	**T2**	**T3**	**T4**
Final weight (g)	377.1	415.6	425.4	436.3
Final biomass (g)	9050	11654	17545	27343
Final length (cm)	26.9	27.9	28.2	28.3
Final condition factor	1.90	1.89	1.86	1.88
Initial density (kg m^{-3})	1.3	1.7	2.4	3.2
Final density (kg m^{-3})	2.3	2.9	4.0	6.8

During the experimental period, the mortality registered was 2.0, 0.8, 0.7, and 2.2% for the fish maintained at Low (T1), Medium Low (T2), Medium High (T3) and High (T4) densities, respectively. This mortality was not related to parasites.

The average number of *N. melleni* eggs weekly collected during the trial tended to decrease ($P=0.08$) with the increase of the culture density (**Figure 25.4.1**). However, the average number of eggs related to the number of fish stocked in the tanks significantly decreased ($P<0.05$) with the increase of the culture density (**Figure 25.4.2**).

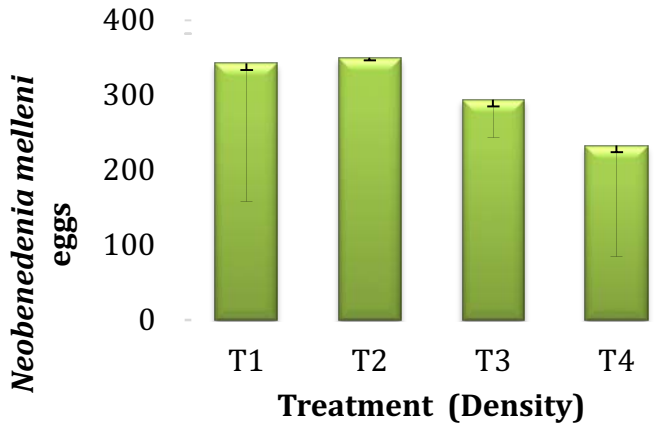


Figure 25.4.1. Average number of eggs from *Neobenedenia melleni* collected at each culture density during the 120-days trial.

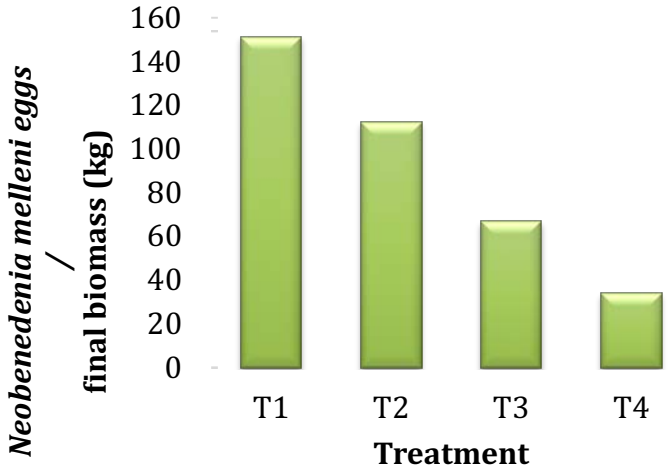


Figure 25.4.2. Average number of eggs from *Neobenedenia melleni* collected with respect to the final biomass of fish at each culture density.

Anti-attachment substances assay

Based on our previous results stated in the 2nd report, different treatments including traditional and experimental (mannose and black cumin oil) anti-attachment substances were assayed in order to determine their effects on the greater amberjack parasitism by *Neobenedenia melleni*.

A total of 51 fish were anesthetized and sampled for weight and length measurements (40.0 ± 20.7 g and 11.9 ± 2.2 cm). The fish were divided in three groups of 17 individuals, stocked at 1 m^3 tanks, and maintained during 106 days with constant seawater renewal and aeration (20.5 ± 1.3ºC and 94.0 ± 0.4% oxygen saturation). The number of eggs of monogenean parasites was sampled weekly using the egg collector device described in the previous report.

Fish groups were subject to the following experimental treatment protocols:

- *Control:* The fish were captured and stocked in a different tank with seawater during 3 minutes and moved to a new tank (S1).
- *Black cumin oil treatment:* The fish were captured and stocked in a tank with seawater and black cumin oil (4 ml l^{-1}) diluted in 60 ml of ethanol. After 3 min, the fish were moved to a new tank (S2).
- *Mannose treatment:* the fish were stocked in a mannose dilution (30 mM) in seawater (5.4 g of mannose l^{-1}) and after 3 min, moved to tank S3.

After the treatments, eggs collector devices were placed in each of the new tanks and checked every 24 h for three consecutive days. Subsequently, the sampling of the parasites eggs collectors were at different frequencies.

The control fish (untreated) increased the number of monogenean eggs during the following 10 days of experiment (532% more eggs than at day 0) whereas both the black cumin and mannose groups showed a reduction in the number of eggs collected during the following days. The effect of the black cumin was maintained exclusively for 2 days, while that of the mannose-treated fish lasted longer. At the end of the experiment, the number of eggs increased by a 48% in the black cumin treated fish, and remained constant for 8 days in fish treated with mannose resulting in a final decrease of about 45% of the initial values (**Figure 25.4.3**).

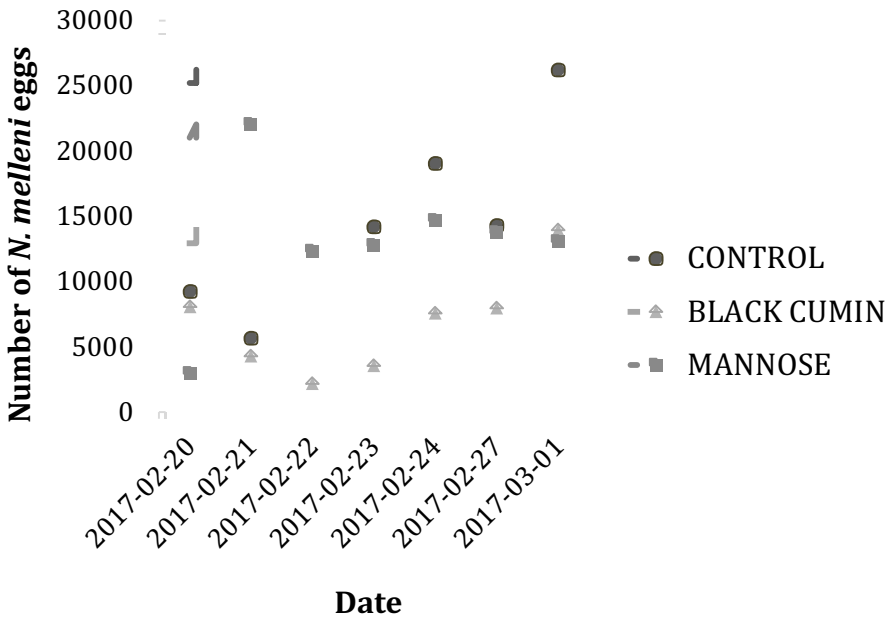


Figure 25.4.3. Evolution of the number of *Neobenedenia melleni* eggs in juveniles of greater amberjack (*Seriola dumerili*) untreated (control) and treated with black cumin oil and mannose. The arrow indicates the day of application of the treatment, and the discontinuous spotted line indicates a trend as there is no available data on the number of eggs in the control fish on the day 2017-02-22.

In order to compare the effects of the experimental treatments with those of the traditional ones, different chemical substances such as copper sulphate, formaldehyde and hydrogen peroxide, frequency of application, and several combinations of them were assayed:

- Copper sulphate and two formaldehyde treatments in Tank S1
- Formaldehyde three times in Tank S2
- Hydrogen peroxide and two formaldehyde treatments in Tank S3

The mortality of fish was registered daily and at the end of the trial (day 106). The final survival recorded was 60, 70 and 40% for tanks S1, S2 and S3, respectively.

The copper sulphate did not reduce the number of parasite eggs collected 8 days after the treatment. However, the two formaldehyde treatments reduced the number of eggs which remained low for 56 days (**Figure 25.4.4**).

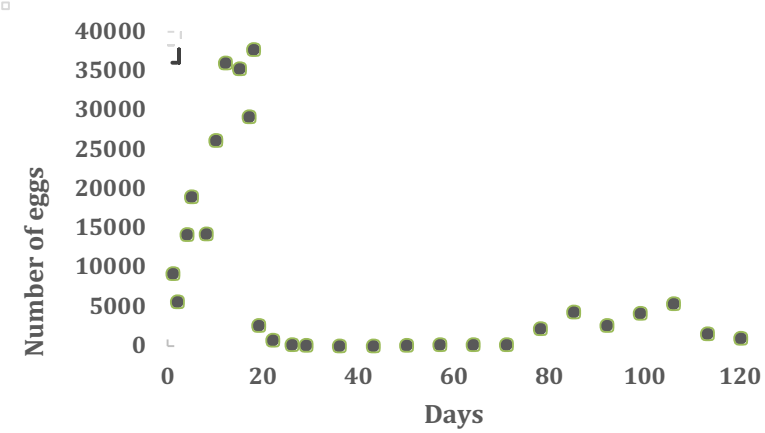


Figure 25.4.4. Evolution of the number of *Neobenedenia melleni* eggs collected in the control tank (S1) of juveniles of greater amberjack (*Seriola dumerili*) untreated and after the application of conventional treatments: copper sulphate (black arrow) and formaldehyde (grey arrows) on specific days.

The application of three successive formaldehyde treatments during 10 days in the black cumin tank (S2) reduced the number of monogenean eggs collected by 90-95%. No increment was registered in the course of the experiment (**Figure 25.4.5**).

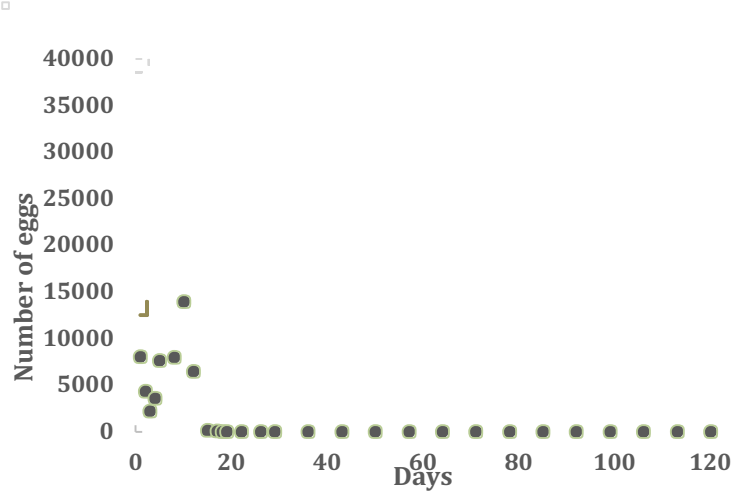


Figure 25.4.5 Evolution of the number of *Neobenedenia melleni* eggs collected in the black cumin tank (S2) of juveniles of greater amberjack (*Seriola dumerili*) under the application of experimental treatment (red arrow) and three successive treatments of formaldehyde (grey arrows) on specific days.

The treatment with hydrogen peroxide did not affect the number of eggs collected, although the two posterior applications of formaldehyde caused a decline in the number of monogenea eggs. This reduction was maintained for 55-60 days where the number of eggs raised (**Figure 25.4.6)**.

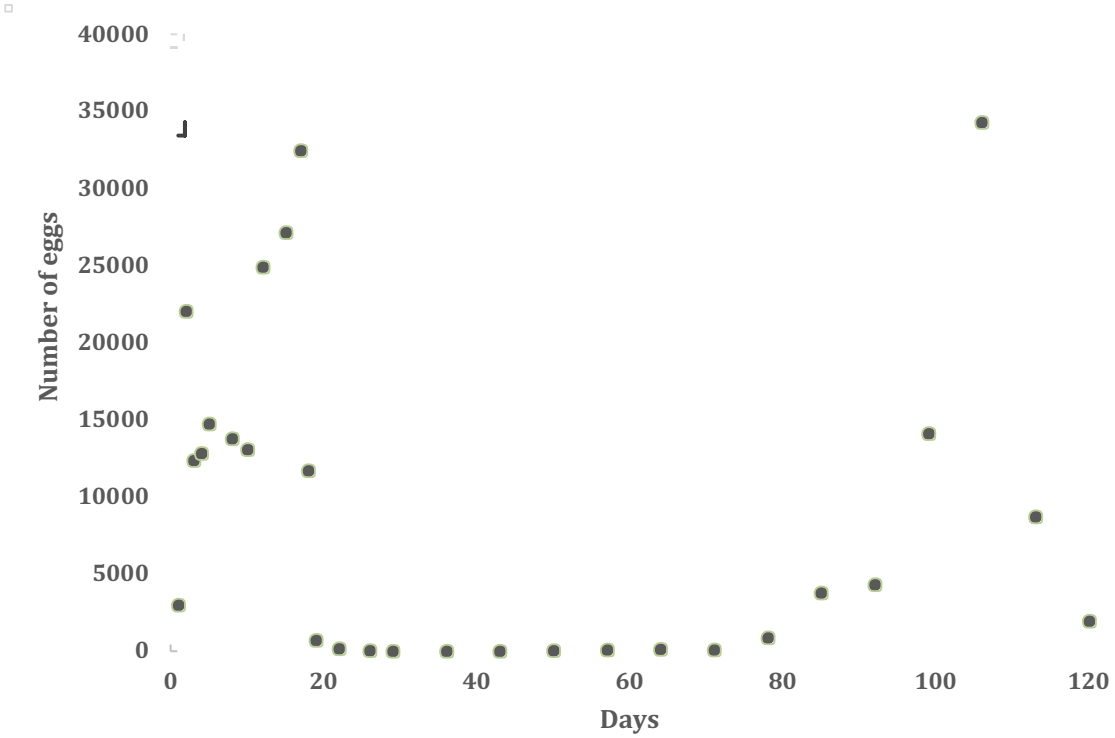


Figure 25.4.6. Evolution of the number of *Neobenedenia melleni* eggs collected in the mannose tank (S3) of juveniles of greater amberjack (*Seriola dumerili*) under the application of the experimental treatment (red arrow) and conventional treatments: hydrogen peroxide (spotted black arrow) and formaldehyde (grey arrows) on specific days.

Mannose caused the detachment of 50% of the monogenean mature adults, while formaldehyde was effective by a 95%. The mannose concentration and the frequency of application could improve the effect of this substance.

Task 25.5. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in amberjack (led by FCPCT, Daniel Montero).

The outbreaks registered in FCPCT facilities during the reporting period are shown in **Table 25.5.1**. Most of the animals died with secondary infection associated with opportunistic pathogens.

Table 25.5.1. Disease outbreaks during the reporting period.

	Weigth	Outbreak	Result
Amberjack	100 g (aprox)	Mortality sporadic	*Vibrio alginolyticus, Vibrio harveyi*
Amberjack	500 g (aprox)	Continuos mortality	*Vibrio alginolyticus*
Amberjack	< 2 g	Mortality	Vibrio spp.
Amberjack	15kg	Mortality	*Vibrio alginolyticus*
Amberjack	6gr		*Photobacterium damselae* spp. *pisicida*

Besides a stress test was conducted with selected diets assayed in tasks 25.2. and samples of liver and spleen were obtained for an study of associated bacterial culture.

Trial design

Seventy two fish (123.18 ± 19.2 g) were disposed in six 500 L tanks with twelve animals in each. Two experimental diets were challenged by triplicate, diet C (*Seriola* base diet as control) and cMOS diet (concentrated MOS, ACTIGEN, developed by ALLTECH). Animals were feed three times per day to apparent satiety.

Sampling procedures

Every 30 days, growth data was collected, until the final sampling at 90 days. At this time, a stress challenge test was conducted by confinement in small cages for 5 days, taking samples in BHI of liver and spleen for bacteria cultures at the start and end of the stress challenge. Samples were incubated for 26 h at 37ºC, bacterial colonies obtained were isolated and identified by biochemical analyses and API 20E.

Results

No differences were obtained for final weight and for specific growth rate (SGR) after 90 days of trial (**Figure 25.5.1**).

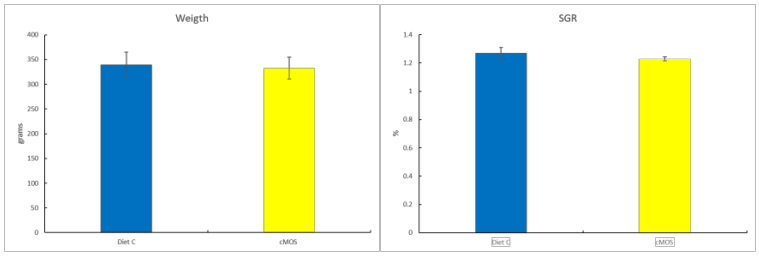


Figure 25.5.1. Final weight and specific growth rate (SGR) after 90 days of trial. No differences were recorded (p>0.05).

Stress challenge test showed differences in prevalence of bacteria colonies in spleen and liver. At the start of the challenge, only 1 fish from 9 was positive for colonies of *Vibrio sp.*1, meanwhile no colonies were isolated for cMOS diet (**Table 25.5.2**). After 5 days of confinement, both diets showed 100% of prevalence for opportunistic bacteria colonies in spleen and liver.

Table 25.5.2. Prevalence (%) of opportunistic bacteria detected in samples from liver and spleen. Three animals per tank, a total of nine per treatment, were sampled.

	T0		5 DAYS	
	Liver	Spleen	Liver	Spleen
Diet C	1/9 (11%)	1/9 (11%)	9/9 (100%)	9/9 (100%)
cMOS	0/9 (0%)	0/9 (0%)	9/9 (100%)	9/9 (100%)

Differences in the quantity of species were also detected in the bacterial cultures, obtaining 2 different colonies of *Vibrio sp.* in cMOS diet and 4 different colonies in control diet (**Table 25.5.3**).

Table 25.5.3. Different species detected in the 5 days BHI cultures of liver and spleen. *Vibrio sp.1* is related with the complex *V. anguillarum, V. damselae, V. splendidus*; *Vibrio sp.* 2 is related with the complex *V. ordalli* and *V. pelagius. Vibrio sp* 3 is related with the complex *V. vulnificus, V. argynolyticus* and *V. harveyi*.

T0		5 DAYS	
Diet C	cMOS	Diet C	cMOS
Vibrio sp. 1		*Flavobacterium sp.*	*Vibrio sp.1*
		Vibrio sp.1	*Vibrio sp.2*
		Vibrio sp.2	
		Vibrio sp.3	

Task 25.6 Diagnostic-recommendation manual for greater amberjack health (led by HCMR, Pantelis Katharios).

In this task we will develop a diagnostic manual for amberjack disease. As for meagre (WP24), this will be a practical diagnostic manual and recommendation guide for meagre health issues targeted to fish health specialists and aquaculture scientists and producers. The manual will be the synopsis of major findings of WP25, and will be published in electronic format (pdf file) and uploaded in the project website, freely available for the public. The task is still in progress and runs throughout the lifespan of the WP.

References

Katharios P et al. 2015. Environmental marine pathogen isolation using mesocosm culture of sharpsnout seabream: striking genomic and morphological features of novel *Endozoicomonas* sp. Sci. Rep. 5:17609. doi: 10.1038/srep17609.

Katharios P, Papadaki M, Papandroulakis N, Divanach P. 2008. Severe mortality in mesocosm-reared sharpsnout sea bream *Diplodus puntazzo* larvae due to epitheliocystis infection. Dis. Aquat. Organ. 82:55. doi: 10.3354/dao01968.

Deviations from Annex I and their impact:

There were no deviations from the DOW

WP 26 Fish health – Atlantic halibut

WP No:	26	**WP Lead beneficiary:**		P7. IMR
WP Title (from DOW):	Fish Health – Atlantic halibut			
Other beneficiaries (from DOW):				
Lead Scientist preparing the Report (WP leader):	Sonal Patel			
Other Scientists participating:	Audun Helge Nerland			

Objectives

1. Determine the effect of delivering recombinant capsid protein during late larval stages on protection to nodavirus (Viral Neural Necrosis, VNN).

Summary of work reported in the previous Reporting Period (1-12 Mo):

During the first period we focused on assessment of the use of several expression systems for production of nodavirus capsid protein. The goal was to assess two eukaryotic expression systems; microalgae and a protozoan (*Leishmania tarentolae*), in addition to *E. coli* and in tobacco plant. Apart from microalgae, all other three systems were assessed.

Expression of the nodavirus capsid protein in all three systems could be achieved. However, it was only in the *E. coli* system that we achieved sufficient and high expression for further use of the protein as antigen for vaccination purposes. Further optimisation for sufficient expression in plant and protozoan systems and a method for purification of the recombinant protein was achieved by the previous reporting period.

There has also been liaison with Targetfish, EU project to consider if amongst the VNN expressed by various systems in their project, the scientist involved could suggest a candidate that can be included in the testing in task 26.2.

Summary of work reported in the previous Reporting Period (13-30 Mo):

Multiple expression systems were tested for production of the capsid protein of VNN during period 1 and 2. There had been a few delays getting the recombinant capsid protein made in tobacco leaves. In addition, issues with wetlab challenge facilities aquarium led to DL26.2 and DL26.3 being pushed back to month 48. Researchers in the TargetFish project were contacted and it was agreed that the best candidate that has shown promising results in sea bass will be possibly delivered to IMR for inclusion in a halibut trial during spring 2017. Since the production of halibut is only once a year, the vaccination and challenge trial was delayed and started in late spring 2017.

Summary of progress towards objectives (31-48 Mo):

VNN capsid protein expressed by varying expression systems in the previous reporting time were delivered to halibut larvae 100 dph either through i.p. injection or through *Artemia*. The juveniles were transported to challenge facility 10 weeks after vaccination, and challenged with NNV. 8 weeks post challenge, the juveniles will be sampled to assess for effect of vaccination and possible adaptive immune response.

Details for each Task

Task 26.1 Production of VNN capsid protein (led by IMR).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 26.1 Assess the use of two eukaryotic expression systems; microalgae and a protozoan (Leishmania tarentolae) for production of nodavirus capsid protein.***

Task 26.2 Monitor and assess the immune response and protection (led by IMR).

The VNN capsid protein expressed in several different systems were delivered to halibut larvae through *Artemia*. The halibut larvae used in this study were 100 days post-hatch. Enriched *Artemia* were produced according to the standard protocol used at IMR, and used for the purpose. The treatment groups were as follows:

Treatment groups at experiment start: 50 larvae/juveniles in each group

1. Pichia expressing VNN – oral delivery through *Artemia*
2. Pichia with empty vector, with no VNN - oral delivery through *Artemia*
3. Purified inclusion bodies of VNN from *E.coli* with mineral oil adjuvant – i.p. injection
4. Purified VLPs from *Pichia* – i.p. injection
5. Purified VLPs from *Pichia* with adjuvant – i.p. injection
6. Purified VNN expressed in tobacco leaves with mineral oil adjuvant – i.p. injection
7. Live *L. tarantolae* expressing VNN - oral delivery through *Artemia*
8. Live *E. coli* expressing VNN - oral delivery through *Artemia*
9. Purified inclusion bodies of VNN from *E. coli* - oral delivery through *Artemia*
10. Purified inclusion bodies of VNN from *E. coli* - oral delivery through *Artemia* – for sampling
11. PBS with mineral oil adjuvant – i.p. injection
12. Negative control – non-treated

<u>For oral delivery (3 days in a row at end of June 2017):</u>

The larvae were starved before the first delivery of feed in the morning. Early morning each day, the enriched *Artemia* were concentrated to 1000 *Artemia* per ml, washed and incubated at 20 °C for an hour so the *Artemia* open their jaws and are ready for feeding (**Figure 26.1.1**). At the end of the incubation, the *Artemia* were mixed well and 35 ml per 50 ml tube was distributed. Either purified protein or live organisms expressing VNN capsid protein were added to the respective treatment tubes, and the tubes were incubated in a water bath with aeration to maintain 20 °C during feeding period of *Artemia*.

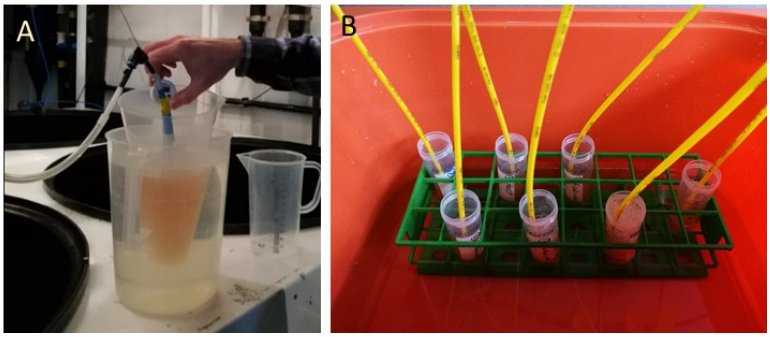


Figure 26.1.1 *Artemia* incubated in water bath before using them for feeding specific VNN protein (A), Tubes with *Artemia* with aeration during incubation for uptake of specific protein or live organisms for oral delivery (B).

At the end of the incubation period, the *Artemia* were filtered through a plankton mesh, washed once with sea water and the specific *Artemia* were added to the respective treatment tanks containing as little as 15 L seawater per tank. The larvae/juvenile halibut were allowed to feed on the *Artemia* carrying VNN capsid protein for 4 h with aeration in the tanks (**Figure 26.1.2**).

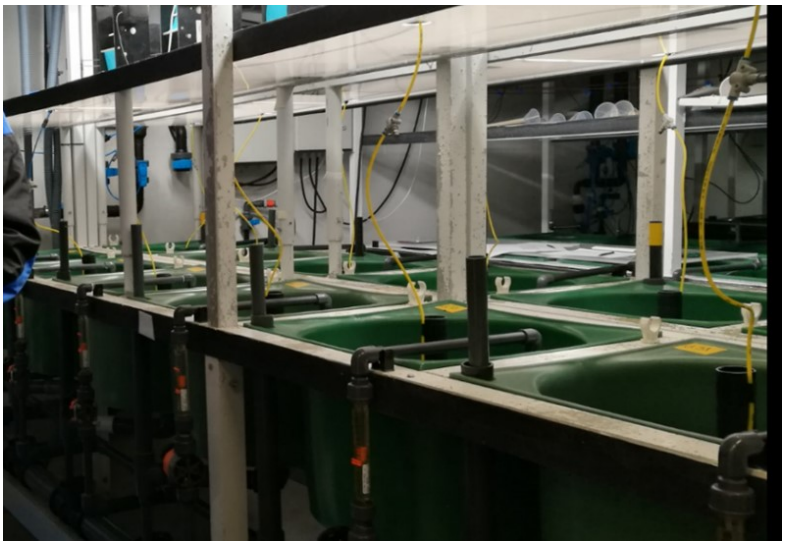


Figure 26.1.2 *Artemia* that had been fed with VNN capsid protein expressed in different systems were fed to halibut larvae/juvenile and the tanks were aerated during the feeding time.

At the end of feeding time, the flow through of water in the tanks with no GMO was started. In the tanks with GMO, flow through was started, while the water from tanks was collected in a specially built extra tank and treated with chlorine following the authorized GMO application connected to this study.

The larvae/juveniles were given one feed portion of routine enriched *Artemia* late in the evening, and starved in the morning to repeat the VNN capsid fed *Artemia* in the afternoon. The process was repeated for 3 consecutive days. One of the two parallel treatment tanks with oral delivery of inclusions bodies of VNN capsid protein expressed by *E. coli* was sampled with 6 larvae/juvenile every second day to analyse uptake of the protein, until the tank was emptied.

On the second day of the experimental, the treatment groups to be i.p. injected received single injections (**Figure 26.1.3**) after sedation, and were then transferred back to their respective tanks. The treatment that included adjuvant for i.p. injection was visible in the peritoneum of the larvae.

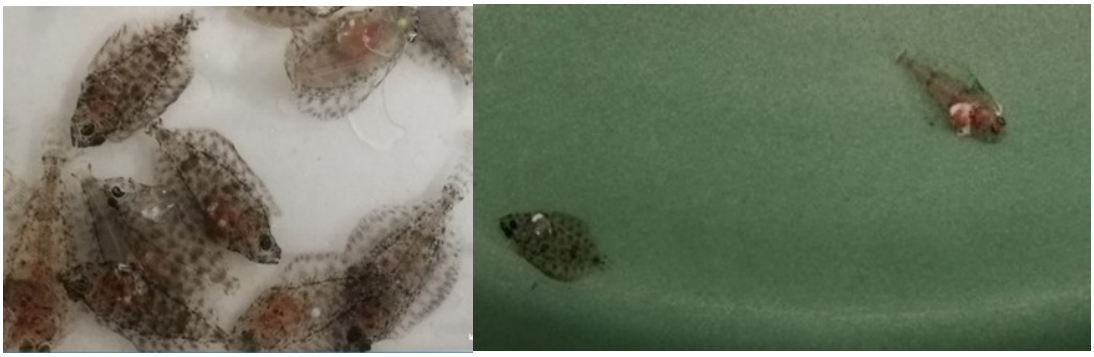


Figure 26.1.3 Halibut larvae/juvenile i.p. injected with VNN capsid protein expressed by different systems formulated with the mineral oil adjuvant.

The larvae/juveniles treated for vaccination were in varying developmental phases, and combined with the treatment and handling, several larvae/juveniles died during the first few days. At the end of 10 weeks of vaccination, the juveniles that had survived within each treatment were transferred to the wetlab challenge facility, IMR, Bergen and acclimatized for 10 days. A few juveniles died due to the transport and handling process. The number of individuals that survived within each treatment group at the time of challenge thus varied from 9 – 20. In the non- treated group, there were 29 individuals that survived.

Half of the individuals in the non-treated groups were sedated and challenged with VNN (nodavirus) by i.p. injection with 1x 50 µl of $1x10^{7.5}$ $TCID_{50}$ /ml, and transferred to a new tank resulting in one non-treated non-challenged group and one non-treated challenged group to enable comparison of the rest of vaccinated challenged treatments. In the rest of the treatment groups, all individuals were i.p. challenged with the same dose of VNN per individual.

The experiment has been terminated in week 50 2017, and all fish have been sampled for brain and spleen. Brain samples will be analysed for VNN using a RNA2 specific real time Q-PCR assay to assess the effect of the different vaccination treatment. If an effect of vaccination is observed, the spleen will be analysed for immune genes involved in adaptive immune response. If possible, a few individuals from each treatment group will be sampled for histology and IHC with VNN specific antibodies. This will depend on the size of the fish.

Deviations from Annex I and their impact:

There has been a delay in the deliverables 26.2 and 26.3 that has been reported previously.

Group Work Packages

Socioeconomics

In this period in this GWP activities have been done in:
- New product development (WP 28)
- Consumer value perceptions and behavioural change (WP 29)
- Business model and marketing strategy development (WP 30)

Work package 27 activities have been finished in the last reporting period.

The work done in WP 28 on the above objectives resulted in no deliverables yet, ince they are both due on month 54. At this stage we have insights in the sensorial perception of the developed fish products and a protocol has been made to analyse objective 3 in WP 28.

Work in WP 29 resulted in Deliverables 29.6, 29.7 and 29.8. So, by the end of the 3rd PR all activities in WP 29 have been finished. These deliverables identified some important parameters for marketing of the new aquaculture products:

a) 'Country of Origin (COO)' and
b) 'Price', followed by
c) 'ASC logo',
d) 'Nutrition claims' and
e) 'Health claims

All these are relevant extrinsic attributes to take into consideration. For a positive attitude towards a new product, with regards to communicated healthiness, taste and traceability, the less processed the product the higher the chance that consumers believe the message. Nevertheless, traceability message works well across all processing levels. All outputs of the previous WPs are used in WP 30.

In WP 30, two deliverables on objective 1 have been realised. However, for two of the species this was impossible, since the technical R&D of these new species is still not of the required level to develop a business model. Further technical research is necessary before this can be done. For the species for which the team developed Canvas Business Models, it was clear that the product input chain parties are good developed, while the market development part was still not available. New supply relationships have to be developed in the upcoming years, to market the products in the market. The deliverables regarding objective 2 and 3 are due in month 52 and further. In this WP a significant change according to the DOW has been made on the approach of task 30.2. Instead of a real market test, an online market test was programmed. This change has been done with approval of the project's Scientific Officer.

The work on objective 2 and 3 in WP 30 have not resulted in a Deliverable yet. The Deliverables regarding objective 2 are due on month 52 and further.

WP 27 Socioeconomics – Institutional and organization context

WP No:	27	**WP Lead beneficiary:**		P6. DLO
WP Title (from DOW):	Socioeconomics – Institutional and organizational context			
Other beneficiaries (from DOW):	P6. DLO	P10. TU/e	P11. AU	P12.APROMAR
Lead Scientist preparing the Report (WP leader):	Gemma Tacken (DLO)			
Other Scientists participating:	Victor Immink (P6), Machiel Reinders (P6), Olga vd Valk (P6), Athanasios Krystallis (P11), Javier Ojeda (P12), K. Grigorakis (P1), M. Keller (P34)			

Objectives

1. To give insight in the competitive field of and market developments in the European aquaculture market with a focus on the species selected in DIVERSIFY (meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet),
2. To assess the obstacles for growth in the current aquaculture production chains and for these selected species,
3. To identify market opportunities for future growth of the European aquaculture sector for the selected species.
4. Propose a certification framework for the species addressed in DIVERSIFY

Summary of work reported in the previous Reporting Period (1-12 Mo):

The first year of the project the activities in WP27 have focussed on identifying the institutional and organizational context in which the new species can be introduced. The macro-environmental context analysis showed that the political, economic, social, environmental and legal environmental factors support introduction of new species in the market. In sustainability certification several certification schemes are identified in the market. Next to legally defined certification schemes, multiple private standards and certification schemes are operational in the EU. Some of these schemes are internationally recognised, such as HACCP, BRC, GLOBALGAP, while others are privately owned, such as the in-house standards of Carrefour and NGO-developed standards such as ACC, ASC, Friends of the Sea and Bioland/Naturland. In some countries supply chain certification schemes are developed, such as Label Rouge in France and Crianza del Mar in Spain. In choosing buyers and selecting a market segment this should be taken into account. Industrial buyers of fish (processors and retail) observe a convergence of consumer preferences regarding fish products within the EU market. Consumers all over the EU are increasingly looking for convenience. Furthermore, consumers in most countries perceive frozen fish as of lesser quality than fresh, which is why most retailers innovate mainly in the fresh fish category. Some consumer preferences still differ between regions; most consumers in Southern countries perceive pre-seasoned fish as being of lesser quality, while consumers in the Netherlands and the UK increasingly purchase these products.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In Period 2, a Porter analysis showed that the 6 DIVERSIFY species are unknown in the market and that some species have specific markets with high brand awareness and recognition, and others where they are completely unknown.

The trend mapping learned that there will be an increasing competition on the EU market for animal proteins on the long run. Another important trend is increasing requirements with respect to quality, traceability, sustainability and animal welfare. The success failure study identified key aspects for a successful introduction in the market and shows that the EU market is very diverse with great variations in market preferences. Before introduction in the market, per product the advantages that meet consumers' needs have to be identified and communicated. Involvement of the industry and the retail is very important as well as a positioning on environmental and ethical issues in the UK and Germany or convenience in Spain and France.

Summary of progress towards objectives (31-48 Mo):

No work was planned during this period, and all activities were completed in the previous Reporting Periods.

Details for each Task

Task 27.1 External environmental analysis (led by DLO, Gemma Tacken)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.1 Report on external environmental factors that affect or will affect the production chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet***, and ***Deliverable 27.2 Report on current certification schemes and standards and their business***

Task 27.2 Competitive analysis (led by DLO, Gemma Tacken)

Sub-task 27.2.1 Competitive analysis (prepared by Victor Immink (P6. DLO) and Javier Ojeda P12. APROMAR)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.3 Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.***

Sub-task 27.2.2 Trend mapping

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.4 Report on trend mapping for the European aquaculture and fisheries sector, and protein market in the (near) future***.

Sub-task 27.2.3 International survey in selected countries (led by SWR/DLO)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.5 Report on the results of international survey on industrial buyers' attitudes and perceptions regarding cultures fish***.

Task 27.3 Opportunities and barriers for growth (led by DLO)

Task 27.3.1 Success-failure study

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.6 List of critical success factors for market acceptance***.

Task 27.3.2 Using the business model Canvas

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.7 Report on the analysis of the business models and supply chains of the participating SMEs***.

Deviations from Annex I and their impact:

There were no deviations from the planning in this work package.

WP 28 Socioeconomics – New product development

WP No:	28	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Socioeconomics – New product development			
Other beneficiaries (from DOW):	P1. HCMR	P6. DLO	P10. TU/e	P11. AU
P15. ULL	P18. CTAQUA	P38. HRH		
Lead Scientist preparing the Report (WP leader):	Luis Guerrero			
Other Scientists participating:	Kriton Grigorakis (P1), Ricard Bou (P3), Athanasios Krystallis (P11), Covadonga Rodriguez (P15), Rocio Robles (P18)			

Objectives

1. To develop new product concepts from selected species, by incorporating consumer and expert input,
2. To select product ideas and develop physical new products from the selected species,
3. To monitor the quality of new products in terms of organoleptic characteristics and nutrition-rearing history,
4. To make a technical assessment of the products.

Summary of work reported in the previous Reporting Period (1-12 Mo):

Two subtasks were started and continued during the 2nd Reporting period within WP28: Sub-task 28.1.1 (led by P11. AU) and Sub-task 28.2.1 (led by P1. HCMR). The main outcomes of these two activities were:

- Design of a series of focus group discussions with consumers and experts in the selected countries of the project (UK, D, ES, F, I). The main objective of this task was to generate a set of ideas to be screened out and further developed into product concepts for testing in subsequent tasks in the new product development process.

- Estimation of optimum fish sizes for developing the selected new products. In this case the activities performed included somatometric measurements for the five species of interest (meagre, greater amberjack, pikeperch, wreckfish and grey mullet) as well as their chemical and sensory characterization.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In WP28 several product ideas have been identified per species on basis of focus group discussion in all selected countries. Some of these product ideas have been worked out to prototypes, that have been sensory tested in the five selected countries. The results will be presented in this (3rd Period) report. During the second period (months 13-30) the following four different activities were completed and/or finished.

In the first activity (Sub-task 28.1.1) new ideas were explored and reported through focus groups with consumers and experts regarding the fish products resulting from the species under study in five focal markets: UK, Germany, Spain, France and Italy. Experts from different countries agreed that the created products were attractive and feasible ideas that have potential in the market. They consider that in overall these ideas could increase profits of fish industry due to the higher diversity of choice. Generally, they stated that these ideas have a possible prospective if they are developed with good coordination between the fish farmers and consumers. In terms of general recommendations for new product development of selected fish

species, the most important drivers and barriers for the choice of the new product ideas most relevant for consumers were analysed and discussed.

The second activity performed (Sub-task 28.1.2) focussed on generation and screening of ideas for new product development based on the market data of WP 27, the results obtained in the focus groups (Sub-task 28.1.1) and the evaluation of the different ideas by the scientists from different scientific areas. Technical limitations and the economic prospects efficiencies (i.e., within a socio-techno-economic study) were used to generate a pool of ideas about potential products. The selected ideas were assessed by means of technical, economic and market assessment criteria, among others.

In the third activity carried out (Sub-task 28.2.1) the optimum fish sizes for developing the new products identified in Sub-task 28.1.2 were described based on basic somatometric measurements and evaluation of losses. In addition, chemical-mechanical and sensory properties of fish species during cutting and minimal processing were obtained, which provided a definition of process solutions for each species based on technological, physical and sensory characteristics.

Finally, the fourth activity completed (Sub-task 28.2.2) focussed on the development of physical prototypes of new products from meagre, greater amberjack, pikeperch and grey mullet. The physical prototypes were developed based on the information provided by WP 27 (market potential of the new species), Sub-tasks 28.1.1 and 28.1.2 (products concept development: technical and consumer driven), Task 29.1 (consumer value perceptions and segmentation), physicochemical characteristics of each raw material (Sub-task 28.2.1), technical properties of the products and the process, and similar product availability in the market. Twelve different prototypes were elaborated based on ten selected ideas (idea numbers 1, 2, 4, 6, 9, 13, 21, 30, 33 and 34). Meagre fish was used for the development of the following ideas “frozen fish fillets with different recipes” (idea 1), “fish burgers shaped as fish” (idea 6) and “ready to eat meal: salad with fish” (idea 4). Pikeperch was used for the development of “fresh fish fillet with different ‘healthy’ seasoning and marinades” (idea 21), “ready-made fish tartar with additional soy sauce” (idea 30) and “fish spreads/pate” (idea 9). Grey mullet was used for the development of “thin smoked fillets” (idea 2), “ready-made fish fillets in olive oil” (idea 33) and “fresh fish fillet with different ‘healthy’ seasoning and marinades” (idea 21). Finally, greater amberjack was used for the development of “frozen fish fillet that is seasoned or marinated” (idea 13), “ready-made fish tartar with additional soy sauce” (idea 30) and “fresh fish steak for grilling in the pan” (idea 34). Information about how to elaborate these new products were provided as well as a number of guidelines, processing conditions, technical specifications and troubleshooting. In addition, basic information regarding the food products packaging, conservation conditions, preliminary product shelf life and consumer handling/cooking specifications were also reported. Since these prototypes have potential as fish product diversification, they will constitute the basis for further tasks in the project, including their consumer acceptability evaluation.

Summary of progress towards objectives (31-48 Mo):

During this period all proximate composition as well as fatty acid analysis of the six developed products has been completed in duplicate. These products have also been characterized by means of sensory descriptive analysis, thus including the training of the panellist, the development of sensory references and the evaluation of their individual performance.

In order to correlate technical quality characteristics with previous nutritional - rearing history, different fish groups from varying farming histories have been identified for sampling. These animals will be analysed for the proximate composition and analytic fatty acid profiles (HCMR and ULL) and sensory characteristics (HCMR and/or IRTA). A detailed sampling protocol has been established and agreed between the partners involved in this sub-task.

Details for each Task

Task 28.1 Product concept development: technical and consumer-driven (led by AU, Athanasios Krystallis)

Sub-task 28.1.1 (led by AU, Athanasios Krystallis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.1 Report with results of focus groups with consumers and experts regarding ideas for new fish products.***

Sub-task 28.1.2 (led by HCMR, Kriton Grigorakis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.2 List of ideas for new product development***.

Task 28.2 New Product Development (led by IRTA, Lluis Guerrero)

Sub-task 28.2.1 (led by HCMR, Kriton Grigorakis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.3 Report on product and process solutions for each species based on technological, physical and sensory characteristics.***

Sub-task 28.2.2 (led by IRTA, Ricard Bou)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.4 Physical prototypes of new products from the selected species meagre, greater amberjack, wreckfish, pikeperch and grey mullet***.

Task 28.3 Monitoring technical quality of the products (led by HCMR)

Sub-task 28.3.1 (led by HCMR, Kriton Grigorakis)

All proximate composition (PC) of the developed products has been completed. The fatty acid analysis of the developed products is currently under investigation and no deviations from schedule are expected. PC and FA analyses were performed on the 6 processed products and for each of the samplings the analysis was performed in duplicates. PC analysis was performed according to the standard AOAC (2005) methods.

Descriptive analysis (Report on results of sensory descriptive analysis of the developed products):

(a) Selection, general training and specific training of panellists included 1. screening of internal panel, 2. determination of sensory acuity (including identification of basic tastes and detection of basic tastes based on ISO 4210), 3. discrimination of levels of intensity in basic tastes, odours and texture, 4. assessment of descriptive ability of a stimuli (Candidates were presented with between five and 10 product-related olfactory stimuli, samples easy to recognize and others less common. The intensity was well above recognition threshold, but not greatly above the levels that might be encountered in the products), 5. odour description (of various odours to be encountered in the products), 6.texture description (with physical references with textural properties similar to products) and 7 flavour description.

(b) Main evaluation procedures of the products:.

All sensory analyses were performed in individual sensory booths in HCMR facilities of Agios Kosmas, Athens, Greece. In all evaluations, questionnaires were filled in by hand, samples were blind-labelled with a three-digit code, mineral water and pieces of green-apple were provided to assessors to cleanse their palates between samples.

The first step of training included a vocabulary development session (1.5h) during which the TP tasted all six products and generated an attribute list that described all main characteristics of their aroma, taste, flavour and texture. The generated consensus list was then divided in sensory modalities, for which presentation followed the 'dynamics of sensory perception', and included in the RATA ballot used for the evaluation of the products (Ares & Jaeger, 2013; Ares et al., 2013). The TP continued with training on the definition and scaling of attributes included vocabulary list generated (see Table 2 for details of the training process). During the training process, the vocabulary list was reduced to 28 attributes, from the initial 34, and one new

attribute, "Sardine flavour", emerged since its inclusion in the list was found essential by the TP (Table 28.3.1). The processed fish product set was then evaluated via generic Descriptive Analysis (DA) (see Table 1 for details of the DA's evaluation process). During training and DA the performance of the TP was evaluated using the Panel Check software V1.4.0.

Table 28.3.1: Summary of the main training elements and sensory evaluation process of the three sensory methodologies.

	Sensory descriptive analysis
Number of assessors	10 trained panellists
Training:	
attribute definition	1.5 h: Vocabulary session, panel discussion & consensus 1.5 h, Physical references
attribute scaling	2*2h, Sample pair-comparisons (150 mm scale), followed by panel discussion 2*1h, sample training in booths (150 mm scale) & panellist feedback
Duration	9.30 h
Evaluation:	
Explanation of task	–
scale	150 mm unstructured line scale ("not at all" to "very much")
Attribute number	29
Attribute order	Fixed within modalities
Sample presentation	Randomized, monadic
Sample replicates	Triplicates (3 sessions- break: 15 min)
Duration	1 h approx.
Total duration	10.30 h approx.

The proximate composition of the six studied products is presented in Table 28.3.2.

Table 28.3.2. Proximate composition of processed fish products (mean values ± standard deviation).

		Proximate composition (%)			
Fish species	**Product**	**Moisture**	**Fat**	**Protein**	**Ash**
G. amberjack	Steak	72.04 ±1.17	3.67 ±1.39	22.40 ±0.80	1.48 ± 0.13
	Burger	71.84 ±0.24	4.82 ±0.15	18.76 ±0.15	2.47 ±0.04
Meagre	Salad	79.81 ±1.09	2.93 ±0.23	12.96 ±1.09	0.80 ±0.04
	Smoked fillets	63.25 ±2.38	2.87 ±0.69	27.41 ±2.08	4.82 ±0.38
Grey mullet	Fillets in Olive oil	57.29 ±6.11	21.85 ±3.51	19.41 ±4.53	1.99 ±0.40
Pikeperch	Pate	65.7 ±0.73	14.94 ±0.45	17.62 ±0.55	1.58 ±0.02

The sensory map of the products, as derived from descriptive analysis is presented in **Figure** 28.3.1.

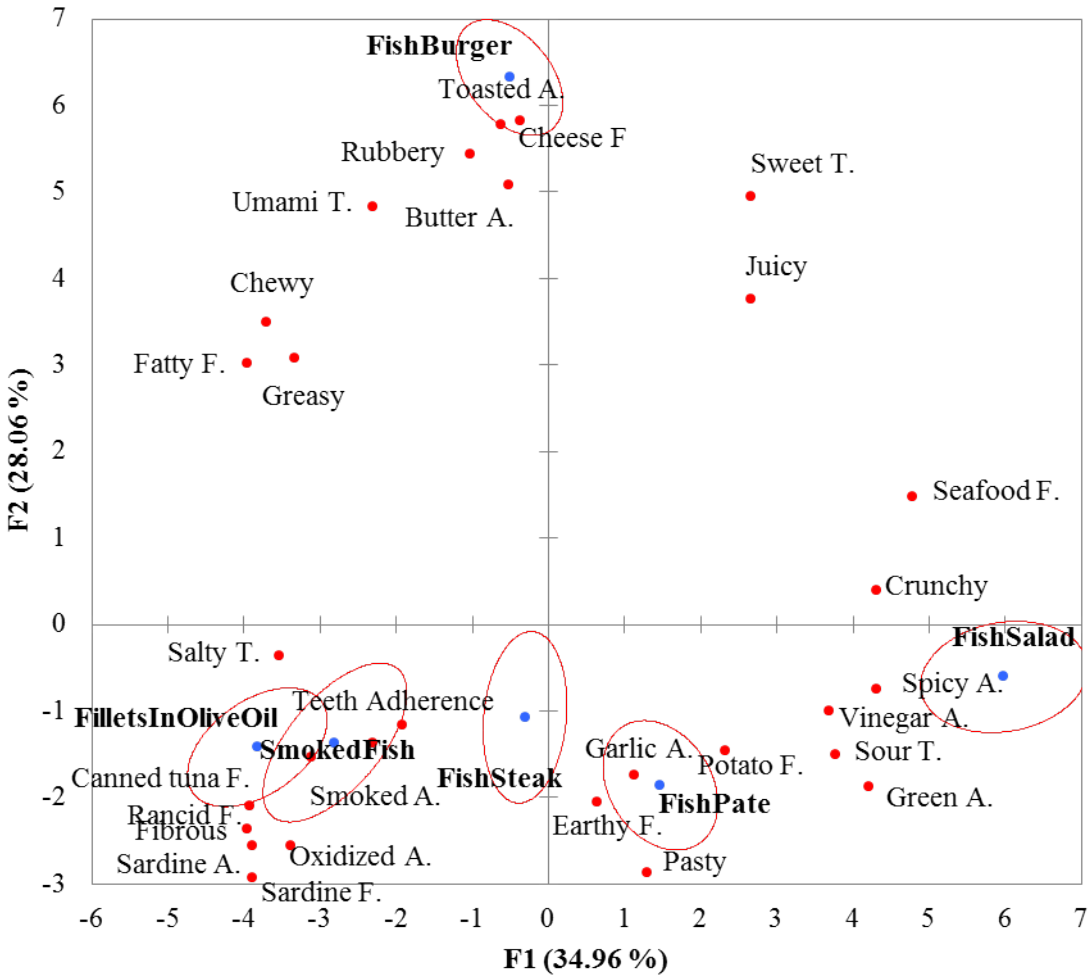


Figure 28.3.1. Principal Component Analysis (PCA) biplot illustrating the sensory characteristics of the developed processed fish products. With A. aroma attributes are denoted and with F flavour attributes are denoted.

The final results of this task are to be published in ***Deliverable 28.6 Report on results of sensory descriptive analysis of the developed products due Month 54***

References

Ares, G., & Jaeger, S. R. (2013). Check-all-that-apply questions: Influence of attribute order on sensory product characterization. Food Quality and Preference, 28(1), 141-153.

Ares, G., Jaeger, S. R., Bava, C. M., Chheang, S. L., Jin, D., Gimenez, A., et al. (2013). CATA questions for sensory product characterization: Raising awareness of biases. Food Quality and Preference, 30(2), 114-127.

Sub-task 28.3.2 (led by ULL, Covadonga Rodriguez)

According to the DOW, a correlation of technical quality characteristics with previous nutritional - rearing history will be addressed by studying the quality of different fish groups from varying farming histories

(ULL, HCMR) and which will provide input for value positioning statement and communication claims (Deliverable D28.7 Report on correlation of technical quality with nutritional - rearing history; month 54).

To this purpose fillets samples from fish receiving different dietary treatments and from different origin and rearing conditions, are being sampled for achieving a specific dossier per species containing both a) the proximate composition and analytic fatty acid profiles (HCMR and ULL) and b) the sensory characteristics (HCMR and/or IRTA). The effect of dietary treatment on the end product quality for each species will be then analysed within its frames, by correlations between individual quality attributes and the dietary history (e.g. dietary fat and protein levels, or fat sources etc.) or other rearing parameters (e.g. different rearing temperature). A strong effort is being performed in order to achieve as much information as possible regarding the sampled fish, which includes a sampling protocol for collaborating partners and companies in order to take all somatometric measurements, instructions on how to proceed for fillet sampling and preservation, yield estimations, etc. As pointed out in the DOW these samplings are dependent upon the Grow out husbandry WP trials, some of them recently finished or still under execution.

In order to achieve this objective a clear protocol has been established:

1. Identify each bag with the name of the species and numbering each sampled fish (1, 2, 3 ... etc.). If the two fillets of the same specimen go in each bag, separate each fillet with aluminium foil or rigid plastic foil to facilitate its subsequent separation during defrosting. If it is decided that each fillet go in separate bags, number them accordingly 1-1 (right side), 1-2 (left side); 2-1, 2-2 ... ..etc.

2. Use the same codes to identify the samples of the little piece of muscle taken for proximate and fatty acid composition.

3. Once all the somatometric measurements and the various indexes have been taken, de-scale the fish as best as possible. Next, each side is threaded, from behind the head to the tail, deepening to the spine and obtaining each complete loin, including its ventral part corresponding to the visceral cavity. Weigh and record the weight data of each fillet and spine for each specimen. (Calculate the performance-IRTA, ULL).

4. Extract about 5 grams of muscle from each loin and specimen, trying to take it from the same area. Store in labelled vials or bags, preferably at -80ºC or -20ºC until shipment. Ship the samples frozen, together with the fillets to IRTA. Also save about 5 grams of the diet. Label the samples and send them frozen to IRTA, together with the fillets and the small muscle sample.

5. Introduce each loin, or every two loins, in one of the opaque bags, practice vacuum (otherwise extract the air) and seal them together with the small sample of fillet and the sample of the diet, and freeze at least -20ºC until it is sent on ice to IRTA (DHL).

Until now it is available all the required information (rearing history, somatometric, proximate and fatty acid analysis and sensorial analysis) concerning samples listed in Table 28.3.3, from Deliverable 28.3.

Table 28.3.3. Origin, season of sampling and fish size information of fish used in Task 28.3

Species	Season	N	Origin – farming conditions	Feed	Fish Size
Greater Amberjack (*Serioladumerili*)	Feb. 2015	10	Farm (Corfu S.A.)-NW Greece -floating sea cages	Commercial extruded feed	1-1.5 kg
Greater Amberjack (*Serioladumerili*)	Apr. 2015	8	Farm (Argosaronikos S.A.) – Attiki, C. Greece - floating sea cages	Commercial extruded feed	15-20 kg
Pikeperch (*Sander lucioperca*)	July 2014	10	France –freshwater intensive farming	Commercial extruded feed	1-2 kg
Grey Mullet (*Mugilcephalus*)	Feb. 2015	10	Wild fish. Bay of Cadiz (Spain) – earthen ponds with sea water	Natural feeding	500g-1 kg
Meagre (*Argyrosomus regius*)	Nov. 2014	10	Farm (Andromeda Group), Burriana, Spain – floating sea cages	Commercial extruded feed	1.5-2 kg
Wreckfish (*Polyprionamericanus*)	Febr. 2015	5	Five specimens: 2 caught in FAO 34.1.2 ATLANTIC N by Canary Islands fishermen and 3 caught in Azores by Galicia´s fisheries	Natural feeding	Three specimens of 2-3 kg Two specimens of 25-30 kg

In addition, two more batches of greater amberjack fillets and diets, taken from Subtask 21.3.2 (IEO) are available for sensorial and proximate and fatty acid analysis at IRTA and ULL, respectively.

Finally, according to a recent meeting held during AE2017 in Dubrovnik (Croacia) and to several conversations maintained in order to coordinate the present task with the others researchers involved in this sub-task, some new fillet samplings are intended to complete information from pikeperch (from Pascal Fontaine-University of Lorraine- own trials/or and companies contacts), and mullets (from Rocío Robles-CTAQUA- companies contacts).

The final results of this task are to be published in ***Deliverable 28.7 Report on correlation of technical quality with nutritional - rearing history due Month 54***

Sub-task 28.3.3 (led by IRTA, Lluis Guerrero)

No work done yet during this period.

Deviations from Annex I and their impact:

There were no deviations from the calendar of activities established.

WP 29 Socioeconomics – Consumer value perceptions and behavioural change

WP No:	29	**WP Lead beneficiary:**		P11. AU
WP Title (from DOW):	Socioeconomics – Consumer value perceptions and behavioral change			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P6. DLO	P18. CTAQUA
	P38. HRH			
Lead Scientist preparing the Report (WP leader):	Athanasios Krystallis (P38)			
Other Scientists participating:	Marija Banovic (P11), Machiel Reinders (P6), GemmaTacken (P6), Luis Guerrero, (P3), Kriton Grigorakis (P1), Rocio Robles (P18), Hellas-Maria Saltavarea (P38)			

Objectives

1. To analyse and understand overall value perceptions of consumers with regard to cultured fish in general and the DIVERSIFY fish species in particular, and undertake a value-based segmentation study,
2. To evaluate consumer sensory perceptions towards the newly developed DIVERSIFY species' products,
3. To optimize the DIVERSIFY species' newly developed products in terms of ideal extrinsic product attribute combinations that have the potential to generate ideal consumer value perceptions,
4. To determine the effectiveness of market communication in consumer behaviour change in relation to the DIVERSIFY species considered and the new raw and other value added products developed.

Summary of work reported in the previous Reporting Period (1-12 Mo):

The first analyses of the consumer survey show that there are differences between the five countries that were selected for the study (*i.e.* UK, Germany, Spain, France and Italy) in values and costs attached to a fictitious new fish species. Consumers in Germany were giving higher scores to functional value, while the southern European countries (Spain and Italy) place more weight on the social values. German consumers tend to provide higher scores on price, whereas Italian consumers give higher scores to performance risk and safety risk. In terms of outcomes (satisfaction, word of mouth and intention to buy) it looks like France and UK are comparatively less enthusiastic, given their scores. Overall, farmed fish is not perceived as significantly better or worse than wild fish. In general, most consumers in the five countries are open to find out more about a new fish species.

A first cluster analysis has given more insights in the market potential for new species in general. This analysis shows that three segments of consumers can be identified:

- Involved traditional consumers (29%): who know relatively more about fish and buy traditional fish products;
- Involved innovators (36%): who know relatively more about fish and who have a more open mind to buy new fish products;
- Ambiguous indifferent (35%): who know relatively less about fish and who are less open to buy new fish products.

Based on the first findings more than 1/3 of the consumers in the five selected countries belong to the segment of 'Involved innovators' and could therefore potentially be open to buy new species. More in-depth analysis in the upcoming year must give insights in the opportunities in the consumer market for the new species and more specific in the five countries.

Summary of work reported in the previous Reporting Period (13-30 Mo):

The 2nd Periodic Report covered Task 29.2 (Consumer sensory perceptions) and the first part of Task 29.3 (Optimization of intrinsic-extrinsic attribute combinations), namely its Sub-task 29.3.1.

The objective of task 29.2 was to develop the actual product samples from the selected fish species for the sensory testing with consumers in the five countries investigated (i.e. France, Germany, Italy, Spain and the UK) (Deliverable 29.3). In this task, the different physical product prototypes developed and tested in Tasks 28.1 - 28.3 related to new product development and the monitoring of technical quality of the products were manufactured according to the amount needed and following strict hygienic conditions.

These product samples were the basis for the acceptability test done in task 29.2 (resulting in Deliverable 29.4). This task provided all the information needed to handle, store and prepare the different samples, the statistical design followed in each location (order of presentation, sample distribution among participants, etc.) as well as some practical recommendations that were necessary to carry out the test and recruit the participants properly. More specifically, participants were recruited in each of the five selected countries (France, Germany, Italy, Spain and UK) based on the consumer segments identified in Task 29.1 (see Deliverable D29.2 report on the segmentation analysis for more information). Further, all the sensory tests were performed under controlled conditions in a central location per country. All the product samples were shipped in advance to each location in the right conditions and guaranteeing the cold chain. Samples were sent with detailed instructions about the right procedure to store them until analysis. Finally, ten tasting sessions were held in each location in two consecutive days. In each tasting session, consumer assessed overall expectations with the different physical product prototypes developed and tested in Tasks 28.1 - 28.3, followed by blind tasting and overall expectation in informed condition (i.e. upon provision of pictures with full description of the product from deliverable 28.2).

In terms of results, products with a higher degree of processing were those who generated lower expected acceptance, although all of them were perceived positively. The most important parameter affecting liking expectations was the expected taste of the product. Health, nutritional and well-being related issues were relevant as well in order to increase individuals' expectations, but to a lower extent. These findings seems to indicate that, in general, consumers are unwilling to sacrifice taste by an improvement in health or functional properties. In a general sense, the perception of these products was similar across countries. Once products were blind tasted, the acceptability results obtained confirmed those previously reported regarding consumers' expectations, and also seems to indicate a tendency to prefer the low processed fish products. Even though the different products were perceived similarly in the different locations regarding acceptability ratings, they were described in a clearly different way when dealing with the main intangible dimensions that might define them (taste, convenience, environmental impact, etc.). …

The full description of this work and results is provided in ***Deliverable 29.3 - Development of the actual product samples from the selected species for the sensory testing with consumers in the five countries investigated and 29.4 - Report on the actual products' sensory profiling in the five countries investigated.***

The objective of sub-task 29.3.1 was to incorporate a number of extrinsic quality attributes (i.e. product labelling elements) into the physical product prototypes developed in WP 28 (see Deliverable 28.2 and Deliverable 28.4) and based on the results from Task 29.2 (and Deliverable 29.4). The main goal was to develop experimental product mock-ups with optimal intrinsic-extrinsic attribute combinations for use in the experimentation with consumers.

Based on a review of secondary data and a detailed literature review of studies dealing with consumer behaviour towards fish product following similar methodologies (i.e. experimentation with product mock ups in simulated choice tasks), sub-task 29.3.1. ended up with the selection of the most appropriate extrinsic quality attributes to be incorporated onto the label of the experimental product mock-ups for further testing

in sub-task 29.3.2. The extrinsic attributes selected were: a) product's country of origin (i.e. EU or domestic), b) a quality guarantee of ethical nature (i.e. ASC logo), c) health claims (i.e. improves cardiovascular function and improves brain function), d) nutrition claims (i.e. rich in Omega 3 and high in proteins), and finally e) three price levels (*i.e.* average, +10% premium and +15 premium).

The full description of this work and results is provided in ***Deliverable 29.4 – Development of the product mock-ups for use in the experimentation with consumers in the five countries investigated.***

Summary of progress towards objectives (31-48 Mo):

The WP29 objectives relevant for the 3rd Periodic Report are Objectives 3 and 4, namely:

5. To optimize the DIVERSIFY species' newly developed products in terms of ideal extrinsic product attribute combinations that have the potential to generate ideal consumer value perceptions, and
6. To determine the effectiveness of market communication in consumer behaviour change in relation to the DIVERSIFY species considered and the new raw and other value added products developed.

In terms of significant results in relation to Objective 3, the most relevant attributes for all three investigated products were 'Country of Origin (COO)' and 'Price', followed by 'Existence of an ASC logo', 'Existence of a nutrition claim' and 'Existence of a health claim'. Consumer preferred the product lower the higher prices were. Higher price sensitivity across the investigated countries has been observed for the case of fish fillets in olive oil compared to the other two products. Results further suggested an increasing probability of choosing a fish product that has been 'produced in own (domestic) country'. Furthermore, fish product alternatives possessing an 'ASC logo' also increased the probability of choice. Nevertheless, consumer preferences for nutrition and health claims varied across products and countries.

Finally, in with respect to Objective 4, the effect of communication on attitude towards the product was significantly higher when the goal message was associated with the lower level of product processing across all three goal messages (i.e. about products' healthiness, tastiness, and traceability). This was evident in the case of low processed product - fresh fish steak and the health goal message. Besides health, the traceability goal message worked well across all three products (i.e. primes). The results further showed that the highest effect on product's purchase probability had the positively and negatively evoked emotions. This finding was evident especially for the experimental conditions with the traceability and taste goal messages primed with medium (i.e. smoked fillet) - and high (i.e. fish burger) - processed products.

Details for each Task

Task 29.1 Consumer value perceptions and segmentation (led by AU, Athanasios Krystallis).

Sub-task 29.1.1 (lead by DLO, Gemma Tacken, prepared by Machiel Reinders)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.1 Dataset of consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the selected species in the five countries investigated***.

Sub-task 29.1.2 (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU))

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.2 Report on the segmentation analysis based on consumer value perceptions about the selected species in the five countries investigated (value-based segmentation task)***.

Task 29.2 Consumer sensory perceptions (led by IRTA, Lluis Guerrero).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.3 Development of the actual product samples from the selected species for the sensory testing with consumers in the five countries investigated*** *and* ***Deliverable 29.4 Report on the actual product's sensory profiling in the five countries.***

Task 29.3 Optimization of intrinsic-extrinsic attribute combinations (led by AU, Athanasios Krystallis).

Sub-task 29.3.1 (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU))

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.5 Development of the product mock-ups for use in the experimentation with consumers in the five countries investigated***

Sub-task 29.3.2 (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU))

The full description of the work and results have been provided in ***Deliverable 29.6 Report on the experimentation with product mock-ups in the five countries investigated and identification of the optimal intrinsic-extrinsic product quality profiles for targeted segments.***

The objective of this sub-task and Deliverable 29.6 was to report on the experimentation with product mock-ups in the five countries investigated (i.e. Germany, France, Italy, Spain and the UK) and the identification of the optimal intrinsic-extrinsic product quality profiles for the target segments (i.e. the "involved innovators" and the "involved traditionals"), established in Deliverable 29.2.

Sub-task 29.3.2 and Deliverable 29.6 provided results of a number of experimental set-ups (i.e., Discrete Choice models) developed in Deliverable 29.5 and established on-line to test three product prototypes developed in WPs 28 and 29 (reported in previous Deliverables 28.1, 28.2, 28.3, 28.4, 29.2, 29.3 and 29.4), mainly:

- Product idea 2: Thin smoked fillet,
- Product idea 33: Ready-made fish fillets in olive oil, and
- Product idea 34: Fresh fish steak.

The experiments were run on consumer samples (approximately 100 participants x 5 EU countries x 3 products), about 300 participants per product. Participants were belonging to the cross-national segments with the highest/best value perceptions per product defined above (i.e. "involved innovators" and "involved traditionals", Action 29.1.1), in order to achieve a best match possible between ideal extrinsic/intrinsic attribute combinations and high-potential market segments. Thus, three on-line surveys (i.e. choice experiments) were undertaken in each of the EU target countries (i.e. France, Germany, Italy, Spain and the UK). For more information see Deliverable 29.6.

The experimental design has been primarily adjusted from Deliverable 29.5 to decrease the number of attribute levels and possible combinations, as well as price premium levels, which were also adjusted as per recommendation from partners using as a reference price average prices from Deliverable 29.5. Selected attributes and their levels were varied according to a orthogonal design producing 36 experimental sets further partitioned into 12 versions of choice set size of three (see examples in **Figure 29.3.1**).

Figure 29.3.1. Example of the product mock-up stimuli used in the choice experiments

Results from the sub-task 29.3.2 and Deliverable 29.6 show that the most relevant attributes for all three investigated products were '*Country of Origin* (COO)' and '*Price*', followed by '*Existence of an ASC logo'*, '*Existence of a nutrition claim'* and '*Existence of a health claim'*. Consumer preferred lower over higher prices, where higher price sensitivity across the investigated countries has been observed for the case of *fish fillets in olive oil* when compared to other two products. Results further suggested an increasing probability of choosing a fish product that has been 'produced in own (domestic) country'. Furthermore, fish product alternatives possessing an '*ASC logo*' also increased the probability of choice. Nevertheless, consumer preferences for nutrition and health claims varied across products and countries.

This sub-task and Deliverable 29.6 were indispensable for determination if new created products from Diversify will be accepted by the consumers (in the investigated countries), as well as to uncover which attributes are playing the major role in this acceptance. It has been shown that it is possible to create new products targeting similar high-profile segments across all big EU markets, where similar pattern in

consumer choice-drivers have been observed, that is: country of origin (COO) and price come first, followed by quality certification, while nutrition/health claims appear to have minimal impact. It has been shown that the higher probability and chances for the investigated products to succeed in the marketplace will depend on the proper use of labelling. Nevertheless, a certain degree of customisation is needed across low- and medium-processed products and across countries, as results further show that these are both product- and country-dependent.

Task 29.4 Communication effectiveness in behavioural change (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU)).

The full description of the work and results is provided in:

Deliverable 29.7 Development of the stimulus (i.e. written and broadcasted information material) that will be used in the communication experiments in the five countries investigated and

Deliverable 29.8 Report on the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers attitudes and behaviour towards the products coming from the selected fish species.

The main objective of Deliverable 29.7 was to develop the stimulus (i.e. written information material) that have been used in the communication experiments in the five countries investigated within Diversify (i.e., Germany, France, United Kingdom, Italy and Spain). Therefore, in the Deliverable 29.7 single communication parameters (i.e. message and process) and their combinations have been selected, as well as experimental design proposed, which were then tested within Task 29.4 and reported in Deliverable 29.8.

The conceptual framework proposed in the Deliverable 29.7 was developed based on the most significant and objective outcomes found within the Diversify project (WP28 and WP29), see **Figure 29.4.1**. It was chosen that the appeals should describe possible benefits of Diversify production method -traceability, -health- and taste-related outcomes resulting from consuming Diversify fish products, see listed messages in Deliverable 29.7. To support priming, three products have been chosen to reflect Diversify aquaculture products: (i) Fresh fish steaks – low processed products; (ii) Smoked fillet – medium processed product; and (iii) Fish burgers – high processed product. The selection of the products has been based on the previous studies done within Diversify, but also on the rationale that the different processing methods should be further explored in light of the proposed communication campaigns.

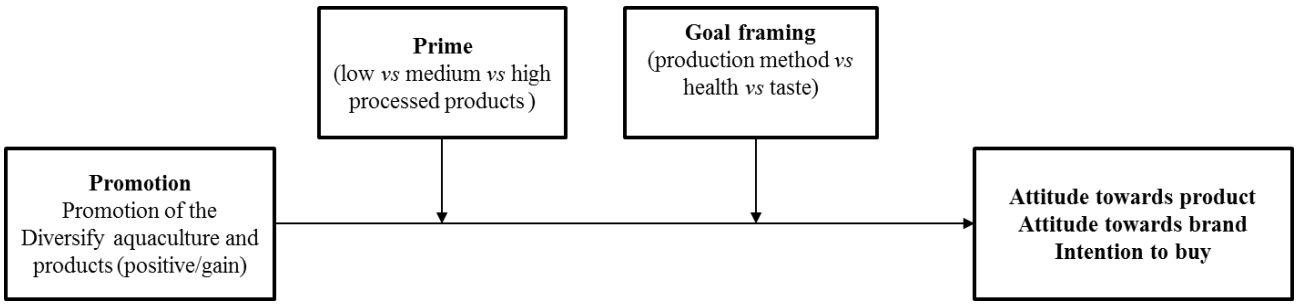


Figure 29.4.1. Conceptual framework

The main aim of the Deliverable 29.8 was to report on the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers' attitudes and behaviour towards the above selected products. The experimental design was based on promoting Diversify production method through an affective appeal of sustainability (i.e. promotion message) and using different product types - levels of processing - as primes and messages as goal frames to assess their effect on the amount of the product

attitude change. Thus, we used 1 (framing: promotion) x 3 (product type: low *vs* medium *vs* high processed product) x 3 (goal frame: traceability *vs* health *vs* taste) between-subjects design. The proposed experimental design is presented in **Table 29.4.1.**

Table 29.4.1. Experimental design

Promotion message with Primes and Goal messages	**Goal messages**		
	Traceability	**Health – wellness**	**Enjoyment while eating - taste**
Promotion message on Diversify aquaculture			
Primes			
Fish steak – low processed product	Message promoting Diversify aquaculture production method and its traceability, consumers primed with image of a low processed product	Message promoting Diversify aquaculture product healthiness, consumers primed with image of a low processed product	Message promoting Diversify aquaculture product taste, consumers primed with image of a low processed product
Smoked fillets – medium processed product	Message promoting Diversify aquaculture production method and its traceability, consumers primed with image of a medium processed product	Message promoting Diversify aquaculture product healthiness, consumers primed with image of a medium processed product	Message promoting Diversify aquaculture product taste, consumers primed with image of a medium processed product
Fish burger – high processed product	Message promoting Diversify aquaculture production method and its traceability, consumers primed with image of a high processed product	Message promoting Diversify aquaculture product healthiness, consumers primed with image of a high processed product	Message promoting Diversify aquaculture product taste, consumers primed with image of a high processed product

The results from Deliverable 29.8 show that the effect of communication on attitude towards the product is higher when the goal message is associated with the lower level of product processing across all three goal messages, see **Figure 29.4.2**. This is evident in the case of low processed product - fresh fish steak and the health goal message. Besides health, the traceability goal message worked well across all three products (i.e. primes).

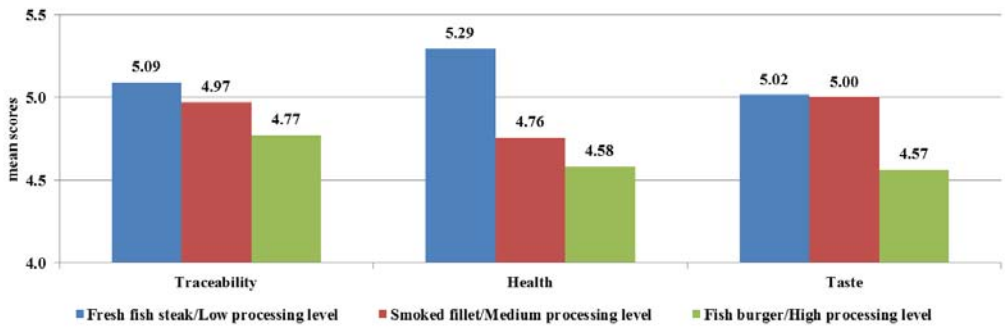


Figure 29.4.2. Product attitude as a function of message goals and primes - processing level.

Similarly, the results from Deliverable 29.8 further showed that the **highest effect** on product's purchase probability had the **positively and negatively evoked emotions**. This finding was evident especially for the experimental conditions with the **traceability and taste goal messages** primed with **medium** (i.e. smoked fillet) - and **high** (i.e. fish burger) - **processed products.**

Deliverable 29.8 considering all the insights from this study and the results from previous tasks (D28.1, D29.2, D29.4 and D29.6) brought forward a comprehensible tactic how to put new Diversify aquaculture products under the spotlight emphasising on the two main areas. First, by **improving the knowledge about "diversity" in aquaculture production** and **Diversify unique production method** to help overrule the mounting criticism about adverse environmental impact of aquaculture. Furthermore, results from Deliverable 29.8 validate this effort showing that if specific aquaculture practice and products are promoted in a unique way, this affects consumers' attitudes and purchase probability in a positive way. Second, the **increase in consumer support** by not only by using positive messages as 'healthy' and 'tasty', but also by adapting these messages to the characteristics and benefits of each product type in a clear, unique and differentiating manner can take a full advantage of the each product and aquaculture method. This is fully supported by the present study, which has shown that by adapting communication efforts to specific product types, not only will consumers learn about the products, but also about the different high-value choices that modern aquaculture systems make available to them, and the option to buy these products if/when available and affordable.

Deviations from Annex I and their impact:

There were no deviations from the calendar of activities established.

WP 30 Socioeconomics – Business model and marketing strategy development

WP No:	30	**WP Lead beneficiary:**		P10. TU/e
WP Title (from DOW):	Socioeconomics – Business model and marketing strategy development			
Other beneficiaries (from DOW):	P3. IRTA	P6. DLO	P11. AU	P12. APROMAR
P18. CTAQUA	P23. ARGO		P25. DOR	P28. CANEXMAR
P39. F2B				
Lead Scientist preparing the Report (WP leader):	Edwin Nijssen			
Other Scientists participating:	Michel van der Borgh (P10), Lluis Guerrero (P3), Gemma Tacken, Machiel Reinders and Mariët van Haaster – de Winter (P6), Athanasios Krystallis (P11), Javier Ojeda (P12), Rocio Robles (P18),			

Objectives

1. To identify business models for sustainable profitability and improved competitiveness of the sector for all the DIVERSIFY species,
2. To devise marketing strategies for the newly developed products from the DIVERSIFY species, aiming to develop a market that is as large and profitable as possible,
3. To come up with policy/strategy recommendations for further development and market expansion.

Summary of work reported in the previous Reporting Period (1-12 Mo):

In the DOW, this WP is not planned to start until project month 43. However some activities were already initiated, because the work for this work package is highly dependent on work done in other work packages (*e.g.*, WP 27, WP 28 and WP 29). A PhD candidate was selected (Maren Vos) who will execute a large part of the work for WP30. Next to that, we consulted with P6. DLO in order to make sure that work executed in Sub-task 27.2.3 and Sub-task 27.3.2 is aligned with work to be executed in WP 30.

Summary of work reported in the previous Reporting Period (13-30 Mo):

In WP 30 no activities were performed in this reporting period.

Summary of progress towards objectives (31-48 Mo):

The results of Task 30.1 show that for several of the species business models are still difficult (greater amberjack and grey mullet) or even problematic (wreckfish and Atlantic halibut). Production problems make process outcomes uncertain and a constant supply difficult. Selling to large retail chains thus will be hard because it requires a controlled and continuous stream of products. Therefore, for the suppliers of experimental species, selling to smaller retailers/parties and local restaurants makes more sense. It generates cash flow, but without the risk of not living up to expectations of being a reliable partner who creates and delivers high quality products on promise. For the producers of the new species collaborating with

innovative channel partners who are willing to co-create and co-invest is their best bet (compare Coviello and Joseph 2012).

The most promising business opportunities and thus models identified concern pikeperch and meagre. For these species, most bottlenecks in production have been subsidised. The challenge now is to grow customer demand and market acceptance. The newly developed products can help give an impulse to these efforts. The products developed for meagre included (i) a fish-burger aimed at children and (ii) a fish salad for consumers who like convenience. By targeting the segment of involved innovative customers (Deliverable 29.2) and in particular those consumers interested in convenience, progress can be made. Unfortunately, while these two species are most production ready they and their products had not been selected for additional consumer research to establish the best value specification and communication message (Deliverable 29.6). Still, suggestions were made towards building of business models for these species and the products developed for them.

Overall, our business model development showed a coherent business story for all four focal species, which is the first litmus test for any business model (Margretta 2002). Although farmers will benefit from continuing to work with their business partners to enhance their production processes and increase product quality/growth and decrease production cost, it is clear that serious investments in marketing and sales/channel management, i.e. market development are important and needed. Only with a buy in from distribution partners and adequate marketing efforts can consumers be reached and convinced to adopt and continue purchasing these new products. It benefits from using country/region of origin branding and health claims (e.g. Omega3), among others. Building a reputation or brand can help create differentiation necessary to prevent or resist price erosion when production begins to increase significantly.

The results of task 30.2 show that most firms are indeed focused on R&D for the species and thus have a partner or alliance portfolio consisting of equipment providers, hatcheries, feed manufacturers, and research institutes. On the one hand, this would appear logical because of the experimental stage of development of most species. However, on the other hand, farmers' (particularly meagre and greater amberjack) limited involvement in marketing and key customer alliances is troublesome. It suggests that the farmers are not very actively cultivating these relationships. Consequently, they may fail to achieve an early buy in, co-development, and other possible roles that customers can play in this process (Coviello and Joseph 2012). Although farmers do recognize the need for creating more market awareness of customers for the new species limited marketing investments and attention could result in involving downstream partners too little and too late.
Firms particularly need to pay more attention to their marketing efforts and relationship building with channel partners in order to succeed. Part of these efforts and channel partner involvement should be the creation of or compliance with a quality/sustainability certificate. Lack of such a certificate has been shown to prevent firms from gaining access to the retail sector and thus the consumer market.

Details for each Task

Task 30.1 Business models (led by TU/e, Edwin Nijssen; Michel van der Borgh).

The full description of the work and results is provided in ***Deliverable 30.1 titled Report on value propositions for the producers and Partners***

Introduction

The objective of this deliverable was to develop business models for the SMEs participating in DIVERSIFY for the focal species and their products. A business model describes the rationale of how an organization and its partners create, deliver, and capture value by accomplishing sustainable competitive advantage in the marketplace (Osterwalder, 2004). A business model has both (i) a coherent business story and (ii) cost and revenue figures that result in a sustainable profit for the parties involved (Magretta 2002). The latter two criteria can be used to check the viability of a business model. In this report, the focus is on the first criterion.

Table 30.1.1 shows the product development funnel of the DIVERSIFY project. For four species, 43 new product ideas were generated (Deliverable 28.2). First, these were reduced by using expert assessments. Ten

ideas were selected. Second, using these ideas 12 prototypes were developed, i.e., three for each of four selected species: grey mullet, greater amber jack, pikeperch and meagre (Deliverable 28.4). Third, based on these results, 6 products were selected for a consumer sensory test using consumer panels in all five target countries. Finally, in subsequent consumer tests the researchers and participating companies involved reduced this set of alternatives to a final set of 3 products for two species. The choice was based on scores of sensory profile attributes (see Deliverables 29.4), but also on scores of overall liking after visual inspection of the different products and purchase intention that were found across the five EU countries (see Deliverables 29.5 and 29.6).

The analysis holds important information for our business modelling effort. First, for meagre and pikeperch no final products were selected and further researched, despite the fact that these species show relatively high production-readiness. Second, for greater amberjack and grey mullet 3 products were developed and studied in subsequent consumer research, i.e. steak, fillets in olive oil and thin smoked fillets. However, characterized by low rather than high production readiness the supply of full-grown fish and products for these species remains uncertain. Farm-based production for these species remains experimental. The uncertain supply makes the farmers and products of these species unattractive for retail stores, who tend to prefer steady and sustainable supply of products for their customers (Helgesen 2007). Even when products deem successful in pre-launch market tests adequate follow up of supply of product will be difficult at best (i.e., ramping up), making continuity hard. Actual market tests and launch for these species should better wait till better control over the production process is obtained.

Finally, the findings summarized above also show that for two species of the DIVERSIFY program no products were created: wreckfish and Atlantic halibut. Although in the biological research progress was made, serious bottlenecks persist hindering serious business model development efforts. With Atlantic halibut availability of juveniles and high mortalities in early life stages have been a bottleneck and a serious challenge for commercial producers.

Table 30.1.2. Overview of focal species and the fish products developed under DIVERSIFY

		Production readiness			
		Low (experimental)		high	
Innovation funnel	*ideas*	***Greater amberjack***	***Grey mullet***	***Meagre***	***Pikeperch***
Stage 'Idea generation'		43 ideas			
Stage 'Prototyping'		12 different prototypes based on 10 ideas selected for the 4 fish species.			
Stage 'Product concepts developed' (29.4)		Grilled steak	Thin smoked fillets Fillets in olive oil	Fish burger Fish salad	Fish pate
Stage 'Consumer test' communications (29.5)		Grilled steak	Thin smoked fillets Fillets in olive oil		
Stage: 'Business model development and market test' (WP30)	 *new product* *market*				

The Canvas framework (Osterwalder 2004) was adopted and used to structure the results and report. It helps (1) explain the business, (2) how to run the business and (3) how to develop the business (Spieth, Schneckenberg and Ricart 2014). In accord with the objective of this task we focus on value proposition, customer relations, target market and resources.

Data collection

Three sources were used to develop the business models and value propositions for the four focal species for which at least some product development efforts were made.

First, from prior deliverables relevant information was identified and compiled. Deliverable D27 contained information about current business models of species and market conditions (e.g. competition and trends). In Deliverable D28 product ideas, concepts, sensor tests but also consumer responses to the newly developed products had been recorded. Finally, in the Deliverable of WP29 (D29) results on market segments, important extra value drivers (country of origin effect, impact of sustainability labels etc) and acceptable price range, and communication were identified. These results provided the core information for the business models.

Second, like for identifying SME's current business models (see D27.7) we used a workshop approach to collect feedback on (i) the current state of fish farming of the species and (ii) the products developed by the new product developers. A workshop approach was chosen because it allows researchers to quickly get input and offers the opportunity for discussion and follow up questions. The presence of business partners helped secure the external validity of data and results. The workshop took place as part of the DIVERSIFY's 3rd Annual Coordination Meeting (ACM) (Barcelona, Spain, 17-20 January, 2017).

Third, we collected data using interviews with a small set of consortium partners engaged in farming efforts for the specific species. The survey questions were derived from the Canvas framework. The aim was to collect complementary data, i.e. fill blank spots. Intermediaries and species leaders helped collect this data.

Results

Analysing and integrating the data, business models were developed for all four remaining species. For illustrative purposes Table 30.1.2 shows a summary of results for pikeperch. Per key element of the business model framework the situation is analysed and suggestions made.

Table 30.1.2. Example of summary result business model for pikeperch (part 1)

General observation: Production of pikeperch remains problematic (e.g., due to high cannibalism). As a result, many producers in the Netherlands, Germany and France gave up their efforts or went bankrupt in the last few years (Eurofish Magazine 2015). It implies that business models for pikeperch remain problematic or fickle. Still, there are also several parties that have invested in pikeperch development. AquaPri A/S (Denmark), for example, invested €7.5 million (3million subsidized by EU and Danish government) in a new 500 metric tons recirculation plant/outgrow facility to diversify its trout business (Fischer, 2013). Fish2be (Belgium) is also betting on pikeperch production. Its main customer, i.e. Migros of Switzerland is investing in a large grow out basin near Dresden. It suggests that business possibilities for pikeperch exist. Below the focus is on business model for intensive recirculation systems for on-growth rather than extensive ponds (see FAO 2017).
Value proposition. Pikeperch is a fresh water fish and resembles rainbow trout and carp. A value proposition has to position this fish in regard to these species. Pikeperch resembles trout; it is low in fat and thus completely lean. The structure of its meat is similar to flat fish species; white with soft texture (e.g. trout, carp, Pangasius). Pikeperch flesh has a mild taste, and thus lends itself to many different forms of preparation. Other USPs are: versatile fish that can be used in many preparations (e.g. salad), excellent taste, recognizable, local, and lean. Its filets can be boneless, unlike carp or (whole) trout. Therefore, it is a good alternative for people who look for an alternative for Pangasius, trout or carp. Due to the low supply, pikeperch is an exclusive fish, and prices are fairly high. Farms sell whole fish at approx. 9-10 euro/kg. The fillets in Germany/Benelux end consumer markets have a going rate of approx. 30-35 euro/kg, while in Switzerland it even is approx. 50-60 euro/kg. Pikeperch is easy to recognize based on its unique skin marks; hence selling fillets with skin on could offer a unique selling point (USP). The growth process takes 15-18 months to reach 800-1,200 kg. For a good fillet the fish should be 1 kg. However, as growth of the fish slows disproportionally after reaching 800 grams the aim should be selling smaller fillets.

Table 30.1.2. Example of summary result business model for pikeperch (part 2)

Target customers. Consumers in the EU countries consuming pikeperch prefer fish sold under local label and are willing to pay higher prices for such products. Developing a local brand is an opportunity for differentiation. The ASC label should be used in combination with this local label. Swiss supermarket chain Migros also plans selling this species using local claims because it positively resonates with consumers and the prices they are willing to pay. So, the customer benefit for the high price could be: '*Delicious fresh water fish from a trustworthy source close to home.'* Currently pikeperch is still a niche product, and relatively unknown in many markets. The niche consists of German speaking countries, like Germany, Austria and Switzerland, where it is well-established and has a serious market share. The fish is also well accepted in Eastern-European countries like Poland and Hungary. Here people mainly eat it for Christmas (festival). In these east European countries people used to eat cheap carp but are now shifting toward the more expensive and therefore exclusive pikeperch. A market for pikeperch also exists in North America and China (Intrafish 2013). After the above-mentioned countries expansion could continue in adjacent countries, for example Denmark, Belgium and The Netherlands, and in Eastern European countries such as Poland, Hungary and Czech Republic (that produce the fish themselves). It is anticipated to be easier to sell here than to consumers in saltwater fish oriented countries like the Mediterranean countries (e.g., Spain). Good international opportunities involve the United States and China (Eurofish Magazin 2015, Fischer 2013). In accordance with Deliverable 29.4 for the traditional (e.g., German speaking) markets traditional segments can be targeted and grown. In contrast, in new markets (e.g., Belgium, Netherlands) involved innovators and traditional fish eaters should be targeted. Involved innovators stand out from other two segments as being more knowledgeable of the healthiness underlying the fish consumption. They are more likely to try new alternatives entering the market. 'Traditionals' are conservative and reserved regarding the new experiences in fish products in general, but hold the strongest positive beliefs regarding the farmed fish production, being also aware of its possibilities both in connection to the environment but also regarding the hedonic aspects of fish consumption.
Distribution channels. Pikeperch is mainly sold as whole fish to restaurants (via wholesalers), and in small amounts to retailers (production lags behind). The out-of-home market seems to be driving penetration in the retail market (BVFi 2017). To sell to large retailers a constant, reliable delivery is important. Retailers sell fillets. Sustainability certification is generally needed to grow this business. Penetrating a market through the out-of-home market would seem to be a good strategy. If consumers get in contact with this new aquaculture species due to preparation by an expert, the chance is higher that they will try it at home. Other products have been successfully introduced in the market this way. The Swiss retailer Migros has shown interest for pikeperch. Referring to Migros' investments in pikeperch other European retailers could be interested also to explore the opportunities for this species. Pangasius is interesting for consumers since it is a cheap product, but all sustainability issues make the product vulnerable. Pikeperch is a more solid and stable choice for distributors. One of the partners in the project has contacts with Carrefour that considers to capitalize and to expand the assortment of fish in France and Belgium. Other retailers could use a similar approach. Collaboration with large super markets is a good idea for quick market expansion and penetration, but farmers /suppliers should be careful to not become too dependent. Restaurants help create a buy-in of customers and facilitate market penetration in the retail /monger market. By developing a portfolio of channel relations and expending its markets growth can be accomplished and sustained.

Table 30.1.2. Example of summary result business model for pikeperch (part 3)

Resources and key partnerships. Pikeperch is a difficult species. It is prone to stress and sensitive to bacterial loading of water. Moreover, it is sensitive to high density resulting in stiff cannibalism. Hence, it needs great attention and quiet conditions. By controlling the temperature and light the fish can spawn four times a year, which allows for continuous supply of the market, e.g. super markets. Close cooperation with research institutes and water management companies is beneficial. An issue in raising pikeperch is cannibalism; approximately 45-50% of product (e.g. larva) is lost. Reduction of cannibalism by good selection and breeding is possible. Only a handful of major farmers of pikeperch exist in Europe. Experimenting with intensive rearing many went bankrupt or stopped their efforts in the past few years. It shows that it is hard for commercial producers to master the process and build a constant revenue stream. Recirculation systems have high energy use. So, energy consumption is high and costly. Reduction of energy cost is vital to success (Fischer 2013). A partnership or deal with an energy company would be desirable for farmers of aquaculture of pikeperch. Stripping the eggs rather than using fish spawning seems to offer the best results. It offers valuable data on numbers of eggs, fertilization and survival rates and thus helps to optimize processes more easily (Eurofish Magazine 2015). So, careful data analyses and active life stock management are needed. Pikeperch has an average market weight of 750-800 g. Fillet yield is approx. 45%. Key to good tasting products is good pre-mortem treatment. Therefore, processing expertise is important for pikeperch farms to achieve necessary product quality. Farmers need adequate financial resources, i.e. slack, to deal with disruptions in production associated with experimentation stage of development of this species. In this regard, adoption of this species may be easier for established firms than start-ups (mono-product). Firms need to step up their marketing, i.e. make serious marketing investments to build channels and develop the market for pikeperch. Selling the fish under local product/country of origin labels resonates with customers and can be used to create a unique, sustainable positioning. Finally, good stakeholder management with, for example, environmental protection agencies, policy makers and government is important. Farmers may benefit from increased awareness for need and opportunity in rural areas to help supply fish in Europe. Farmers may benefit from Brexit due to negative consequences for fresh water fish supply which may increase possibilities for farming. As pikeperch aquaculture is getting established it can positively affect employment in rural regions.
Key activities (and related resources). In summary, the following activities are needed for the successful launch and commercialization of pikeperch in the market: Human: strong production knowledge required (spawning, post mortem treatment etc.) Constant supply, e.g. using multiple breeding groups. Sustainable certificate. Marketing knowledge (e.g., country of origin claims/brand), channel relations management, international marketing activities (leveraging large retailers' relations) Financial slack to be able to overcome incidents Good data and livestock management for optimal results Energy cost management Relationships with research and water treatment/equipment suppliers

Finally, also value propositions and suggestions for market development using business partners per new product were developed. Table 30.1.3 shows an example for the new fish burger developed for meagre. The key elements of the business model are shown in the first column. The last column (right hand side) lists the suggestions that were developed.

Table 30.1.3. Detailing of value proposition and marketing mix for meagre/fish burger

	Meagre (D27.3)	*Fish Burger*	*Value proposition based on Results D29.6*	*Suggestions*
Value proposition	• Sold whole or as fillets. • The largest fillets can be smoked in some cases. • An application for quality labelling has been filed (Label Rouge and Indication Geographique Protégé - IGP) • Good quality flesh, low in lipids with a high proportion of polyunsaturated fatty acids • Appropriate size for processing (filleting and smoking) • Long shelf life • Scarcity (high demand, low volume)	• Frozen product (D28.2, p.6) • Convenience • Fish shape for children • Premium product, • Sustainable, EU origin • Position as healthy AND playful/fun • Vacuum packed, transparent packaging • Low fat; high preservation quality (D28.2, p.24-26), nutritious (D28.2, p.34) • Competing products include e.g. fish fingers and hamburgers (substitute). • Many new fish burgers launched all countries in 2011-2016 (D29.5, p.8)	• **[no information]** • Burger with shape of fish • Healthy and fun meal for children (up to 8 years) • Nutritious and low fat, comparing favourably to fish fingers/hamburger • Add Omega 3 claim and ASC certificate • Price: 5.73€ (reference price per 300 gr.)	• Most innovative new product; • Better sold as fresh than frozen to stress value and legitimize price premium
Customer segments	• **Niche market**: Dealers & Chefs • Market not sufficiently developed as species is poorly known by the general public. • Main markets Southern France and Italy.	• Specific mass segment; mothers with children • Health conscious parents (D28.2, p.34) • Convenience/variety seeking parent (p.34) • Innovative yet familiar product characteristics (D28.2 p.16) • Favourable purchasing probability in all countries: >4.7 (lowest in UK/It; highest in France, i.e. 6.2 (29.4, p.25)	• Sell to parents that like fish/variety in diet	• Consider advertising, e.g. in super markets folder. • Advertise aimed at parents and children • Low cost options are use of social media and YouTube
Customer relationship	• Short & long term relationships (building)		• Consider developing brand to build relationship with audience/children. • Use cartoon	• Brand relationship • Maintain relationship actively
Distribution channels	• **Partner channels**: Small set of distributors (face/ phone)	• Supermarket	• Super markets • Food services, e.g. schools	

Conclusions and recommendations

The results from this report have implications for food policy makers and managers in the aquaculture industry interested in developing the business opportunity for the focal fish species and stimulating market launch of the newly developed products. The results show that for several of the species business models are still difficult (greater amberjack and grey mullet) or even problematic (wreckfish and Atlantic halibut). Production problems make process outcomes uncertain and a constant supply difficult. Selling to large retail chains thus will be hard because it requires a controlled and continuous stream of production. Therefore, for the suppliers of experimental species, selling to smaller retailers/parties and local restaurants makes more sense. It generates cash flow, but without the risk of not living up to expectations of being a reliable partner who creates and delivers high quality products of promise. For these producers, collaborating with innovative channel partners who are willing to co-create and co-invest is their best bet (Coviello and Joseph 2012).

The more promising business opportunities and thus models concern pikeperch and meagre. For these species, most bottlenecks in production have been subsidised. The challenge now is to grow customer

demand and market acceptation. The newly developed products can help give an impulse to this effort. The products developed for meagre were a fish-burger aimed at children and a fish salad for consumers who like convenience. By targeting the segment of involved innovative customers (Deliverable 29.2) and in particular those interested in convenience, progress can be made. Unfortunately, while these two species are most production ready they and their products had not been selected for additional consumer research to establish the best value specification and communication message (Deliverable 29.6). Still, suggestions were made towards building of results for the products developed for the less-production-ready species.

Overall, our results of the business model development showed a coherent business story for all four focal species, which is the first litmus test for any business model (Margretta 2002). The results showed that firms particularly need to pay more attention to their marketing efforts and relationship building with channel partners. Lack of such a certificate can prevent access to the retail sector. Although farmers will benefit from continuing to work with partners to enhance their production processes to further increase quality/growth and decrease cost, it is clear that serious investments in marketing and sales/channel management are needed. Only with a buy in from distribution partners and adequate marketing efforts can consumers be reached and convinced to adopt and continue purchasing these new products. It benefits from using country/region of origin branding and health claims (e.g. Omega3), among others.

Key references

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Sub-task 30.1.3 (led by TU/e, Maren Vos).

According to the DOW, cost structures and possibilities to further drive down costs will be analysed together with the SME Partners. The way different companies along the value stream are involved and will get an income from cooperation or customer segments will be described and analysed (TU/e, APROMAR). It will be linked to price decisions to allow for estimating revenue streams. Several ways to generate revenue streams will be explored. The effort will draw on market data and trends from Task 27.1. Deliverable D30.4 and will be delivered in month 48 (November 2017). Revenue (pricing & costs structures) model per species will present the results of this Sub-task.

The revenue model describes the way a company makes money. It includes (i) revenue streams, (ii) cost structure, (iii) pricing mechanisms and price level, (iv) profitability, i.e gross sales -/- cost. **Figure 30.1.1** shows the revenue model including these elements and their interrelationships.

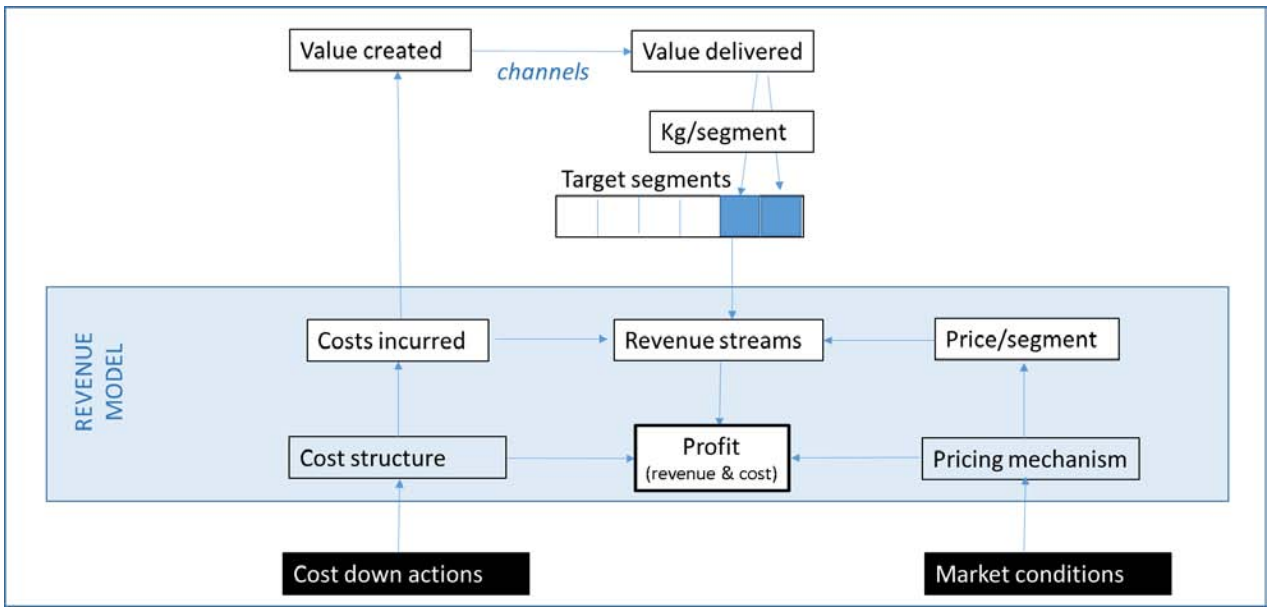


Figure 30.1.1. Visual representation of the financial aspects of the business model.

Methodology

To develop revenue models and learn more about prices, cost structures, and sales of the SME's currently developing and marketing pikeperch, greater amber jack, and meagre, we used a survey to collect data. We collected qualitative data on their current revenue streams and pricing mechanisms, which together make up the revenue model. We also collected data on current costs structures and expected cost reductions. The survey was sent to all SME's participating in the project, as well as a Portuguese SME and Spanish SME's connected to CTAQUA and APROMAR. In addition, we collected data through a qualitative questionnaire offered to project leaders and a director of a producer association.

Despite a personalized letter and several reminders the response was limited (n<10). Consequently, the data were analyzed and interpreted using simple tabulations and qualitative analyses. To increase reliability and validity of the results, we triangulated the survey data with data from other sources (i.e., qualitative questionnaire and secondary reports) and insights gained in other subtasks of WP30 (*e.g.* in D30.3). Since no data on grey mullet were received, we cannot offer conclusions for this species.

Results are affected by the fact that the farming of these species is still in the experimental stage and products are in the conceptual stage rather than the physical market testing stage. While some SME's are optimistic, the average expectation is that particularly farming of meagre and greater amberjack will take several, i.e. > 4, years at best before being fully established. The outlook regarding time to market for pikeperch is more optimistic, i.e. 2 years.

Results

For each species the deliverable will discuss the different parts of the revenue model in order to derive profits and estimate the break-even point. We supplement this with a discussion of possibilities to drive down costs in the future.

Due to space limitations we only show (preliminary) results for greater amberjack:

Business model of greater amberjack

Revenue streams. SMEs involved in farming greater amberjack currently depend on two revenue streams: (i) revenue from fish sold as consumable fillets or whole fish, and (ii) revenue from juveniles. The former is the more important one. The whole fish and fillets are typically sold to restaurants and supermarkets. Because amberjack is a large species, it is easy to process (fillets, portions, etc.) and highly marketable. It is

generally considered to be of good quality and thus well received by the market. Compared to European sea bass and Gilthead sea bream, it grows fast. Large harvesting sizes (>3kg) can be achieved with very reasonable production cycle time.

Consumable fish revenues. SMEs sell fish sized up to 3-5 kg to both restaurants and wholesalers, while smaller fish (about 2 kg) are sold to supermarkets. At the moment, SMEs indicate that they sell on average 50-100 kilograms per month, at a price of €10/kg to €20/kg. Greater amberjack has an excellent, i.e. high yield of up to 50%. The revenues from greater amberjack can be considered transaction revenues from asset sales using fixed pricing mechanisms; that is, it is based on product features such as the size and level/type of processing.

Juvenile revenues: Revenues from greater amberjack juveniles sales to on growing farms occurs also. These can be considered as transaction revenues from asset sales, with fixed pricing mechanisms.

Cost structure.
Variable costs per kilo:
15-20% of all costs
Energy: 10-15% of all costs
Feed: 55% of all costs
Medicine: 10% of all costs
Running costs:
Fixed costs/ investments made: € 50.000 (for established farms)
Fry: 10% of the running costs.
Possible other future costs: Promotion and marketing. (tbd).
Loss:
Cannibalization and illness: 45%, reducing to 20%.

Costs differ depending on the production system. For land-based systems (ponds) costs depend mainly upon the size of the farm. Availability in the production area of appropriate/exploitable processing facilities helps, as most SME's are only prepared for boxing bass and bream with almost no processing capacity.

Profitability. New ventures pioneering greater amberjack farming expect to require 5 years or more to break even, while established firms using this species for diversification purposes may reach profitability more quickly. Respondents of SMEs already farming other fish species indicate that 2-4 years should be attainable. Investments involve resources such as, buying breeding stock/juveniles, hardware, health treatments, and marketing campaigns. Variable costs are expected to remain constant. In earlier production phases, 45% of fish is lost due to cannibalization and illness. This is expected to decrease to 20% in the next 2-3 years, as farmers become more familiar and better master the reproduction and outgrow processes.

Revenue model. We estimate the revenue per SME farming greater amberjack (**Table 30.1.4**) as equal to the price per kilo (10) * the amount of kilo's sold (50), i.e. 500 euros per month. We also assume that profit equals price per kilo (10) – variable costs per kilo * Sales in Kilo's (50) – fixed costs (50.000+0.1).

Using a conservative estimate, we thus it will take over 9 years to recover the investment made. In the most optimistic scenario of a price of €20 per kilo and sales of on average 100 kilos per month, the firms would break even after approximately 2.5 years. The break-even period can be seriously decreased by mastering the farming process and stepping up production levels. However, it should be complemented with sufficient customer relationship development and marketing efforts to ensure adequate market demand to absorb the extra product.

SMEs farming greater amberjack should involve customers during their new farming efforts to ensure adequate channel access and market potential. If firms are able to grow the amount of kilos they need to grow their number of customers and/or the repurchase frequency of existing customers.

Because greater amberjack is a fish that can be easily processed value creation may be more with the processing firms/partners than with the farmers. Processing firms may add more value than farmers to the product and decide to protect it using a brand. As a result they might own the customer rather than the fish farmers. Selling a rather homogeneous products prices could fall and margins decrease. By selling fillets but also products that help exploit the fish and its discards to a maximum profits can be optimized and even maximized.

Table 30.1.4 Summary of the main parameters of the revenue model of greater amberjack

Greater Amberjack	Whole fish	Fillets	Juveniles
Price	€10-20/kg	€25-30/kg	
# Amount of kilo's/pieces sold Monthly	50-100		
Buyers	Restaurants, fish mongers, supermarkets, wholesalers		Ongrowing farms
# of Buyers	Tbd.		
Average amount of kilo's per purchase	Tbd.		
Purchase frequency	Tbd.		
Loss (cannibalization, illness)	20-45%		
Variable costs per kilo	%		
Labor	15-20		
Energy	10-15		
Feed	55		
Medicine	10		
Fry	10-15		
Fixed costs (investments made)	€ 25.000 - 50.000		

Cost reduction opportunities

As **Table 30.1.5** shows, SME's indicate that there are several production costs that are expected to change as production becomes mature. Costs incurred due to cannibalization and illness are expected to decrease over time, which will greatly drive down overall costs and increase firms' viability. Furthermore, energy costs are expected to decrease, as farming becomes more efficient and SME's are able to find more sustainable energy resources. It seems that pikeperch farming is expected to require more labor and feed as production intensifies. Since labor costs comprise the largest cost component for farming pikeperch, increasing production by automating parts of the farming process can help to substantially reduce the variable costs per kilo. Fry costs are only expected to decrease for meagre. SME's farming meagre face serious challenges with becoming profitable, but may be able to reduce feed costs by selling smaller, younger fish. Only SME's farming Greater Amberjack expect their variable costs to remain stable over time.

Table 30.1.5. Expected changes in production costs.

Expected changes in production costs per species			
	Pikeperch	Meagre	Greater Amberjack
Loss (cannibalization, illness)	-	- 15%	- 25%
Labor	+	o	o
Energy	-	-	o
Feed	+	o	o
Medicine	o	o	o
Fry	o	-	o

Note: o: costs are expected to remain stable, +: costs are expected to increase, -: costs are expected to decrease.

General conclusions

SMEs should be able to use the revenue models developed in this report to calculate their revenue after each year by taking the price per kilo and multiplying it by the amount of kilo's sold. In order to increase revenues, special attention still needs to be payed to increasing the number of buyers and the average amount per purchase, or purchase frequency. Since early investments are relatively high, it will take SMEs several years to recoup their initial investment. When we take variable costs into account, this time-span increases. The main limitation for most species seems to be the low production numbers. It matches the observation that the farming of these species is still largely experimental. In addition, firms will need to begin investing in marketing to grow their number of customers and stimulate market demand. It should also include pursuit of a quality /traceability label to ensure access to the retail market.

Task 30.2 New product marketing strategy development (led by TU/e, Edwin Nijssen).

Sub-task 30.2.1 (TU/e)

According to the DOW the aim of this sub-task is the development of a new product marketing strategy including actionable product-market combinations, new product launch, new market entry and timing, stimulating consumer adoption and encouraging diffusion across EU markets (TU/e, IRTA, AU, DLO, APROMAR, CTAQUA, HRH), drawing on the results of the segmentation of the market (Sub-task 29.1.2), the experimental studies (Sub-tasks 29.3.2 and 29.4.1) and the sensory evaluation (Sub-task 29.2.1). The focus will be on the five countries selected for the Tasks in WP7. Results from market tests (Sub-task 30.2.2) conducted will be used to further improve these strategies. These strategies will be reviewed by the involved SME's. In Deliverable D30.3 Guidelines to cultivate buyer-supplier relationships per species, the results of the product market phase are presented which will be delivered in month 48 (November 2017).

Preparations (e.g., building initial simulation model; defining parameters; make short-list of products) for this sub-task start in month 34 (September 2016). Currently we are waiting for additional results from deliverables, e.g. the virtual market test. These results, i.e. parameters can be entered into the model. For the execution of the modelling task we collaborate with expert from TU/e in system dynamics (B. Walrave).

All details of the outcomes will be presented in ***Deliverable 30.3 regarding Cultivating buyer-seller relationships.*** This deliverable is about to be submitted according to plan, so you will find below a brief summary of its contents.

Many of a company's supplier and customer relationships are vital for its continuing competitive survival, and each may involve a substantial commitment of resources that cannot be easily used elsewhere. A company's identification of the right partners and decisions regarding what actions to take in each relationship are of great importance to the development of its overall portfolio of relationships and its competitive success, particularly when extending its market using new business development.

By involving partners, firms create an ecosystem that helps them create value but also shape their business model. The partners and relationships help to jointly create value and deliver this value to the market i.e., to target customers. The resulting sales and cash flow will help the firms' earn a profit but also to grow their business through further investments. These ecosystems and their partnerships are important because firms are typically unable to perform and control all tasks involved in value creation and delivery themselves.

Building the ecosystem requires developing alliances and a healthy alliance portfolio. Consequently fish farmers engaging in developing a new species should develop relationship building and alliance management capabilities. It refers to abilities concerning acquiring and retaining partners, but also portfolio management capabilities.

The objective of this deliverable was to develop guidelines for fish farmers to develop their alliance portfolio, and offer suggestions which buyer-supplier relationships to cultivate. We will also identify key challenges and potential bottlenecks and offer some suggestions on how to address these challenges.

To learn more about the alliance portfolio of the farmers involved in developing and bringing to market product of the four species of the DIVERSIFY project, i.e. pikeperch, grey mullet, greater amber jack and meagre, we used a survey to collect data. In addition, we collected data through a qualitative questionnaire offered to the species leaders and a director of a producer association.

Despite a personalized letter and several reminders the response was limited (n<10). Consequently, the data were analysed and interpreted using simple tabulations. To increase reliability and validity of the results, we triangulated the survey data with data from other sources (i.e., qualitative questionnaire and reports) and insights gained in other subtasks of WP30 (*e.g.* in D30.1). No data on grey mullet were received. As a result data could not be analysed and we cannot offer conclusions for this species.

Results are affected by the fact that species are still in the experimental stage and products are in the conceptual rather than physical market testing stage. While some firms are optimistic, the average expectation is that particularly farming of meagre and greater amberjack will take several, i.e. > 4 years at best. Outlook regarding time to market for pikeperch is more optimistic, i.e. 2 years.

What stands out from the data, is the fact that firms enter and leave the market for developing these new species almost constantly. In most cases, firms pursue the farming of new species in an attempt to diversify their portfolio. This 'waxing and waning' confirms that most species are still in an experimental stage. It may explain the difficulties encountered in collecting data and information from firms, including project partners, on issues such as cost structure and alliance portfolio development.

Table 30.2.1. Fish farmers' current partners (level of involvement)

	Meagre	Greater amberjack	Pikeperch
R&D alliance			
Equipment providers	●●	●	●●
Feed manufacturers	●●●	●●	●
Hatcheries	●●	●	●
Research institutes, incl. health	●●	●●	●●
Marketing and key customer relations alliance			
Government	●	●	●
Customers (wholesalers, mongers and local restaurants)	●		●●
Customers (retailers)			●●
Other alliance			
Investors	●	●	●

● – ●●●: some –(moderate) –high involvement; no entry = no involvement

In accordance with the fact that emphasis remains on mastering the farming process of the species firms' current partners mainly involve R&D relationships. As **Table 30.2.1** shows, their alliances focus on: (i) equipment suppliers, (ii) hatcheries, (iii) feed suppliers and (iv) research institutions. This is true for all three species involved. Except for pikeperch, firms have limited marketing and key customer alliance. The better position of pikeperch farmers can be explained by the fact that they are further in the process of bringing the fish to market. These stronger relationships with key customers also increase the chance that they will succeed. Involving and closely working with these key customers and also by having better developed marketing relationships (e.g. on branding) they have ensured market access and key customer support, sometimes with key customers also making investments in value adding activities (e.g., by investing in market tests).

Some firms (particularly meagre and greater amberjack) mentioned worrying about how to keep current relationships strong. It is probably explained by the long development process of getting the fish production ready. In the process, alliance partners (including investors) may become impatient and drop out.

Table 30.2.2 lists the results regarding the biggest challenges of relationship development that managers of the firms see. It confirms firms' awareness regarding the need to invest in marketing and key customer relationship development in the future for meagre and greater amberjack.

Table 30.2.2. Fish farmers' perceived major challenges regarding developing relationship alliance portfolio (size of challenge)

	Meagre	Greater amberjack	Pikeperch
Equipment providers			●
Feed manufacturers/food treatment	●●		
Marketing	●●	●●	●
Key customer relationships/wholesalers	●●	●●	●
Hatcheries	●●	●	
Research institutes, incl. health	●		
Investors/financial resources	●		●
Government			

● – ●●●: low –(moderate) –high efforts; no entry = no effort

Apart from the relational challenges listed, firms also mentioned several important bottlenecks. **Table 30.2.3** provides a brief overview and categorizes these bottlenecks based on the stage in the relationship development process, i.e. initiation, building, maintenance/retention, and termination. The bottlenecks include: (i) inability to raise wholesaler/retailer interest, (ii) not being able to offer enough and rapid benefits for partners or still having to work out deals, and (iii) not knowing how to ensure enduring relationships. Another issue was that some firms had few or no partners yet.

The categorization confirms that most bottlenecks pertain to initiation/building of partnerships and thus acquisition capabilities. Particularly on the commercial size of the business development process problems exist. A solution would be for firms to invest better in developing their marketing sales capabilities. By extending the amount and quality of sales/marketing personnel more time and resources are available for these activities. It fits the notion that it is a firm's task to not just create products but actually to create customers. Another option would be, as some farmers also suggested and hoped for, to pull together and organize market development at the fish industry (fish producers associations) or even governmental level. It may help generate awareness for fish consumption in general and new species in particular. It can enhance the success of new launches. However, such campaigns should better be considered complementary measures that enhance existing marketing investments by the firms themselves. Therefore, to ensure success farmers should better step up rather than reduce their own marketing expenditures in the light of these national campaigns. These efforts are complements and not substitutes.

Table 30.2.3. Bottlenecks fish farmers experience regarding development of relationship alliance portfolio

Bottlenecks mentioned	Related capabilities
Raising retailer/wholesaler interest **Engaging in market development** **Partners need benefits quickly** **Working out a deal for continuous supply at a profitable level** **No partnerships yet, no experience yet**	Initiation/building
Make sure partnerships last	Maintenance/retention

Conclusions

The results from this report have implications for food policy makers and managers in the aquaculture industry interested in developing the business opportunity for DIVERSIFY's focal fish species. The results show that most firms are focused on R&D for the species and thus have a partner or alliance portfolio consisting of equipment providers, hatcheries, feed manufacturers, and research institutes. This would appear logical because of the experimental stage of development of most species. However, farmers' (particularly meagre and greater amberjack) limited involvement in marketing and key customer alliances is troublesome. It suggests that the farmers are not very active cultivating these relationships. Consequently, they may fail to achieve an early buy in, co-development, and other possible roles that customers can play in this process (Coviello and Joseph 2012). Although farmers do recognize the need for creating more market awareness of customers for the new species limited marketing investments and attention could result in involving downstream partners too little and too late.

Sub-task 30.2.2 (TU/e)

According to the DOW the aim of this sub-task is the test of the proposed market strategy. In cooperation with the SMEs involved, a market test will be performed in the 5 countries selected (i.e. UK, D, ES, F, I). The network of the SMEs will be used to perform this test. These small-scale consumer tests will run in mid-size to large cities of these countries (TU/e, DLO, IRTA, AU, HRH). Locations will be chosen in accordance with the target segment, channel selection and using the marketing (e.g., communications, packaging) guidelines from WP29, and options available. Based on test market guidelines for actual rollout will be optimized. Furthermore, market simulations will be performed to estimate and facilitate the market launch and diffusion. The modelling will happen using data from WP27 and using system dynamics modelling. It will help SMEs to make decisions regarding their sales & operations planning (so called S&OP). As the choice of value products developed will impact the nature of the markets that will be targeted and parties involved, the efforts of market testing and simulation should be expected to vary by species, product and country. In Deliverable D30.5 New product marketing strategies per species and product, the definite marketing strategies are presented, while in Deliverable D30.6 Report on results of test markets per species are summarised. Both deliverables will be delivered in month 52 (March 2018).

This description of work suggests that a market test with physical products in physical stores will be done. However, the production and processing resources, capabilities, and processes needed for testing the Diversify species are not yet at the necessary level to conduct a real life market test with physical products. To date the production and processing of 4 of the 6 species only is executed in a research environment or at small scale in regular production environments.

More specifically:

- There are no production facilities that can sell processed fish species from the Diversify project to regular stores, and/or
- the scale of the production is too small to do a physical market test, and/or
- only fresh sales are possible in the current chain process (partly wild catch), what makes distribution in 5 countries expensive to reach the objected preservation time.

Given these drawbacks, an online market test will be performed, in which an online supermarket is imitated and in which consumers do their groceries based on an assignment. The nationality of participants and the language of these online environments will match the five countries selected.

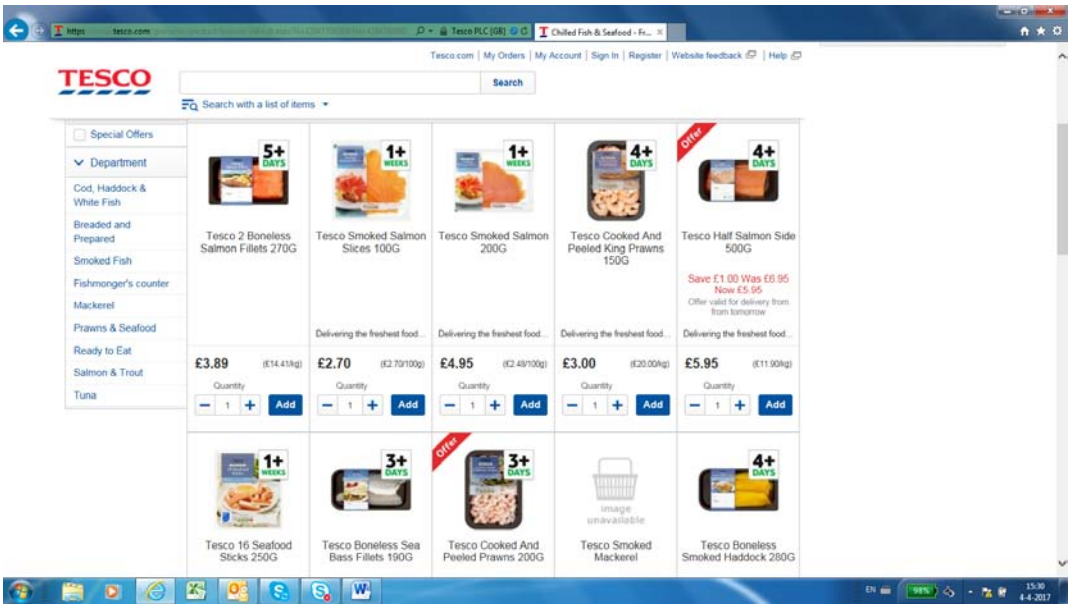


Figure 30.2.1. Example of national retailer's fish counter/category

Currently we are preparing this online market test. Based on results and ideas developed in WP28 and further tested in WP29 a project proposal has been designed. It involves an experiment with several manipulations and a retail web store setting (see **Figure 30.2.1** for an example of the online fish counter/category of a national retailer). The plan was discussed during the 2nd promotional workshop with business partners in Spain on 28 September 2017. Now it is reviewed by partners within the project and detailed further to an experimental design. In the meantime our Greek partner (HRH) developed the web store environment to be able to run the experiment in our five target countries. Below some screenshots from the presentation of the set up for the virtual market test (see **Figure 30.2.2**).

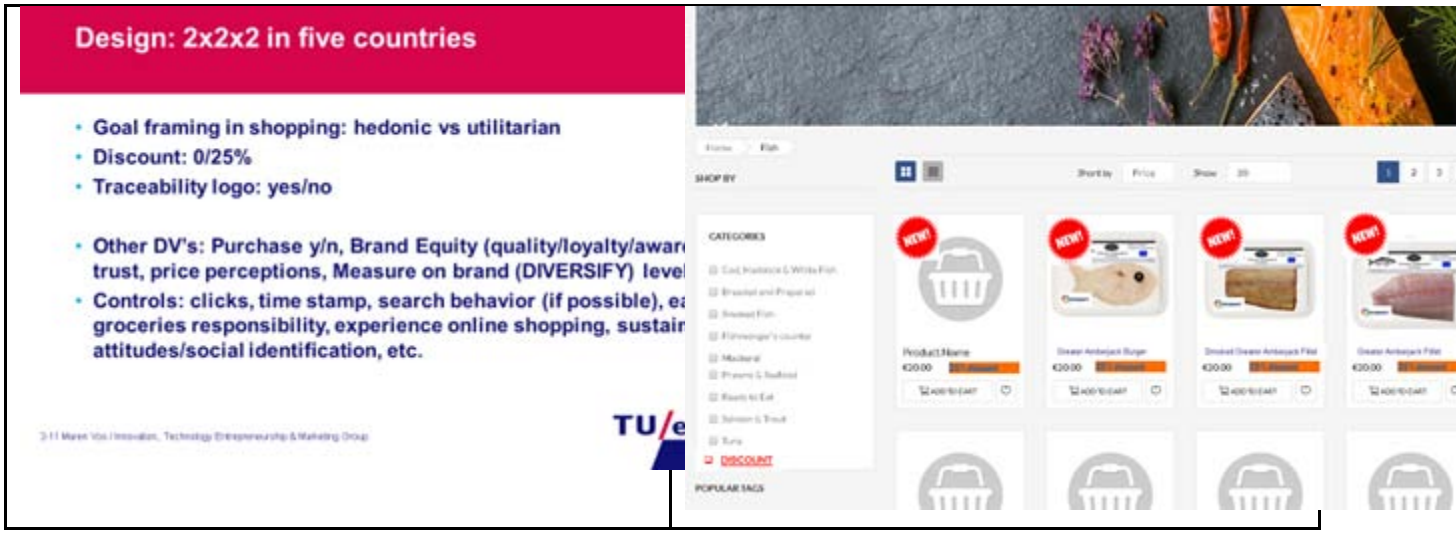


Figure 30.2.2. Screenshots virtual market test presentation

In the upcoming months the webstore will be developed and the test planned and executed. The plan is to run the experiment in 5 countries, with n=300 per country. Classification criteria will be used similar to those used in the prior consumer studies of the project. It will be complemented with the criterion that customers had at least minimal online experience. Results are expected to be delivered in Month 53.

Task 30.3 Recommendations for industry development and international market expansion (led by DLO, Gemma Tacken).

Sub-task 30.3.1

According to the DOW the aim of this sub-task is to conduct a feasibility study. In the feasibility study, an analysis on basis of the technical assessment (WP 28), market information (WP 29), resource and cost analysis (Task 30.1) and the results of the tested strategies (Task 30.2) will be delivered (DLO, IRTA, TU/e, HCMR). This study covers a financial analysis, an assessment of return on investment and a definition of efforts needed, a risk assessment, technological assessment (WP 28), political analysis of potential risks of implementation, environmental impact assessment (with information from GWP5 Grow out husbandry), a sociological and market impact assessment and a stakeholder identification to introduce the products in the market. This feasibility study will be reviewed by the participating SMEs (ARGO, ITICAL, DOR, CANEXMAR and ASIALOR). The results will be presented in Deliverable D30.7 Feasibility study and will be delivered in month 60 (November 2018).

Given the fact that the preparation work of Task 30.1.3 first had to be finished, only limited work is done on this task yet. Wageningen Economic Research (|DLO, partner 6) has done some internal search for a model and a methodology that can be used for this feasibility study. As base model is developed founded on models used before in EU project ORAQUA and models for developing business models with respect to aquaculture in Africa. All these models are validated and published.

In 2018 we object to visit all business companies to find out how feasible the new species are for the European market or submarkets within the EU.

Sub-task 30.3.2

According to the DOW the aim of this sub-task is to design a global market approach. The global market approach will be developed based on input from Task 30.2. Based on market similarities and existing contacts of the EU fish industry in foreign countries, opportunities for the new products developed in WP 28

will be identified and suggestions developed on how to further promote growth and market expansion (DLO, AU, HRH). The development of these plans will involve experts from the industry and the respective countries, as well as the experience (and networks) of the SMEs involved in DIVERSIFY. On the basis of the analysis, policy (macro-level) and strategy (micro-level) recommendations will be provided (DLO, AU, APROMAR, CTAQUA, HRH) with the potential to make the European aquaculture sector more competitive, and to provide a level playing field with respect to production in developing countries. Based on the above input we will again develop system dynamics simulation models that help predict the diffusion, of the EU produced fish species of this study, internationally. The models will factor in SMEs' international relations and other (e.g., cultural) linkages between geographical markets. The results will be published in Deliverable D30.8 Report on EU and international market development plans and recommendations and will be delivered in month 58 (January 2018).

The format of this Deliverable is dependent on the final report that we aim at in this project. In the ACM of January 2018 we would like to discuss with the project coordinator and the socio economic team about a potential approach.

Dissemination – WP 31

WP No:	31	**WP Lead beneficiary:**		P18. CTAQUA
WP Title (from DOW):	Dissemination			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P6. DLO	P7. IMR
P8. IEO	P9. UL	P10. TU/e	P.11 AU	P12. APROMAR
P13. UNIBA	P15. ULL	P19. CMRM	P33. FGM	P34. BVFi
P35. MASZ	P36. ANF	P37. EUFIC		
Lead Scientist preparing the Report (WP leader):	Rocio Robles			
Other Scientists participating:	Constantinos C. Mylonas, Maria Papadaki and Ioannis Fakriadis (P.1), Alicia Estévez (P.3), Neil Duncan (P.3), Luis Guerrero (P.3), Mathias Keller (P.34), Maria Banovic (P.11), Javier Ojeda (P.12), Blanca Álvarez (P.8), Covadonga Rodriguez (P.15), Gemma Tacken (P.6), Aldo Corriero (P.13), Fátima Linares (P.19), Laslo Varadi (P.35), Martiña Ferreira (P.36).			

Objectives

1. Disseminate the knowledge acquired to the scientific community, to promote further research,
2. Disseminate the knowledge acquired to the aquaculture sector, to enhance feed back acquisition,
3. Promote implementation of new husbandry methods, protocols and products developed by DIVERSIFY by the aquaculture industry and the seafood processors,
4. Enhance awareness of the diversification efforts of the project to the general public, with special attention to the food industry and consumer's organizations,
5. Promote investment opportunities making available the species feasibility studies to the industry,
6. Provide documented information to fish producers, fish processors and consumers on the new farmed aqua products from DIVERSIFY.

Summary of work reported in the previous Reporting Period (1-12 Mo):

According to Task 31.1 (Project website and brochure), Task 31.2 (Annual Coordination Meetings), Task 31.3 (Presentation of DIVERSIFY at the AQUA EUROPE meetings), and Task 31.7 (Dissemination to the food industry and consumers), the following Deliverables were reported in the Reporting Period (1-12 Mo):

- D31.1 Establishment of the Project website (www.diversifyfish.eu) including information on the objectives and main tasks of the project. Tabs: News, Summary, Partners, Species, Research Area and Dissemination.
- D31.2 Project logo and brochure
- D31.3 Publication of the first of two articles in Food Today
- D31.4 and D31.7 Production and release of audiovisual material
- D31.5 Collaboration agreement with food industry and consumer organization; linkage of websites.
- D31.6 Annual presentation of DIVERSIFY (Y1) at a relevant conference (Aqua Europe 2014).

Summary of work reported in the previous Reporting Period (13-30 Mo):

During the 2nd Reporting Period and according to Task 31.1 (Project website and brochure), Task 31.2 (Annual Coordination Meetings), Task 31.3 (Presentation of DIVERSIFY at the AQUA EUROPE meetings), and Task 31.7 (Dissemination to the food industry and consumers), the following Deliverables were reported in the previous Reporting Periods (13-30 Mo):

- D31.1 Establishment of the Project website (www.diversifyfish.eu) including information on the objectives and main tasks of the project and the adaptions of the web structure.
- D31.4, D31.7, D31.8, D31.12 and D31.13 Production and release of audiovisual material
- D31.9 Annual presentation of DIVERSIFY (Y2) at a relevant conference (Aqua Europe 2015)
- Presentations of diversify at the aqua Europe meetings (Diversification Sessions by the Species leaders (Y2).

Summary of progress towards objectives and details for each Task (31-48 Mo)

As it was mentioned in the previous report, the Deliverables corresponding to this Task have been already reported in the previous reporting periods (the full description of the work and results has been provided in ***Deliverable 31.1 Establishment of the Project website*** and ***Deliverable* 31.2 *Project logo and brochure).*** However, the web page of the project has continued to be updated, providing essential information on project activities. Moreover, the structure of the website has been adapted to the evolution of the project, incorporating a new page structure.

The website structure is as follows:

1. **News** this page remains as front page of the web, incorporating the recent activities of the project and **the latest video of the project** of 3 minutes duration compiling all the project information. This page is updated often, with the objective of providing up-to-date information to partners, but also to interested people visiting the web site, regarding the most recent activities of the project.

2. The general information of the project, i.e. Summary of the project, Partner description, Species and the different Research Areas are now under the page "**About Diversify**".

3. **Scientific articles (New page)**: this page includes the scientific publications in peer-reviewed journals as a result of the research done in the different WPs of the project (**Figure 31.1.1**). These publications are available online or by direct contact with the authors. At the time of the preparation of this report (December 2016), a total of 20 (twenty) scientific articles in ISI-Index journals have been published. These articles have been uploaded in the appropriate site of the ECAS site. More scientific results are expected to be published in the coming months, since several manuscripts are under preparation.

Dissemination: These pages keep being updated with all the dissemination activities done in the project, including articles in magazines and other type of popular publications. Magazine articles in the language of some project partners are also included here (Greek, Spanish, German, Hungarian…). There are two new sections incorporated in the Dissemination page:

- The section of the **Promotional Workshops (New)** of the project where the resume of the workshops are included (see Task 31.6). Two of the workshops have been already organized, the first one in Bremen Germany (complete description has been submitted as *Deliverable 31.16 Promotional workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) 1st Workshop)*) and the second one, in El Puerto de Santa Maria Cádiz- Spain (complete description has been submitted as *Deliverable 31.18 Promotional workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) (2nd Workshop)*. The information is uploaded in the web page: http://www.diversifyfish.eu/promotional-workshops.html

- The **Aquaculture Europe magazine (New)** publications on the DIVERSIFY research. There are already 7 articles about the project published in the magazine. Last issue (September 2017 issue) includes a special extended article on the project (http://www.diversifyfish.eu/aquaculture-europe-magazine.html).

Other sections included in the Dissemination page are:

- Articles in magazines and in the internet with project information in other languages of the consortium; latest update includes the publication of an article about the featured article on DIVERSIFY published in the magazine Aquaculture Europe, translated to Hungarian by Dr. Laslo Varadi (P.35, MASZ) from the Hungarian Association of Fish producers (**Figure 31.1.2**). Our P.37 EUFIC keeps the link to DIVERSIFY web page in their web, within the section EU initiatives, helping to drive traffic to the DIVERSIFY website (http://www.eufic.org/article/es/show/eu-initiatives/rid/diversify/).

- The project Newsletter is also included in this page: already five issues have been published and are available at the website www.diversifyfish.eu/newsletter.html ; the sixth one is in progress at the moment of writing this report.

- Presentations and posters: all the presentations presented at the Special Diversify Session during AE 2017 in Dubrovnik, Croatia and during the AE 2015 in Rotterdam, Netherlands, have been included in this section.

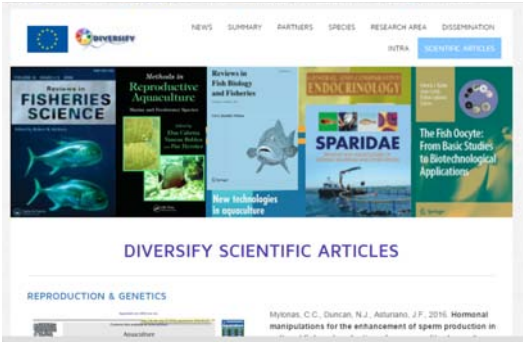


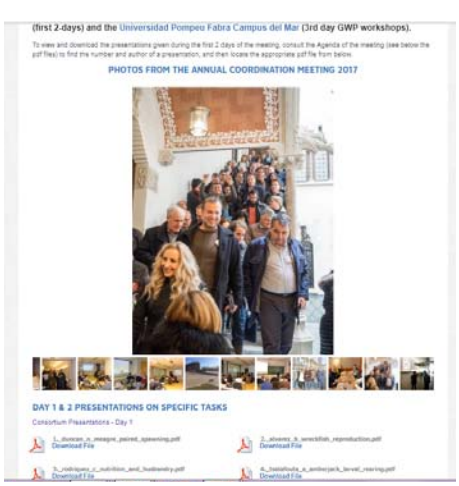
Figure 31.1.1. New page included in the web of DIVERSIFY, were all the scientific articles resulting from the project are being listed, with a copy of the first pages of the published article and a link to the corresponding author.

4. **Intranet:** with all the useful information for the partners with regard to specific information on meetings, documents, deliverables, etc…. This section is updated regularly including the documents and Deliverables produced within the project tasks. The page is very useful to provide specific information to the consortium, different official documents and other internal project documents. The page is updated with the incorporation of the new Deliverables and any other document of interest for the consortium. The website keeps being used extensively for the organization, agenda and logistics of the Annual Coordination Meetings (**Figure 31.1.3)**

5. **Species Workshops (New)**: this is a new page designed to present the information of the upcoming full-day seminars on "Know-how Transfer" of the state-of-the-art of the aquaculture for each of the six species of DIVERSIFY, which are planned for 2018 (**Figure 31.1.4)** (http://www.diversifyfish.eu/species-workshops.html).

6. **Meeting and activities**: public information on the organization of the annual coordination meetings of the project, as well as other relevant activities.

Figure 31.1.2. Desktop view with the Hungarian translation of the last article about DIVERSIFY published in Aquaculture Europe magazine (September, 2017).



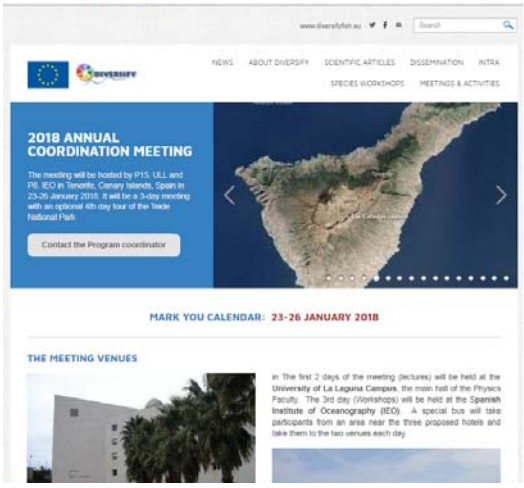


Figure 31.1.3. Right**:** Desktop captures of the DIVERSIFY page "Intranet- Meetings &Activities", including the information of the past ACM 2017 held in Barcelona, Spain, with the summary documents of the different sessions; left: the announcement of the next ACM 2018 to be held in Tenerife, Spain.

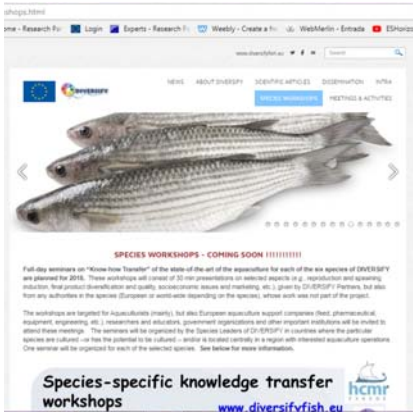


Figure 31.1.4. Desktop capture of the web page with the announcement of the Species Workshops to be organized during 2018 in different locations.

DIVERSIFY BROCHURE AND BOOKMARK

The project brochure and bookmark have been widely distributed with the occasion of several aquaculture conferences and events as it was included in the previous reporting documents. During the present reporting period, additional distribution of DIVERSIFY dissemination material has been carried out during the following events:

- AQUACULTURE EUROPE (AE 2016) conference (Edinburg, Scotland, UK), September, 2016.
- EUROTIER conference (Hannover, Germany), November 2016.
- ACM in Barcelona, Spain, 2017.
- Sectorial meeting FEDEPESCA, (Madrid, Spain), January 2017.
- Seafood Expo Global and Seafood Processing Global (Brussels, Belgium), April 2017.
- 1st Promotional Workshop DIVERSIFY, (Bremen, Germany) May, 2017
- 2nd Promotional Workshop DIVERSIFY, (El Puerto de Santa María, Cádiz, Spain), September 2017.
- CONEXMAR, International Frozen Seafood Exhibition ANFACO, (Vigo Spain) October, 2017.
- AQUACULTURE EUROPE (AE 2017) conference (Dubrovnik, Croatia), Special Diversify Session during the conference.

PRODUCTION AND RELEASE OF DOWNLOADABLE AUDIOVISUAL MATERIAL

All the videos of the project are included following a chronological order in the "News" page of the project website (http://www.diversifyfish.eu/). A summary of the videos from previous reporting period includes:

- February 2015, two videos: The first video presents the summary of the first year work of DIVERSIFY, including the state of the research at that moment of the project life (https://www.youtube.com/watch?v=hO67fbvMX3k). The second video shows a sampling of wreckfish done by researchers from the P15. University of La Laguna (Tenerife).
- May, 2015 a short video on the evaluation of the reproductive condition and induction of spawning of several broodstock specimens of greater amberjack born in captivity (F1) at the P8. Instituto Español de Oceanografia in Tenerife (Canary Island, Spain) https://www.youtube.com/watch?v=W1G9hAk4x3E
- July 2015 short video including an interview of the PC commenting on the progress and results of the project after the first 18 months. https://www.youtube.com/watch?v=SUba5STbFz8
- January 2016, a video compiling the presentations given during the Special Diversify Session at Aquaculture Europe 2015. https://www.youtube.com/watch?v=I_JTnaPskK8 .
- A methodological video with comprehensive explanations on motility of wreckfish sperm has been produced by Dr. Christian Fauvel (P14. IFREMER), presenting the application of the standardized method for activation and analysis of spermatozoa movement https://www.youtube.com/watch?v=taHt2_dYYbQ
- An additional video on the greater amberjack gill parasite *Zeuxapta seriolae*, elaborated by Dr. Pantelis Katharios from P1. HCMR has been uploaded in March 2016. The video documents the main characteristics of the parasite and its life cycle.
- In May 2016, a video summary of the ACM held in Nancy (France) was released (https://youtu.be/juk8_bOlm0I). The video includes interviews to the project Coordinator, Dr. C. Mylonas (P.1); to Mr. Alistair Lane, Executive Director of the European Aquaculture Society; to the DIVERSIFY Dissemination leader, Dr. Rocio Robles (P.18) and to Prof. Pascal Fontaine from University of Lorraine (France) (P.9), who was the hosting organization of the ACM 2016.

During the period June 2016 to November 2017, a video released in November 2016 documented the major advances on the three years of research with the DIVERSIFY fish species (https://youtu.be/uB1xqmih8aM).

Protocols for the control reproduction and broodstock management and larviculture optimized protocols for meagre, success on the reproduction control and larviculture of greater amberjack, improved larval feeding methodology for halibut and spawning induction protocol for grey mullet are documented in the video (**Figure 31.1.6**). Detailed description of this video has been submitted *as Deliverable 31.15: production and release of audiovisual material*.

Figure 31.1.6: Desktop capture of video compiling the major results of DIVERSIFY after 3 years of research.

In August 2017, a new video summary of the project has been finalized. It is a short video of 3 minutes in which the main aspects of the project as well as main research findings are very well summarized (**Figure 31.1.7**).

Click here to see the video: DIVERSIFY in 3 minutes

The video has had a considerable impact and plenty of positive reactions have been directly transmitted to the project Coordinator and to the Dissemination leader.

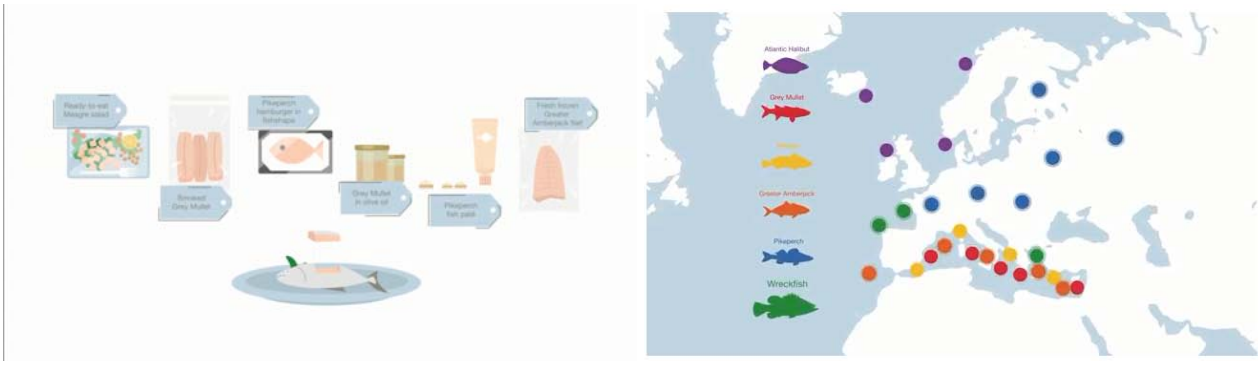


Figure 31.1.7: Desktop captures of the newest released video of DIVERSIFY showing the value added products developed within the project and evaluated by consumers (left) and the regional distribution of the research work per species (right)

ADDITIONAL DISSEMINATION ACTIVITIES

Several dissemination activities have been implemented to broadcast project activities to the scientific community and the aquaculture industry, as well as the general public (**Table 31.1.1**).

Table 31.1.1: Dissemination activities of DIVERSIFY during 2016-2017 uploaded on the "Dissemination activities" site of the SESAM application of the Participants Portal.

DIVERSIFY
KBBE2013
Dissemination articles

No	Date	Discipline	Work Package	Title	Type	Language
149	02/06/2016	All	All	Video summary of the annual coordination meeting of Diversify held at the University of Lorraine (Nancy, France)	Video	English
150	06/06/2016	All	All	THE EFFECTS OF DIETARY INCLUSIONS OF VITAMIN D3 ASSOCIATED TO CYP27A1, ANTIOXIDANT ENZYMES AND NON-INFECTIOUS SYSTEMIC GRANULOMATOSIS IN MEAGRE (Argyrosomus regius).	Oral presentation	English
151	06/06/2016	All	All	The importance of dietary content of vitamins k and d for meagre (Argyrosomus regius) larvae	Oral presentation	English
152	06/06/2016	All	All	Dietary combinations of vitamin k, e and c affect the incidence of systemic granulometrosis in ongrowing meagre (Argyrosomus regius)	Oral presentation	English
153	20/06/2016	All	All	Characterising the mucosal immune response in the Greater Amberjack (S. dumerili)	Poster	English
154	27/06/2016	All	All	Effect of temperature on growth performance and immunological parameters of greater amberjack Seriola dumerili juveniles	Poster	English
155	29/06/2016	All	All	Diversify project seeking to solve bottlenecks and expand EU aquaculture	Popular press	English
156	29/06/2016	All	All	Effect of GnRHa therapy on spawning performance of Atlantic halibut (Hippoglossus hippoglossus)	Poster	English
157	01/09/2016	All	All	Advances in Atlantic halibut (Hippoglossus hippoglossus) research: the Diversify project	Popular press	English
158	21/09/2016	All	All	DIVERSIFY	Flyers	English
159	21/09/2016	All	All	Divesification of fish species and products in European aquaculture "DIVERSIFY"	Oral presentation	English
160	21/09/2016	All	All	Early weaning in meagre A. regius: effects on growth, survival, digestion and skeletal deformation	Poster	English
161	21/09/2016	All	All	Description of the wreckfish (*P. americanu* s) reproductive cycle in captivity	Poster	English
162	21/09/2016	All	All	Influence of broodstock nutrition of Wreckfish (P. americanus) on the oocytes fatty acid composition	Poster	English
163	21/09/2016	All	All	First experiences of wreckfish (*P. americanus*) larval husbandry in the Atlantic and East Mediterranean	Poster	English
164	22/09/2016	All	All	Exploring the biological and socioeconomic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry	Oral presentation	English
165	22/09/2016	All	All	Effect of background color and expression of genes related to the GH/IGF axis at early development of Greater Amberjack (S. dumerili)	Oral presentation	English

166	22/09/2016	All	All	Evaluation of common husbandry practices in Greater Amberjack (*S. dumeril* i)	Oral presentation	English
167	22/09/2016	All	All	The stress response of Greater Amberjack (S. dumerili)	Poster	English
168	22/09/2016	All	All	Influence of broodstock nutrition of Wreckfish (*P. americanus*) on the oocytes fatty acid composition	Poster	English
169	22/09/2016	All	All	Ontogeny of Greater Amberjack digestive system	Poster	English
170	22/09/2016	All	All	Combined effect of LC-PUFA- rich lipids and carotenoids in rotifers enrichment products for Greater Amberjack larvae	Poster	English
171	22/09/2016	All	All	Exploring the biological and socioeconomic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry: major result after two years of research	Oral presentation	English
172	22/09/2016	All	All	DIVERSIFY New species for European Aquaculture	Flyers	English
173	22/09/2016	All	All	Solving bottlenecks in commercial production of Atlantic halibut- The Diversify project	Oral presentation	Norwegian
174	22/09/2016	All	All	Impact of environmental attributes on consumer perceptions of aquaculture products in the UK	Oral presentation	English
175	22/09/2016	All	All	The role of involvement and innovativeness on consumer perceived value of new aquaculture products	Oral presentation	English
176	22/09/2016	All	All	Comparison between the quality of natural and induced spawns, using GnRHa injections and implants, of the greater amberjack (Seriola dumerili) broodstock kept in captivity	Poster	English
177	22/09/2016	All	All	Effect of dietary vitamin c and e in larval performance and incidence of bone animalies in meagre (Argyrosomus regius)	Oral presentation	English
178	22/09/2016	All	All	Influence of dietary combinations of vitamin e, c and k in the development of systemic granulomatosis in meagre (Argyrosomus regius)	Oral presentation	English
179	22/09/2016	All	All	Determination of vitamin k dietary requirements in meagre larvae (Argyrosomus regius)	Oral presentation	English
180	22/09/2016	All	All	Effect of stocking density on greater amberjack (Seriola dumerili) larval performance	Oral presentation	English

181	22/09/2016	All	All	Feeding rates for greater amberjack Seriola dumerili; effects on growth, feed utilization and welfare indicators	Poster	English
182	22/09/2016	All	All	Effects of broodstock nutrition on larviculture of greater amberjack (Seriola dumerili, Risso 1810)	Oral presentation	English
183	28/09/2016	All	All	Kveite mest interessant som oppdrettsart i fremtiden	Interwiews	Norwegian
184	15/11/2016	All	All	Diversifying aquaculture to develop new markets- Building a solid foundation for Europe	Popular press	English
185	17/11/2016	All	All	DIVERSIFY New species for European Aquaculture at Eurotier 2016	Oral presentation	English
186	30/11/2016	All	All	We take a look at the EU project exploring the biological and socio-economic potential of new/emerging candidate finfish species for the expansion of the European aquaculture industry	Popular press	English
187	30/11/2016	All	All	DIVERSIFY_ New aquaculture Species-Consumer´s perception of new fish products	Popular press	English
188	02/12/2016	All	All	DIVERSIFY results after 2,5 years of work	Video	English
189	02/12/2016	All	All	Control de la reproducción de la corvina (Argyrosomus regius)	Oral presentation	Spanish
190	08/01/2017	All	All	DIVERSIFY Article in aqua feed International translated and presented to APROMAR members	Web	English
191	08/01/2017	All	All	DIVERSIFY Article in aqua feed International translated and presented to BVFi members	Web	English
192	08/01/2017	All	All	DIVERSIFY Article in aqua feed International translated and presented to MASZ members	Web	English
193	12/01/2017	All	All	Building a solid foundation for Europe´s aquaculture industry	Popular press	English
194	24/01/2017	All	All	DIVERSIFY New species for European Aquaculture	Flyers	English
195	01/03/2017	All	All	Investigadores avanzan en la domseticación de la cherna (Polyprion americanus)	Popular press	Spanish
196	22/02/2017	All	All	Commercial farming of Atlantic wreckfish closer to reality	Press release	English
197	28/03/2017	All	All	Annual meeting of COST Action Cephalopod Science from Biology to Welfare	Flyers	English
198	30/03/2017	All	All	Advnces in wreckfish (Polyprion americanus)research: the Diversify project. AE Magazine, March 2017	Popular press	English
199	05/04/2017	All	All	Investigador danésDr. Ivar Lund visita la Universidad de la Laguna y presenta los avances en técnicas de acuicultura más limpias	Popular press	Spanish
200	05/04/2017	All	All	Entrevista: Investigador danésDr. Ivar Lund visita la Universidad de la Laguna y presenta los avances en técnicas de acuicultura más limpias	Popular press	Spanish

201	20/04/2017	All	All	DIVERSIFY: a program of the EU for the Evaluation of the biological and socioeconomic potential of new/emerging species for the enhancement of the European Aquaculture	Popular press	English
202	24/05/2017	All	All	1st Promotional Workshop- Bremen Germany	Workshop	English
203	27/06/2017	All	All	DIVERSIFY at WAS meeting Cape Town, South Africa	Oral presentation	English
204	16/08/2017	All	All	Video: DIVERSIFY in 3 minutes	Video	English
205	04/09/2017	All	All	Effects of broodstock on nutrition larviculture of greateramberjack during the first 15 days of life	poster	English
206	04/09/2017	All	All	Recent advances in greater amberjack culture in aquaculture research group (GIA)	Oral presentation	English
207	04/09/2017	All	All	Effects of broodstock nutrition on greater amberjack egg quality	poster	English
208	05/09/2017	All	All	Importance of dietary phosphoglycerides andHUFa levels for pikeperh	Oral presentation	English
209	28/09/2017	All	All	2nd Promotional Workshop,El Puerto de Santa Maria-Cádiz,Spain	Workshop	Spanish/English
210	03/10/2017	All	All	DIVERSIFY at CONXEMAR, Vigo Spain	Exhibition	Spanish
211	17/10/2017	All	All	Evaluation of wreckfish growth in Galicia. Croatia 2017	poster	English
212	17/10/2017	All	All	Effect of dietary ffatty acids on spawn quality in greater amberjack broodstock. Croatia 2017	poster	English
213	17/10/2017	All	All	Preliminary studies on the relationship of temperature and tie of digestion on enzymatic activity and growth of seriola	poster	English
214	17/10/2017	All	All	Requirements for n-3 HUFA of meagre fingerlings	Oral presentation	English
215	17/10/2017	All	All	Dietary use of prebiotics in greater amberjack juveniles	Oral presentation	English
216	18/10/2017	All	All	Broodstock management and spawning induction of greater amberjack reared in tanks and sea cages	Oral presentation	English
217	18/10/2017	All	All	DIVERSIFY: exploring the biological and socioeconomic potential of new/emerging species for the enhancement of the European Aquaculture industry	Oral presentation	English
218	18/10/2017	All	All	spawning kinetics of greater amberjack in response to multiple GnRHa injections or implants	Oral presentation	English
219	18/10/2017	All	All	Description of the endocrine reproductive cycle of the wreckfish Polyprion americanus in captivity	Oral presentation	English
220	18/10/2017	All	All	The effect of different stimuli on meagre Argyrosomus regius feeding behavior	Oral presentation	English

221	18/10/2017	All	All	Construction of the first genetic linkage map in meagre (Argyrosomus regius) and identification of growth-related loci to be used in marker assisted selection programs	Oral presentation	English
222	18/10/2017	All	All	Recent advances in the study of systemic granulomatosis in meagre (Argyrosomus regius)	Oral presentation	English
223	18/10/2017	All	All	Progress in the wreckfish intensive culture. New candidate species for aquaculture	Oral presentation	English
224	18/10/2017	All	All	Reproductive development in wild and captive-reared greater amberjack Seriola dumerili (Risso, 1810)	Oral presentation	English
225	18/10/2017	All	All	Gamete quality and management for in vitro fertilisation in meagre (Argyrosomus regius) to facilitate the implementation of genetic breeding programs	Oral presentation	English
226	18/10/2017	All	All	Light environment affecting endocrine and immune circadian rhythms in pikeperch (Sander lucioperca)	Oral presentation	English
227	18/10/2017	All	All	Fish for the future: what could influence European consumer choice of new aquaculture products? Evidence from an experimental study with low and medium processed products	Oral presentation	English
228	18/10/2017	All	All	Requirements for n-3 hufa of meagre (Argyrosomus regius, Asso, 1801) fingerlings	Oral presentation	English
229	18/10/2017	All	All	Improvement of rearing conditions for juvenile pikeperch (Sander lucioperca) production in RAS	Oral presentation	English
230	18/10/2017	All	All	Lysine optimization of a diet with low fish meal inclusion for greater amberjack (Seriola dumerili, Risso 1810)	Oral presentation	English
231	18/10/2017	All	All	Designing weaning diets based on the ontogeny of digestive tract enzyme activity during the carnivorous-omnivorous transition in grey mullet Mugil cephalus juveniles.	Oral presentation	English
232	18/10/2017	All	All	Improvement of reproductive performance of F1 generation greater amberjack (Seriola dumerili) with succesive implants of gonadotropin-releasing hormone agonist (GnRHa)	poster	English
233	18/10/2017	All	All	Effects of stocking density on growth performance and health of greater amberjack (Seriola dumerili) juveniles.	poster	English
234	18/10/2017	All	All	Hematological and plasma biochemical parameters in F1 generation greater amberjack (Seriola dumerili) during spawning induction with GnRHa delivery systems	poster	English
235	18/10/2017	All	All	Effect of male rotation on induced pair spawning of meagre Argyrosomus regius	poster	English

236	18/10/2017	All	All	Population genetic structure of greater amberjack (Seriola dumerili) in the Mediterranean Sea and eastern Atlantic Ocean	poster	English
237	18/10/2017	All	All	Effect of different ratios of DHA, EPA and ARA on ontogeny of digestive activites and larval development of pikeperch larvae (Sander lucioperca)	poster	English
238	18/10/2017	All	All	Proximate, fatty acids and volatile compounds composition of reared vs. wild greater amberjack (Seriola dumerili) as affected by fish size.	poster	English
239	18/10/2017	All	All	Combined effect of immune-stimulant enrichment products and feeding frequency on greater amberjack larval performance.	poster	English
240	18/10/2017	All	All	Recirculation (RAS) vs. flow-through (FT) systems during yolk sac and first feeding stages: effects of rearing system bacteriology, and survival, quality and growth of Atlantic halibut, Hippoglossus hippoglossus larvae	Oral presentation	English
241	18/10/2017	All	All	Effect of background color and expression of genes related to the GH/IGF growth axis at early development of greater amberjack (Seriola dumerili)	Oral presentation	English
242	28/10/2017	All	All	Presentation of the activities of HCMR to a delegation from the 1st Institute of Oceanography of China	Flyers	English
243	20/09/2017	All	All	New Species for Eu Aquaculture: What new Methods have been Developed?	Popular press	English
244	04/10/2017	All	All	Calidad de los gametos y su gestión en la fertilización artificial de la corvina (Argyrosomus regius) para facilitar la realización de programas de mejora genética	Oral presentation	Spanish
245	17/11/2016	All	All	"DIVERSIFY - New species meet markets" - Host of discussion at fair "Eurotier	Oral presentation	English
246	24/05/2017	All	All	First promotional workshop of DIVERSIFY	Oral presentation	English
247	25/04/2017	All	All	Seafood Expo Brussels 2017 Distribution of flyers and bookmarks	Flyers	English
248	17/11/2016	All	All	EUROTIER- AQUACULTURE FORUM	Flyers	English
249	24/05/2017	All	All	First promotional workshop of DIVERSIFY	Flyers	English
250	28/09/2017	All	All	2nd Promotional Workshop of DIVERSIFY	Flyers	English

PRESS RELEASES

In this section it has been included the information on the visit of Dr. Ivar Lund (P.21, DTU)to the University of La Laguna, (Tenerife, Spain) where he offered an interview to the media (**Figure 31.1.8**) and presented together with Dr. Covadonga Rodriguez (P.15, ULL) the advances on clean aquaculture production techniques.

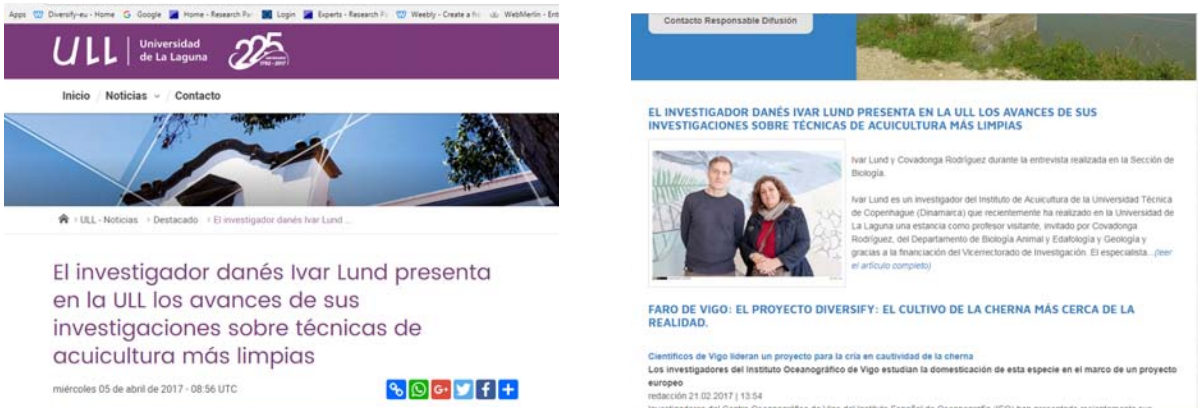
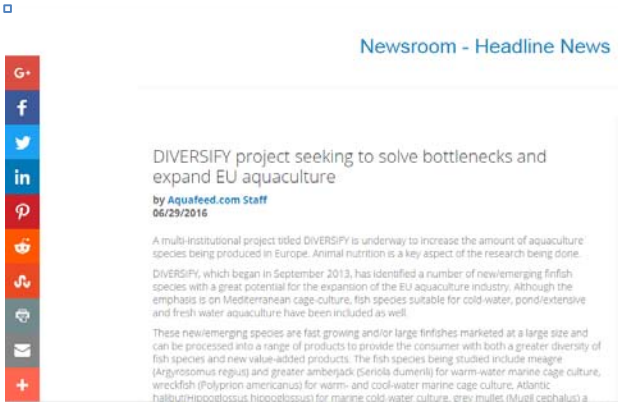


Figure 31.1.8: Desktop capture of the press release about the visit of Dr. Ivar Lund at the university of La Laguna.

Wreckfish research has been also reported in the digital newspaper Faro de Vigo, in which researchers from the Instituto Español de Oceanografia (P8. IEO) explain the program for the domestication of the species and their activities within the Area of Reproduction and Genetics of wreckfish (**Figure 31.1.9)**.

Figure 31.1.8: Desktop capture of the press release about DIVERSIFY project in the Faro de Vigo.

A press note on the project has been also published in Aquafeed.com (**Figure 31.1.9)**
http://www.aquafeed.com/news/headline-news-article/6721/DIVERSIFY-project-seeking-to-solve-bottlenecks-and-expand-EU-aquaculture/?utm_source=Aquafeed+English+Newsletter&utm_campaign=a0a97b4216-af-nl-06-30-16&utm_medium=email&utm_term=0_0e7f7c0399-a0a97b4216-2429


Newsroom - Headline News

DIVERSIFY project seeking to solve bottlenecks and expand EU aquaculture

by Aquafeed.com Staff
06/29/2016

A multi-institutional project titled DIVERSIFY is underway to increase the amount of aquaculture species being produced in Europe. Animal nutrition is a key aspect of the research being done.

DIVERSIFY, which began in September 2013, has identified a number of new/emerging finfish species with a great potential for the expansion of the EU aquaculture industry. Although the emphasis is on Mediterranean cage-culture, fish species suitable for cold-water, pond/extensive and fresh water aquaculture have been included as well.

These new/emerging species are fast growing and/or large finfishes marketed at a large size and can be processed into a range of products to provide the consumer with both a greater diversity of fish species and new value-added products. The fish species being studied include meagre (Argyrosomus regius) and greater amberjack (Seriola dumerili) for warm-water marine cage culture, wreckfish (Polyprion americanus) for warm- and cool-water marine cage culture, Atlantic halibut(Hippoglossus hippoglossus) for marine cold-water culture, grey mullet (Mugil cephalus) a

Figure 31.1.9. Press note published in the www.aquafeed.com web.

DIVERSIFY is also present in the social media, via a twitter account and facebook account. Twitter account counts with 333 followers (December 2017).

ARTICLES

Beginning of 2017, a dedicated article about DIVERSIFY has been published in the magazine Impact Science entitled "Building a solid foundation for Europe´s Aquaculture industry" (**Figure 31.1.10**). In this article the project Coordinator and the Dissemination leader offer a complete view of the project objectives and highlighted the progress made at the time of publication. The full article is included in the web of the project and can be consulted at
http://www.diversifyfish.eu/uploads/1/4/2/0/14206280/diversify_impact__publication_p14-16_02.pdf


Impact Objectives

- Build on recent/current national initiatives for species diversification in aquaculture in order to overcome the documented bottlenecks in the production of these species
- Support the diversification of the aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets

Building a solid foundation for Europe's aquaculture industry

Drs Constantinos C. Mylonas and Rocio Robles are the Coordinator and Dissemination Leader, respectively, of a five-year project that seeks to expand the EU's aquaculture sector through diversification of farmed fish species. Below, they discuss the species investigated and highlight the progress they have made so far

Figure 31.1.10. Impact Science publication on DIVERSIFY project.

Moreover, seven articles have been published by the European Aquaculture Society. The collaboration established with the editor includes the publication of one article about DIVERSIFY each semester during the project duration. After the publication of the initial article on the general objectives and program of DIVERSIFY, the subsequent articles are focusing on each of the project species. Each Species Leader has been responsible for the preparation of the article.

At the moment of writing this report, 5 of the 6 DIVERSIFY species have been documented in the corresponding articles in the Aquaculture Europe magazine. Last issue of the publication (September, 2017) includes an extended article with the updates of the research results of the project including socioeconomics. **(Figure 31.1.11).** In 2018, the feature article will be devoted to grey mullet.

Figure 31.1.11. Publications of DIVERSIFY project in the Aquaculture Europe magazine.

During 2017, three articles on DIVERSIFY have been published in the magazine Aquafeed International (**Figure 31.1.12**). The first one was a feature article published in December 2016 and in the same issue, there is a second article documenting the consumers' perception of the new fish products developed within the project.

The third article, published in April 2017, is a compilation of the interviews made by the magazine editor to the project Coordinator Dr. C. Mylonas and to the Dissemination leader Rocio Robles during the ACM held in Barcelona in January 2017 (**Figure 31.1.13**.).

Figure 31.1.12. Front page of the International Aquafeed magazine issue including the DIVERSIFY extended article and the article documenting the consumers' perception of new fish products.

Figure 31.1.13. International Aquafeed article published in April 2017 documenting the last ACM of DIVERSIFY held in Barcelona, Spain (January, 2017).

WORKSHOPS

In November 2016, DIVERSIFY partners Matthias Keller (P.34, BVFi), Javier Ojeda (P.12, APROMAR) and the Dissemination leader, Rocio Robles (P.18, CTAQUA) participated in the Eurotier Fair in Hannover Germany, in the section "Aquaculture Forum" organized by the DLG (German Agricultural Society) . Eurotier is a well know fair in Europe and it attracted more than 2531 exhibitors in 2016 .

Aquaculture section concentrated on the world's fastest-growing food sector (Germany is only 12 percent self-sufficient on seafood, leaving huge room for growth in the sector). Neutral experts were on-hand to provide independent advice. The DLG Aquaculture Forum was part of the InfoCenter and had almost achieved cult status within the sector. During four days, it offered presentations from more than 40 industry experts from Germany and the rest of the world on relevant topics and experiences. Furthermore, visitors could gain valuable perspective and advice by talking to independent experts from leading professional

institutions. The "growth in the water" showcase also highlighted the information on a diverse range of topics from fish, mussels and crabs to microalgae and macroalgae.

During the event of 2016, the Dissemination leader gave a presentation on DIVERSIFY project and the three partners CTAQUA, APROMAR and BVFi participated later on in the Aquaculture discussion forum organized after the different presentations of the event. **(Figure 31.1.14**). The need for diversification and intensification of the aquaculture industry together with the must to provide information to the consumers on aquaculture products were the main points of the debate.

Figure 31.1.14. Presentation of DIVERSIFY and participants of the Aquaculture Forum in the Eurotier fair and expo last November 2016.

In April 2017, the Dissemination leader Rocio Robles attended the Seafood Expo Global held in Brussels, Belgium (**Figure 31.1.15.).** DIVERSIFY dissemination material (brochures and bookmarks) was distributed and displayed in the EU stand. The day of the EUMOFA report presentation, dissemination material was distributed to the attendees of the event. Information on the project was provided to the visitors and some interesting discussions on the use of the marine resources for fish feeds were generated with some of them. Full description of the event and participation has been submitted as *D31.21. Presentation of DIVERSIFY at the Seafood Expo.*

Figure 31.1.15. Left: The Dissemination leader, Rocio Robles, at the EU stand distributing the dissemination material of DIVERSIFY. Right: folder and bookmarks on the shelfs of the EU stand at Seafood Expo organized in Brussels (April 24-26, 2017).

DIVERSIFY was also present at the International Frozen Seafood Exhibition (CONXEMAR), held in Vigo (Spain) from 3rd to 5th October 2017. This exhibition is a world reference and meeting point for the entire fish and seafood processing sector: wholesalers, importers, exporters, transformers, manufacturers, distributors, cold storages, machinery, and auxiliary industry, with a clear focus on the market of frozen fish and seafood. In this 2017 edition, CONXEMAR received 31.370 visitors from 104 countries.

ANFACO-CECOPESCA participated in CONXEMAR to promote the products and services offered by their associated companies. An additional task this year was the dissemination of DIVERSIFY, by the exhibition and distribution of promotional leaflets to visitors (**Figure 31.1.16**).

Figure 31.1.16. DIVERSIFY folders on the stand of ANFACO (P36) at the Conxemar fair in October 2017.

Task 31.2 Annual Coordination Meetings (led by HCMR, Constantinos Mylonas).

The full description of the work and results of the ACM 2017 has been submitted as ***Deliverable D1.9 Annual Coordination Meeting for Y4***. A brief presentation is also provided here.

The ACMs were planned in the DOW to consist of 2-days of open presentations and 1 for consortium activities. However, the previous ACM 2014 (Bari, Italy) and ACM 2016 (Nancy, France) contained only 1 open day and 2 days reserved for consortium activities. This was considered necessary because of the large number of Work Packages in the project, and the need for as much time as possible to be allocated to the discussion of obtained results and future planning of the work, as well as the preparation of the 1st and 2nd Periodic Reports (Mo 12 and Mo 36). However, as this time there was no periodic report due, until a year later (January 2018), we returned to the originally planned format of having 2-days of open presentations and 1 for consortium activities and planning of upcoming activities. This decision was already taken after the previous ACM (2016) and was reported in ***Deliverable 1.6 Annual Coordination Meeting for Y3***. This format allowed all Partners to have a detailed view of the progress of the project after 3 years and will disseminate the information to a larger invited guest audience.

There were no other major deviations from the DOW at this time. Some delays in the uploading of the Deliverables have been discussed (and mentioned in the minutes of the GWP Workshops), but they are not considered major in kind. Also, there are a number of expected delays in some of the upcoming deliverables, but so far there is no expectation of any Deliverables not been completed within the lifespan of the project. These expected delays have been mentioned within the minutes of the specific GWP workshops reported in the previous pages.

The ACM 2017 was hosted by Dr. Alicia Estevez of the Instituto de Recerca y Tecnologia Agronomica (P3. IRTA) and was held at two venues between 17-19 January 2017. The task-specific presentations during Days 1 and 2 took place at Palau Macaya (**Fig. 31.X**). The Group Work Package (GWP) workshops took place at the Campus Del Mar of the University Pompeu Fabra. In addition, a half day meeting took place at

the Hotel Ayre Rosellon on Friday 20 January 2017, for the participants of WP 30 Business model and marketing strategy development. The 3-day meeting was attended by 85 persons: 78 coming from the DIVERSIFY consortium and 8 invited guests from outside the consortium.

As for all previous ACMs, information regarding the meeting was uploaded continually on the project's web site (http://www.diversifyfish.eu/2017-annual-coordination-meeting-jan.html) to ensure that all participants had access to the most updated information. The Agenda (**Tables 1 and 2**) was developed with assistance from GWP leaders and consisted of:

(a) DAY 1 and 2: a common session for all participants (including invited guests) presenting Task-specific presentations from various WPs, and presentations from invited guests,
(b) DAY 2: a presentation of the WP 31 Dissemination presenting the dissemination activities of the consortium, and organizing the preparation of Deliverables as well as of manuscripts for scientific articles, and
(c) DAY 3: a common session dealing with Dissemination, Scientific and Financial Reporting, and Management.
(d) In addition a brief meeting of WP 30 meeting was held on Friday 20 January

Figure 31.2.1. A group photo of some of the participants of DIVERSIFY ACM 2017 at the beautiful staircase of Palau Macaya, Barcelona, Spain.

Table 31.2.1 Agenda of DAY 1 of the Annual Coordination Meeting 2017, which took place on the 16-19 January 2017, at the Palau Macaya, Barcelona, Spain.

DAY 1		17-Iαv	Tuesday (Open Day presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration		Pick up badges
9.00	9.30		Welcome-Logistics	Alicia Estevez & CC Mylonas	
9.30	9.50		Welcome	Dr Sergi Tudela Casanovas	Director of Fisheries, Cataluna
9.50	10.10	1	Induced spawning of paired meagre with male rotation	Duncan, Neil	IRTA
10.10	10.30	2	Wreckfish reproduction status in Spain	Alvarez, Blanca	IEO
10.30	10.50	3	Some approaches to improve the nutrition and husbandry of DIVERSIFY´s target species. A U La Laguna collaborative contribution	Rodriquez, Covadonga	ULL
10.50	11.30		Coffee		
11.30	11.50	4	Effect of background color and photophase on performance of larval greater amberjack and expression of genes related to the GH/IGF axis	Tsalafouta, Aleka	HCMR
11.50	12.10	5	Prospects for probiotics with Atlantic halibut larve	Berg, Øivind	IMR
12.10	12.30	6	The effect of algal turbidity on larval performance and the ontogeny of digestive tract functionality in grey mullet	Koven, Bill	IOLR
12.30	12.50	7	Wreckfish ontogeny of the major organs related to feeding and digestion	Papadakis, Ioannis	HCMR
12.50	13.10	8	COLUMBUS Project – Knowledge Transfer for Blue Growth: Aquaculture knowledge outputs and case studie	Christofilogiannis, Panos	AQUARK (Invited)
13.10	13.30	9	Physical prototypes of new producs from the selected DIVERSIFY species	Bou, Ricard and Robles, Rocio	IRTA/CTAQUA
13.30	15.00		Lunch		
15.00	15.20	10	Epigenetics in aquaculture	Piferrer, Francesc	ICM (Invited)
15.20	15.40	11	How can CFeed copepods help bring new marine species to the table	Remman, Tore	C-Feed (Invited)
15.40	16.00	12	Results on mullet grow out in farm conditions: a multi-partner trial	Robles, Rocio	CTAQUA
16.00	16.20	13	Parasitic infections in greater amberjack in Greece	Katharios, Pantelis	HCMR
16.20	16.40	14	Construction of a genetic linkage map in meagre and identification of genetic markers related to growth for use in marker-assisted breeding programs through QTL mapping	Tsigenopoulos, Costas	HCMR
16.40	17.00	15	Consumer sensory perceptions of the selected new products from DIVERSIFY species	Guerrero, Lluis	IRTA
17.00	17.30		Coffee		
17.30	17.50	16	What do we know about the immune system of meagre and amberjack?	Milne, Douglas	UNIABD
17.50	18.10	17	Behavioral analysis of intra-cohort cannibalism in young pikeperch	Colchen, Tatiana	UL
18.10	18.30	18	Wreckfish larval rearing trials	Vilar, Antonio	MC2
18.30	18.50	19	Feeding pattern for greater amberjack: effects on growth, feed utilization and welfare	Montero, Daniel	FCPCT
20.00			Dinner at Ayre Rosellon Hotel (consortium dinner)		

Table 31.2.2. Agenda of DAY 2 of the Annual Coordination Meeting 2017, which took place on the 16-19 January 2017, at the Palau Macaya, Barcelona, Spain.

DIVERSIFY					
7FP-KBBE-2013-603121					
Meeting Agenda			2017 Annual Coordination Meeting	Barcelona 17-19 January 2017	Palau Macaya
DAY 2		18-Ιαν	Wednesday (Open Day presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration		Pick up badges
9.00	9.20	1	Protocol for the strip spawning of meagre females and in vitro fertilization	Ramos, Sandra	IRTA
9.20	9.40	2	Spawning kinetics of greater amberjack in response to multiple GnRHa injections or implants	Fakriadis, Ioannis	HCMR
9.40	10.00	3	Effects of phosphoglycerides and HUFA levels on ontogenetic development and performance of pikeperch larvae	Lund, Ivar	DTU
10.00	10.20	4	Sensory characterization of DIVERSIFY species	Grigorakis, Kriton	HCMR
10.20	10.40	5	Influence of dietary combinations of vitamin e, c and k in the development of systemic granulomatosis in meagre	Montero, Daniel	FCPCT
10.40	11.00	6	Systemic granulomatosis in meagre	Katharios, Pantelis	HCMR
11.00	11.30		Coffee		
11.30	11.50	7	Meagre behaviour and response to feeding training stimuli	Papadakis, Ioannis	HCMR
11.50	12.10	8	The effect of cage depth in the performance of meagre	Tsalafouta, Aleka	HCMR
12.10	12.30	9	Experimental consumer test of the new products from DIVERSIFY	Krystallis, Thanassis	HRH/AU
12.30	12.50	10	Spermatogenesis and sperm characteristics in captive greater amberjack	Zupa, Rosa & Fauvel, Christian	UNIBA/IFREMER
12.50	13.10	11	Why I have come to hate meagre and why amberjack is a jinxed species: 25 years of feelings & experiences from health diagnostics	Padros, Sito	Uni Autonoma Barcelona (invited guest)
13.10	15.00		Lunch		
15.00	15.20	12	Launching the new DIVERSIFY products: business models, market tests and market diffusion	Nijssen, Ed and vd Borgh, Michel	TU/e
15.20	15.40	13	Comparison of programmed and auto-demand type feeding in tanks	Duncan, Neil	IRTA
15.40	16.00	14	Multifactorial nutrition experiment in pikeperch	Kestemont, Patrick	FUNDP
16.00	16.20	15	Maturation and spawning induction of grey mullet	Rosenfeld, Hanna	IOLR
16.20	16.40	16	Atlantic halibut larval nutrition and drivers of asymmetric pigmentation and eye migration in flounders	Hamre, Kristin	NIFES
16.40	17.30		Coffee		
17.30	17.50	17	Induction of gonadal maturation in teleosts by recombinant gonadotropins	Gimenes, Ignacio	Rara Avis Biotech (invited guest)
17.50	18.10	18	Nodavirus in Atlantic halibut and possible vaccine strategies	Patel, Sonal	IMR
18.10	18.30		Dissemination activities, articles and uploading on ECAS system - Rocio Robles		
18.30	18.50		Dissemination activities, articles and uploading on ECAS system - Rocio Robles		

Task 31.3 Presentation of DIVERSIFY at the AQUA EUROPE meetings (led by HCMR, Constantinos Mylonas).

During this reporting period, two presentations have been made in the Aquaculture Europe conferences (2016 and 2017), organized by the European Aquaculture Society.

The AQUACULTURE EUROPE 2016 conference was held between 20-23 September 2016 in Edinburgh, U.K (**Fig. 31.3.1**). This year the WP 31 Dissemination leader Dr Rocio Robles gave a presentation in the Special Session "Diversification in finfish production". The session was chaired by Dr. Rocio Robles, who gave the first presentation focused on the results of the project during the first 30 months of the project.

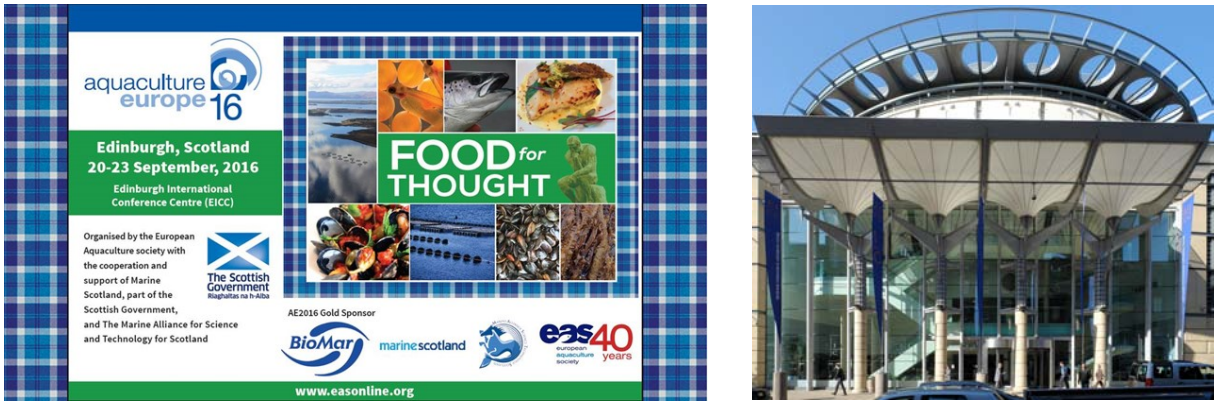


Figure 31.3.1. The announcement poster of the AQUACULTURE EUROPE 2016 (left) organized year by the European Aquaculture Society, and a view of Edinburgh International Conference Center (right).

The session "Diversification in finfish production" opened with a summary presentation for DIVERSIFY, given by the WP Dissemination leader of the project. The session lasted for the whole morning time schedule (10:30 to 12:50). The first presentation was given by the Dissemination leader of DIVERSIFY (**Fig. 31.3.2**) and an estimated 80 attendees were present in the room. Due to the fact that the second presentation was a not-show-up, the first speaker had some minutes extra and more time for questions from the audience. Some of the major achievements of the project up-to-date, in the six different scientific disciplines after two years of research were presented. In **Reproduction and genetics**, it was highlighted the successful spawning obtained with greater amberjack, Atlantic halibut, wreckfish (*Polyprion americanus*) and grey mullet (*Mugil cephalus*). A major breakthrough has been the production of 50 kg of greater amberjack eggs that have been distributed to 7 commercial hatcheries in Greece and Cyprus, as well as the production of 150.000 juveniles that have been distributed to 5 commercial grow out sites in Greece. With regard to the Research Area of **Nutrition**, main focus was the development of live feed enrichment protocols and weaning diets for meagre and greater amberjack larviculture and the importance of taurine inclusion in larval diets for grey mullet. A description of the biochemical composition and nutrient content of wild wreckfish filet and the gonads were reported. In the area of **Larval husbandry**, major achievements have been the first larval rearing studies on wreckfish (Spain and Greece) and the study of the effect of environmental factors on pikeperch larviculture in RAS. With regard to **Grow out and husbandry**, very promising results have been achieved on meagre feeding behaviour in response to physical/optical stimuli. Production of VNN capsid protein for vaccine development in Atlantic halibut and identification of important parasites in greater amberjack are some of the most relevant results from the **Fish health** research area. Concerning **Socioeconomic** results, clear consumer segmentation in regards to their attitude towards new fish products from the DIVERSIFY species has been established. A list of more than 40 different product ideas has been elaborated and 12 of them have been evaluated in terms of production feasibility and food safety. Six of those ideas have been produced and tested with consumers in five different countries.

Figure 31.3.2. Representative slides of the summary presentation of DIVERSIFY during the session "Diversification in finfish production" at AQUACULTURE EUROPE 2016, presented by Rocio Robles (P18. CTAQUA).

In the same conference, the Project Coordinator (PC) participated in the "EU Session: Research and Innovation supporting European Aquaculture". Dr. Mylonas presented a summary of the major achievements of the project. The session was well attended and the PC summarized the most relevant results of DIVERSIFY and the main breakthrough in the culture of greater amberjack last summer. The presentation begun with an introduction of the world aquaculture status, the current seafood consumption in the EU and the underlining reasons for the implementation of this project **(Fig. 31.3.3).** Then, the presentation explained the justification of the project's species selection. Then, there was a brief description of the bottlenecks of each of the selected species, and a brief mention of some of the major achievements of the project in the six scientific disciplines, which are Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health and Socioeconomics, including final product quality (**Fig. 31.3.4**). There were many opportunities to publicize DIVERSIFY during the conference, and to inform colleagues on the project's objectives, already implemented and planned work. People were encouraged to follow the website of the project, where we have been uploading regularly news regarding the project. Interested researchers and industry managers were informed of the open component of the ACMs, and various researchers expressed an interest to coordinate their research activities with DIVERSIFY and perhaps carry out joined experiments.

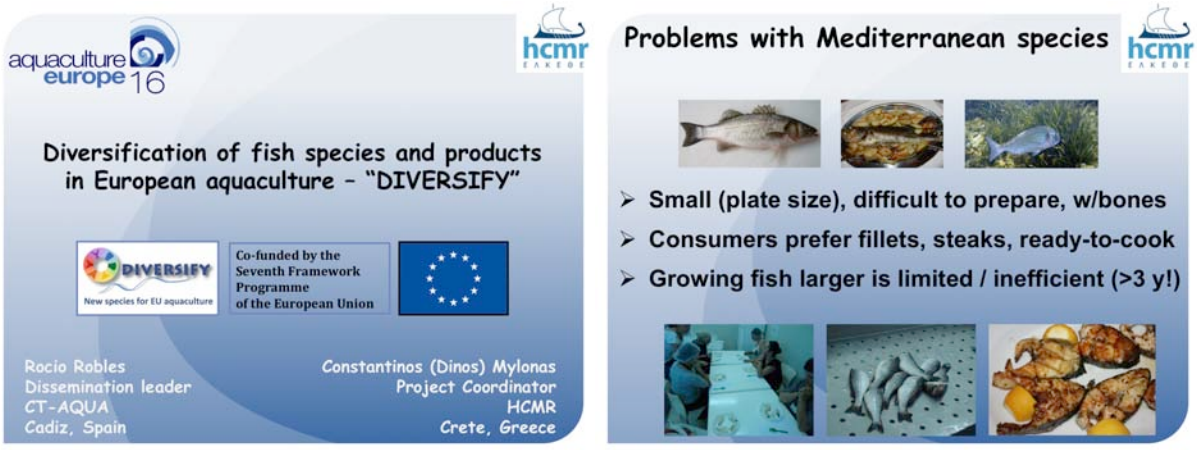


Figure 31.3.3. Representative slides of the summary presentation of DIVERSIFY given by the Project Coordinator at the "EU Session: Research and Innovation supporting European Aquaculture" at AQUACULTURE EUROPE 2016.

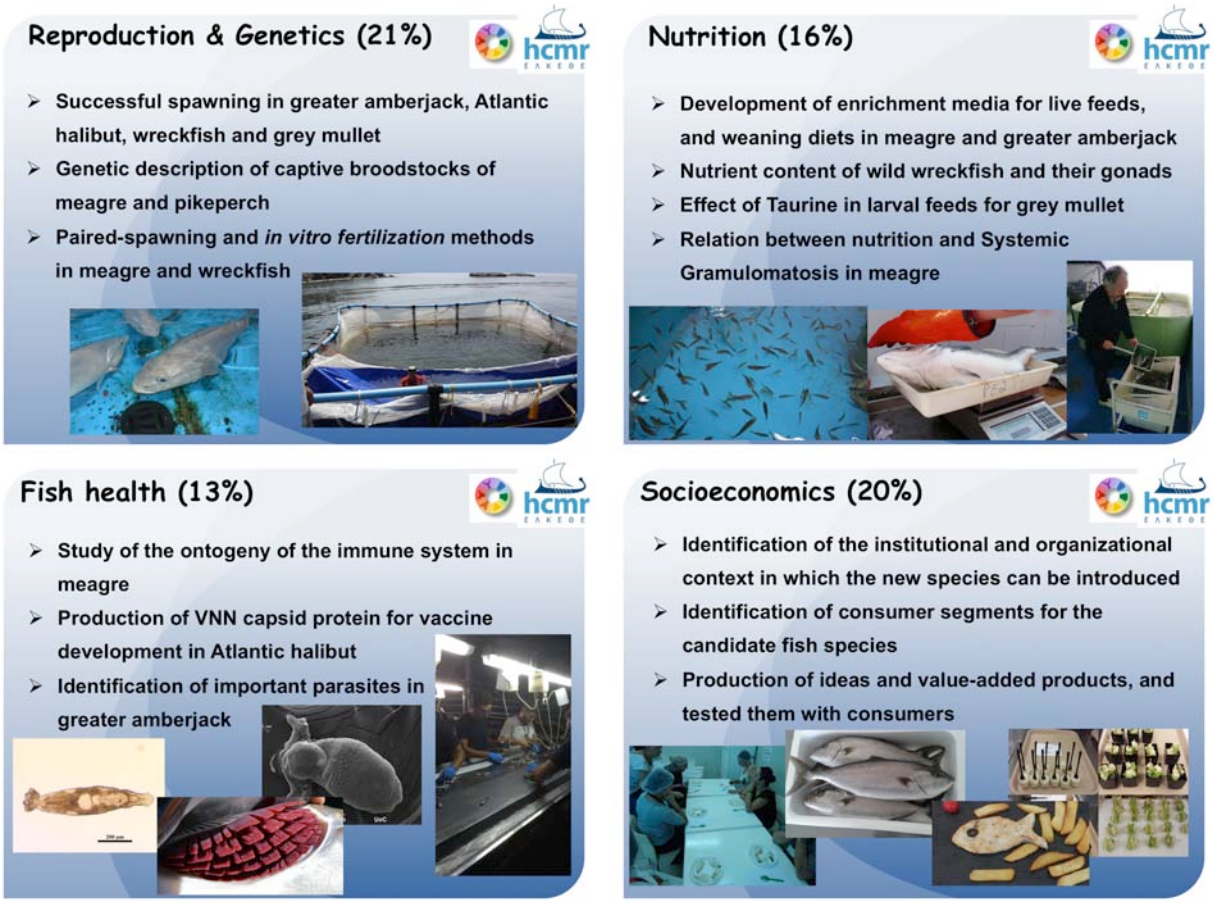


Figure 31.3.4. Representative slides of the DIVERSIFY presentation with a mention of the major achievements of DIVERSIFY in the six scientific disciplines.

The AQUACULTURE EUROPE 2017 conference was held between 17-20 October 2017 in Dubrovnik, Croatia (**Fig. 31.3.5**). The conference was attended by 1700 persons, including the visitors to the Trade show. This year the Project Coordinator (PC) Dr. Constantinos C. Mylonas gave a presentation in the Special Session "DIVERSIFY - New/emerging finfish species (EU Project)" (See D31.20 Presentations of DIVERSIFY (Y4) at the Aqua Europe meetings (Diversification Sessions). The session was chaired by Drs Rocio Robles (WP31 Dissemination leader) and Constantinos C. Mylonas, the latter opening the full-day session with the first presentation, which focused on some of the more interesting results of the project after almost 4 years.

Figure 31.3.5. The announcement poster of AQUACULTURE EUROPE 2017 (left) organized every year by the European Aquaculture Society, and a view of the spectacular old town of Dubrovnik, Croatia (right).

The "DIVERSIFY - New/emerging finfish species (EU Project)" session ςασ held on Wednesday, October 18, 2017. The DIVERSIFY presentation of the PC was well attended with more than 100 people in the room, which is indicative of the interest that this project has for the Aquaculture sector (**Fig. 31.3.6**). The PC summarized the most relevant results of DIVERSIFY according to species studies and in the area of socioeconomics. The presentation begun with a very brief mention of the project and its consortium, and the underlining reasons for the support of this project by the European Union **(Fig. 31.3.7).**

Figure 31.3.6. A full room with ~100 people attended the opening presentation of the Special Session DIVERSIFY (EU project)-New/emerging finfish species.

Figure 31.3.7. Representative slides of the summary presentation of DIVERSIFY during the special session at AQUACULTURE EUROPE 2017, presented by the PC.

The presentation explained the justification of the project's species selection. Then, there was a brief description of the major achievements of the project in the six species examined (**Fig. 31.3.8**). In meagre, for example, we have completed the genetic characterization of many of the existing broodstocks in Europe, and begun work on a genetic linkage map and QTL analysis. We have developed methods necessary for the implementation of selective breeding (*in vitro* fertilization, paired spawning). We studied the feeding behaviour in order to improve grow-out in cages and we also undertook a significant amount of work to try to understand the cause of Systemic Granulomatosis and its relation to nutrition, and we begun work on the immune system characterization of the species. In greater amberjack, we have succeeded in the development of broodstock management and spawning induction methods, including the first spawning of F1 stocks. We have developed larval rearing methods for the production of juveniles, and have initiated the first commercial on-growing trials in sea cages in a number of facilities in Greece, and of course we dedicate significant effort in issues of health management (parasites) and immune system characterization.

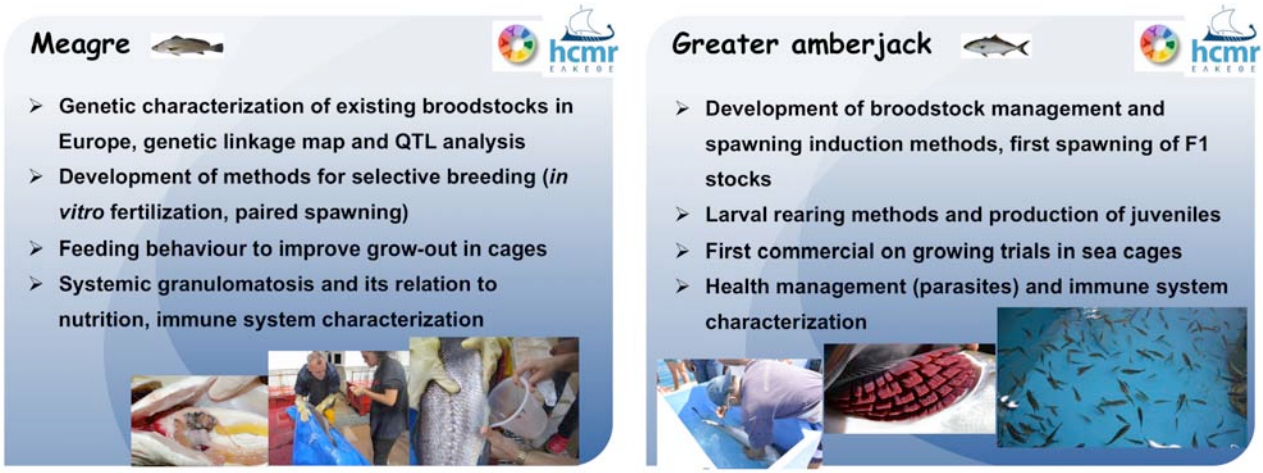


Figure 31.3.8. Representative slides of the Species achievements in the project.

As in the previous EAS meetings, there were many opportunities to publicize DIVERSIFY during the conference, and to inform numerous colleagues on the project's objectives, already implemented and planned

work. People were encouraged to follow the website of the project, where we have been uploading regularly news regarding the project. Interested researchers and industry managers were also informed of the open component of the Annual Coordination Meetings, and a special mention was made of the upcoming **Species-specific knowledge transfer workshops**, which are planned for 2018 (**Fig. 31.3.9**).

These **Species-specific knowledge transfer workshops** will be constituted of 30 min presentations on selected aspects (*e.g.* reproduction and spawning induction, larval rearing, grow out, nutrition, final product diversification and quality, etc.) given by DIVERSIFY researchers, but also from any authorities in the species (European or world-wide, depending on the species), whose work was not part of the project. Aquaculturists (mainly), but also European aquaculture support companies (feed, pharmaceutical, etc.), researchers, government organizations and other important institutions will be invited to attend these meetings. The cost of the invited speakers and the registration of the participants will be covered by the programme (50-100 participants). The seminars will be organized by the Species Leaders from DIVERSIFY in countries where the particular species are cultured --or has the potential to be cultured. One seminar will be organized for each of the selected species (**Fig. 31.3.9**). In the same Special Session for DIVERSIFY, where this presentation was given by the PC, a number of specific presentations from the project were also presented, both as oral and poster presentations, and have already been reported in ***D31.20 Presentations of DIVERSIFY to the Aquaculture Europe meetings (Diversification Sessions) by the Species Leaders in Y4***.

Figure 31.3.9. The announcement of the **Species-specific knowledge transfer workshops** of DIVERSIFY, which will take place in 2018.

Task 31.4 Scientific presentations and submission of manuscripts (led by HCMR, Constantinos Mylonas).

Scientific presentations

A Special Session was organized at the AQUACULTURE EUROPE 2017 conference (**Fig. 34.4.1**), titled "DIVERSIFY- New/emerging finfish species (EU Project) chaired by the WP31 Dissemination leader and the PC of DIVERSIFY. Instead of summary presentations by the six Species Leaders (SL) of DIVERSIFY - as described in the DOW, reporting on species studied in the project, it was decided to allow as many researchers as possible from the consortium to present their work undertaken in the various specific tasks of the project. The Special Session lasted for the whole day and an estimated of 50-120 persons were present at the different presentations in the designated room (**Fig. 31.4.1**). A total of 16 presentations were given, 15 from DIVERSIFY work (**Fig. 31.4.2**). In addition, a number of Posters were presented under this Special Session, being also from DIVERSIFY tasks

Figure 31.4.1. Dr. Marija Banovic (AU) presenting at the DIVERSIFY Special Session of AE 2017

15.30 **Konstantin D. Matishov**, Ulyana S. Aleksandrova
DEVELOPMENT OF INTEGRATED INNOVATIVE TECHNOLOGIES FOR PRODUCING ENVIRONMENTALLY FRIENDLY PRODUCTS OF AQUARICULTURE IN A RECIRCULATING SYSTEM

15.50 **Benz Kotzen**, Mohammed Khandaker
THE POTENTIAL FOR COMBINING LIVING WALL AND VERTICAL FARMING SYSTEMS IN AQUAPONICS

16.10 **Maja Turnšek Hančič**, R.I. Thorarinsdottir, Agnes Joly
FROM DREAM TO REALITY: DIFFICULTIES ENCOUNTERED BY AQUAPONIC START-UPS IN EUROPE

16.30 **Daniel Malilic**, Paul Kledal, Vesna Milicic, Maria Dos-Santos, Joao Cotter
AQUAPONICS: THE UGLY DUCKLING IN EUROPEAN ORGANIC REGULATION

16.50 **Kyra Hoevenaars**, Matej Leskovec
EU POLICIES: OPPORTUNITIES FOR AQUAPONICS

DIVERSIFY – NEW / EMERGING SPECIES (EU PROJECT)
Wednesday, October 18 10.30 - 17.30 Olipa 1 & 2
Chairs: Rocio Robles, Constantinos Mylonas

10.30 **Constantinos C. Mylonas**, Rocio Robles
"DIVERSIFY": EXPLORING THE BIOLOGICAL AND SOCIO-ECONOMIC POTENTIAL OF NEW/EMERGING CANDIDATE SPECIES FOR THE EXPANSION OF THE EUROPEAN AQUACULTURE INDUSTRY

10.50 **Aldo Corriero**, Constantinos C. Mylonas, Rosa Zupa, Chrysovalentinos Pousis, Ioannis Fakriadis, Maria Papadaki, Caterina De Virgilio, Nicoletta Santamaria, Letizia Passantino
REPRODUCTIVE DEVELOPMENT IN WILD AND CAPTIVE-REARED GREATER AMBERJACK *Seriola dumerili* (RISSO, 1810)

11.10 **Ioannis Fakriadis**, Francesca Lisi, Irini Sigelaki, Maria Papadaki, Anastasios Raftopoulos, Constantinos C. Mylonas
SPAWNING KINETICS OF GREATER AMBERJACK *Seriola dumerili* IN RESPONSE TO MULTIPLE GnRHa INJECTIONS OR IMPLANTS

11.30 **Maria Papadaki**, Jose Benito Peleteiro, Blanca Alvarez-Blázquez, J.L. Rodriguez Villanueva, Fatima Linares, Antonio Vilar, Evaristo Pérez Rial, Nuria Lluch, Ioannis Fakriadis, Constantinos C. Mylonas
DESCRIPTION OF THE ENDOCRINE REPRODUCTIVE CYCLE OF THE WRECKFISH *Polyprion americanus* IN CAPTIVITY

11.50 **Neil Duncan**, Sandra Ramos, Wendy Gonzalez, Gilbert Dutto, Constantinos Mylonas, Christian Fauvel
GAMETE QUALITY AND MANAGEMENT FOR *IN VITRO* FERTILISATION IN MEAGRE *Argyrosomus regius* TO FACILITATE THE IMPLEMENTATION OF GENETIC BREEDING PROGRAMS

12.10 **Sebastien Baekelandt**, Syaghalirwa N.M. Mandiki, Patrick Kestemont
LIGHT ENVIRONMENT AFFECTING ENDOCRINE AND IMMUNE CIRCADIAN RHYTHMS IN PIKEPERCH *Sander lucioperca*

19

WEDNESDAY

WEDNESDAY

12.30 **Torstein Harboe**, Sonal Patel, Audun H. Nerland, Nina Sandlund, Øivind Bergh, Birgitta Norberg
RECIRCULATION (RAS) VS. FLOW THROUGH (FT) SYSTEMS DURING YOLK SAC AND FIRST FEEDING STAGES: EFFECTS ON REARING SYSTEM BACTERIOLOGY, AND SURVIVAL, QUALITY AND GROWTH OF ATLANTIC HALIBUT *Hippoglossus hippoglossus* LARVAE

12.50 **LUNCH**

14.30 **William Koven**, Enric Gisbert, Oriya Nixon, Iris Meiri-Ashkenazi, Aviad Gaon, Mikhail Solovyev, Amos Tandler, Hanna Rosenfeld
DESIGNING WEANING DIETS BASED ON THE ONTOGENY OF DIGESTIVE TRACT ENZYME ACTIVITY DURING THE CARNIVOROUS-OMNIVOROUS TRANSITION IN GREY MULLET *Mugil cephalus* JUVENILES

14.50 **Ioannis Papadakis**, Nikos Papandroulakis, Alkioni Sfendouraki, Veronica Camporesi, Manolis Vasilakis, Constantinos Mylonas
THE EFFECT OF DIFFERENT STIMULI ON MEAGRE *Argyrosomus regius* FEEDING BEHAVIOUR

15.10 **Pascal Fontaine**, Tatiana Colchen, Ledoré Yannick, Soumaya Hmilla, Enric Gisbert, Daniel Zarski, Alain Pasquet
IMPROVEMENT OF REARING CONDITIONS FOR JUVENILE PIKEPERCH *Sander lucioperca* PRODUCTION IN RAS

15.30 **Blanca Álvarez-Blázquez Fernández**, J. Luis Rodríguez Villanueva, A. Vilar, C. Mylonas, N. Papandroulakis, Evaristo Pérez Rial, Nuria Lluch, Gemma Pazos, Fátima Linares
PROGRESS IN THE WRECKFISH *Polyprion americanus* INTENSIVE CULTIVATION: NEW CANDIDATE SPECIES FOR AQUACULTURE

15.50 **M.I. Tsertou**, S. Chatzifotis, R. Fontanillas, E. Cotou, E. Fountoulaki, M. Smyrli, E. Antonopoulou, P. Katharios
RECENT ADVANCES IN THE STUDY OF SYSTEMIC GRANULOMATOSIS IN MEAGRE *Argyrosomus regius*

16.10 **Yannis Kotzamanis**, Ramon Fontanillas, Emmanouil Kouroupakis, Vassiliki Ilia, Sofia Vardali, Efthimia Antonopoulou
LYSINE OPTIMIZATION OF A DIET WITH LOW FISH MEAL INCLUSION FOR GREATER AMBERJACK *Seriola dumerili* (RISSO, 1810)

16.30 **Alvaro Fernández-Montero**, María José Caballero, Silvia Torrecillas, Douglas Milne, Chris Secombes, Maria Soledad Izquierdo, Daniel Montero
DIETARY USE OF PREBIOTICS IN GREATER AMBERJACK JUVENILES: EFFECTS ON GROWTH PERFORMANCE, IMMUNE GENE EXPRESSION AND DISEASE RESISTANCE AGAINST *Neobenedenia girellae*

16.50 **Marija Banović**, Athanasios Krystallis
FISH FOR THE FUTURE: WHAT COULD INFLUENCE EUROPEAN CONSUMER CHOICE OF NEW AQUACULTURE PRODUCTS? EVIDENCE FROM AN EXPERIMENTAL STUDY WITH LOW AND MEDIUM PROCESSED PRODUCTS

17.10 **Philip James**
THE EU URCHIN PROJECT; UTILISING THE ARCTIC SEA URCHIN RESOURCE – PROGRESS AND PITFALLS

20

Figure 31.4.2. The program pages of the DIVERSIFY Special Session of AQUACULTURE EUROPE 2017.

Overall, this Special session demonstrated that significant progress has been achieved in the study of new/emerging species for the EU aquaculture industry. The knowledge acquired so far, will now be more widely disseminated to the scientific community through the publication of scientific articles, and to the aquaculture industry through the upcoming **Species-specific knowledge transfer workshops**, with the objective of increasing their annual production with the inclusion of new species that offer significant biological (faster growth and better FCR) and market advantages (flesh quality, consumer acceptance and world-wide distribution).

DIVERSIFY oral presentations

A number of Oral and Poster presentations from DIVERISFY work were also presented in other Special Sessions of the AE 2017 conference. Below is the full list of Abstracts submitted to AE 2017.

1. Álvarez-Blázquez, B., Rodríguez, J. L., Vilar, A., Mylonas, C., Papandroulakis, N., Pérez Rial, E., Lluch, N., Pazos, G. and Linares, F. Progress in the wreckfish intensive culture. New candidate species for aquaculture (oral).
2. Corriero, A., Mylonas, C.C., Zupa, R., Pousis, C., Fakriadis, I., Papadaki, M., De Virgilio, C., Santamaria, N., Passantino, L. Reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810) (oral).
3. Ramos, S., Gonzalez, W., Dutto, G., Mylonas, C.C., Fauvel, C and Duncan, N. Gamete quality and management for *in vitro* fertilisation in meagre (*Argyrosomus regius*) to facilitate the implementation of genetic breeding programs (oral).
4. Fakriadis, I., Lisi, F., Sigelaki, I., Papadaki, M, Raftopoulos, A., and Mylonas, C.C. Spawning kinetics of greater amberjack S*eriola dumerili* in response to multiple GnRHa injections or implants (oral).
5. Mylonas, C.C. and Robles, R. "Diversify": exploring the biological and socio-economic potential of new/emerging candidate species for the expansion of the European aquaculture industry (oral).
6. Mylonas, C.C., Fakriadis, I., Papandroulakis, N., Raftopoulos, A., Iakovopoulos, G., Papadaki, M. and Sigelaki, I. Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared in tanks and sea cages in Greece (oral).
7. Papadaki, M., Peleteiro, J.B., Álvarez-Blázquez, B., Rodríguez Villanueva, J.L., Linares, F., Vilar, A., Pérez Rial, E., Lluch, N., Fakriadis, I., Mylonas, C.C. Description of the endocrine reproductive cycle of the wreckfish *Polyprion americanus* in captivity (oral).
8. Papadakis, I.E., Papandroulakis, N., Sfendouraki, A., Camporesi, V., Vasilakis, M., and Mylonas, C.C. The effect of different stimuli on meagre *Argyrosomus regius* feeding behavior (oral).
9. Manousaki, T., Chatziplis, D., Tsakogiannis, A., Tzokas, K., Villa, J., Estevez, A., Mylonas, C.C., Duncan, N., Tsigenopoulos, C.S. Construction of the first genetic linkage map in meagre (*Argyrosomus regius*) and identification of growth-related loci to be used in marker assisted selection programs (oral).
10. Tsertou, M., Chatzifotis S., Fontanillas R., Cotou E., Fountoulaki E., Smyrli M., Antonopoulou E., Katharios P. Recent advances in the study of systemic granulomatosis in meagre (*Argyrosomus regius*) (oral).
11. Baekelandt, S., Mandiki, S.N.M., Kestemont, P**.** Light environment affecting endocrine and immune circadian rhythms in pikeperch (*Sander lucioperca*) (oral).
12. Banović, M., and Krystallis, A. Fish for the future: what could influence European consumer choice of new aquaculture products? Evidence from an experimental study with low and medium processed products (oral).
13. Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M. Requirements for n-3 hufa of meagre (*Argyrosomus regius*, Asso, 1801) fingerlings (oral).
14. Fernández-Montero, A., Caballero, M.J., Torrecillas, S., Milne, D.J., Secombes, C.J., Izquierdo, M., Montero, D. Dietary use of prebiotics in greater amberjack juveniles: effects on growth performance, immune gene expression and disease resistance against *Neobenedenia girellae* (oral).
15. Colchen, T., Ledoré, Y., Hmilla, S., Gisbert, E., Zarski, D., Pasquet, A., Fontaine, P. Improvement of rearing conditions for juvenile pikeperch (*Sander lucioperca*) production in RAS (oral).
16. Kotzamanis, Y. Fontanillas, R., Kouroupakis, E., Ilia, V., Vardali, S., Antonopoulou, E. Lysine optimization of a diet with low fish meal inclusion for greater amberjack (*Seriola dumerili,* risso 1810).
17. Harboe, T., Patel, S., Nerland, A.H., Sandlund, N., Bergh, O., Norberg, B. Recirculation (RAS) vs. flow-through (FT) systems during yolk sac and first feeding stages: effects of rearing system bacteriology, and survival, quality and growth of Atlantic halibut, *Hippoglossus hippoglossus* larvae.
18. Koven, W., Gisbert, E., Nixon, O., Meiri-Ashkenazi, I., Gaon, A., Solovyev, M., Tandler, A., and Rosenfeld, H. Designing weaning diets based on the ontogeny of digestive tract enzyme activity during the carnivorous-omnivorous transition in grey mullet *Mugil cephalus* juveniles.
19. Tsalafouta, A., Pavlidis, M., Papandroulakis, N. Effect of background color and expression of genes related to the GH/IGF growth axis at early development of greater amberjack (*Seriola dumerili*).

DIVERSIFY posters

1. Jerez, S., Fakriadis, I., Martin, M.V., Felipe, B.C., Papadaki, M., and Mylonas, C.C. Improvement of reproductive performance of F1 generation greater amberjack (*Seriola dumerili*) with succesive implants of gonadotropin-releasing hormone agonist (GnRHa) (poster).
2. Jerez, S., Martin, M.V., Santamaría, F.J., Felipe, A., Lago, M.J. Effects of stocking density on growth performance and health of greater amberjack (*Seriola dumerili*) juveniles.
3. Martin, M.V., Fakriadis, I., Jerez, S., Misol, A., and Mylonas, C.C. Hematological and plasma biochemical parameters in F1 generation greater amberjack (*Seriola dumerili*) during spawning induction with GnRHa delivery systems (poster).
4. Sigelaki, I., Nogueira França, M.C., Karamanlidis, D., Fakriadis, I., Duncan, N., and Mylonas, C.C. Effect of male rotation on induced pair spawning of meagre *Argyrosomus regius* (poster).
5. Kolios, E., Tsaparis, D., Ekonomaki, K., Fakriadis, I., Papadopoulos, V., Corriero, A., Ilgaz, S., Mylonas, C.C. and Tsigenopoulos, C.S. Population genetic structure of greater amberjack (*Seriola dumerili)* in the Mediterranean Sea and eastern Atlantic Ocean (poster).
6. El Kentaoui, N., Lund, I., Mandiki, S.N.M., Kestemont, P. Effect of different ratios of DHA, EPA and ARA on ontogeny of digestive activites and larval development of pikeperch larvae (*Sander lucioperca*).
7. Almeida, A.S., Fernández-Montero, A., Montero, D., Izquierdo, M. Preliminary studies on the relationship of temperature and time of digestion on enzymatic activity and growth of *Seriola dumerili*.
8. Alexi, N., Byrne, D.V., Nanou, E., Grigorakis, K. Proximate, fatty acids and volatile compounds composition of reared vs. wild greater amberjack (*Seriola dumerili*) as affected by fish size.
9. Rodríguez Villanueva, J.L., Álvarez-Blázquez, B., Pérez, E., Martínez, , J.M., Méndez, J.C., Acuña, I., Linares, F. Evaluation of wreckfish *Polyprion americanus* growth in Galicia (Spain).
10. Martín, M.V., Pérez, J.A., Jerez, S., Chaves-Pozo, E., Lorenzo, A., Arízcun, M., Bolaños, A., Rodríguez, C. Combined effect of immune-stimulant enrichment products and feeding frequency on greater amberjack larval performance.

Presentations of DIVERSIFY work have also been given in other International conferences held in Europe and around the world as it is shown in **Table 31.1.1.**

Submission of manuscripts to scientific journals

The following articles have been published (or are in press) so far in international journals with an ISI-index.

Alexi, N., Byrne, D.V., Nanou, E., Grigorakis, K., Investigation of sensory profiles and hedonic drivers of emerging aquaculture fish species. Journal of the Science of Food and Agriculture, in press.

Andree, K.B., Roque, A., Duncan, N., Gisbert, E., Estevez, A., Tsertou, M.I., Katharios, P., 2015. Diplectanum sciaenae (Van Beneden & Hesse, 1863) (Monogenea) infecting meagre, Argyrosomus regius (Asso, 1801) broodstock in Catalonia, Spain. A case report. Veterinary Parasitology: Regional Studies and Reports 1–2, 75-79.

Baekelandt, S., Redivo, B., Mandiki, S.N.M., Bournonville, T., Houndji, A., Bernard, B., El Kertaoui, N., Schmitz, M., Fontaine, P., Gardeur, J.-N., Ledoré, Y., Kestemont, P., 2017. Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pikeperch *Sander lucioperca*. General and Comparative Endocrinology.

Banović, M., Krystallis, A., Guerrero, L., Reinders, M.J., 2016. Consumers as co-creators of new product ideas: An application of projective and creative research techniques. Food Research International 87, 211-223.

Campoverde, C., Estevez, A., 2017. The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae. Aquaculture 478, 16-24.

Campoverde, C., Milne, D.J., Estévez, A., Duncan, N., Secombes, C.J., Andree, K.B., 2017a. Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*). Fish & Shellfish Immunology 69, 200-210.

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estevez, A., 2017b. Early weaning in meagre Argyrosomus regius: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research 48, 5289-5299.

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Task 31.5 Full-day seminars on "Know-how Transfer" of the aquaculture for each of the studied species (led by CTAQUA and the Species Leader Partner)

Planning has already begun for the organization of the **Species-specific knowledge transfer workshops (Fig. 31.3.9.).** These will be constituted of 30 min presentations on selected aspects (*e.g.* reproduction and spawning induction, larval rearing, grow out, nutrition, final product diversification and quality, etc.) given by DIVERSIFY researchers, but also from any authorities in the species (European or world-wide, depending on the species), whose work was not part of the project. Aquaculturists (mainly), but also European aquaculture support companies (feed, pharmaceutical, etc.), researchers, government organizations and other important institutions will be invited to attend these meetings. The cost of the invited speakers and the registration of the participants will be covered by the programme (50-100 participants). The seminars will be organized by the Species Leaders from DIVERSIFY in countries where the particular species are cultured --or has the potential to be cultured. One seminar will be organized for each of the selected species

Task 31.6 Promotional workshops (led by CTAQUA, Rocio Robles).

Two Promotional workshops have been already organized during the 3rd Reporting Period:

- 1st Promotional Workshop held in Bremen Germany
- 2nd Promotional Workshop held in El Puerto de Santa Maria, Cádiz,Spain.

The complete description of the work and results have been submitted as ***Deliverable 31.16 Promotional Workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) 1st Workshop*** and as ***Deliverable 31.18 Promotional Workshops (2nd) for specialized audience in fish market sector (Spain, Greece, UK or Italy).***

A brief presentation of both events is provided in the following pages.

1st PROMOTIONAL WORKSHOP BREMEN, GERMANY

Initially four countries were listed in the DOW as relevant locations for the organization of this activity: Spain, UK, Italy and Greece. From these locations, UK has been changed for Germany due to market and consumer reasons. DIVERSIFY partner BVFi (Matthias Keller) from Germany is a very active partner in terms of contacts with the German fish markets, German fish processors and big supermarket chains. Moreover Matthias Keller, is the managing director of the following professional organizations: Bundesmarktverband der Fischwirtschaft e.V. (Federal market association of fisheries), Bundesverband der deutschen Fischindustrie und des Fischgroßhandels e.V. (German fish processing and wholesale association), Fisch-Informationszentrum e.V. (Fish-Informationcenter) and "Stiftung seeklar" – Verein zum Schutz der Meere e.V. ("Foundation seeklar" – Foundation for the protection of the sea). The federation BVFi has contacts with several Governmental organizations, the fishing industry, NGOs and the media, in order to support its members and to promote the image of the German fish-processing sector. On the EU-level BVFi is a member of AIPCE/CEP. The federation informs its members about relevant issues for fish processing and wholesaling, such as import tariffs and tariff contingents, EU-policies, quality standards and certification initiatives.

All the above mentioned information and the lack of such a relevant counterpart in UK made much impacting and straightforward for the purpose of the promotional workshops to organize the 1st DIVERSIFY Promotional Workshop in Germany so we could guarantee the right broadcasting of the project activities and evaluate their impact with an adequate representation of fish processing, fish markets and consumers.

The agenda of the event was distributed to fish processing and fish industry stake holders in Germany. The event was organized in very close collaboration with the P34. BVFi. The meeting was organized including six presentations from DIVERSIFY partners and also from Jürgen Pauly, manager of Globus SB-Warenhaus, a very strong German supermarket group with more than 160 hypermarkets and superstores in Germany and 20 more in Europe. After the presentations and the following debate, it was organized a degustation session

with two of the products developed within the project: grey mullet in olive oil and pikeperch pate (WP 28 Socioeconomics: New product development).

The invitation sent to the participants including the agenda and useful information of the event is included here:

April 5, 2017

On behalf of the **DIVERSIFY** project consortium, we would like to invite you to the 1st Promotional Workshop on May 24, 2017 at the

FAIR BREMEN,
Business Lounge (Hall 7, 1st floor), Theodor-Heuss-Strasse
in Bremen, Germany.

This is a half-day workshop intended to disseminate the project results and to provide a forum for discussion on market and consumer attitude towards aquaculture products. The meeting is hosted by the DIVERSIFY partner, Bundesverband der Deutschen Fischindustrie und des Fischgross-handels E.V. (BVFi) from Hamburg.

WORKSHOP AGENDA

10:00 *Presentation of the project DIVERSIFY*.
Rocio Robles, Dissemination leader. Technical Director, CTAQUA, Spain.

10:20 *The German fish market in figures: Update on valuable data.*
Matthias Keller, Managing Director of BVFi, Germany.

10:40 *German markets: consumer attitude to new fish products*.
Jürgen Pauly, Category Manager Fresh, Globus SB-Warenhaus, Germany.

11:00 Coffee break

11:30 *Aquaculture products for the long run: Consumer-driven product idea development from Diversify.*
Marija Banovic, MAPP Centre, Department of Management, Aarhus University, Denmark.

11: 50 *Traceability, labelling and certification of fish products*. Javier Ojeda, APROMAR, Spain.

12: 10 *Cross-cultural consumer perception of new fish products.* Luis Guerrero, IRTA, Monells, Spain.

12:30 Debate: *Consumer attitude to diversification in aquaculture fish products: trust of consumers in aquaculture products, sustainability and health-related behaviour.*

Moderator: Marija Banovic.

Panel:

- Matthias Keller, BVFi
- Jürgen Pauly, Globus
- Birgit Schmidt-Puckhaber, Fachzentrum Landwirtschaft
- Javier Ojeda, Apromar
- Luis Guerreo, IRTA
- Machiel Reinders, WUR

Please reply by sending us your name and the name of your company by

E-mail or fax until May 19, 2017 to the following address:

Bundesverband der deutschen Fischindustrie und des Fischgrosshandels

Tel. +49 (0)40 38 18 11

Fax +49 (0)40 389 85 54

E-Mail: info@fischverband.de

For parking please use the parking space at Bürgerweide near Hall 7 of the fair.

We are looking forward to meet you for an interesting exchange of news.

With kind regards

Rocio Robles — Dissemination leader

Dr. Matthias Keller — Project partner

The meeting had an attendance of 23 people including professionals from the fish processing industry, quality control in fish processed products, frozen fish industry, State veterinary officer, representative from the Ministry of Agriculture and the Environment, etc.

A poster with a summary of all the relevant information about the project was designed and displayed in the conference room during the meeting (**Figure 31.6.1**).

Figure 31.6.1. Poster summarizing the main information of the DIVERSIFY project and displayed during the meeting in Bremen for the 1st Promotional Workshop (left) and the Dissemination Leader Rocio Robles giving the presentation on the project (right).

2nd PROMOTIONAL WORKSHOP EL PUERTO DE SANTA MARIA, SPAIN

The 2nd Promotional Workshop has been organized in Spain, at the facilities of the P18. CTAQUA located in El Puerto de Santa María, Cádiz. The building counts among other areas, with two meeting rooms and a dining room with food preparation equipment which was needed for the degustation session of DIVERSIFY products developed in WP28 and WP29.

The agenda of the event was distributed to fish processing and fish industry stake holders in Andalusian region (South of Spain) as well as to all the members of CTAQUA. The meeting was organized including five presentations from DIVERSIFY partners and a presentation from Ulises Ameyugo, Head of the Food Safety Department of Regional Government of Andalusia. After the presentations and the following debate, it was organized a degustation session with two of the products developed within the project: grey mullet in olive oil and pikeperch pate (WP 28 Socioeconomics: New product development) (**Figure 31.6.2**).

The meeting had an attendance of 22 people including fish producers, fish purchasers from big wholesaler in Spain (Makro), professionals from the fish processing industry, representatives from the Regional Andalusian Government (Dept. Agriculture and Fisheries), University of Cádiz etc.

Figure 21.6.2. Left: Summary presentation of DIVERSIFY by Rocio Robles (P18. CTAQUA). Right: DIVESIFY products, grey mullet preserved in olive oil (together with salad) and meagre pate (with bread crackers), prepared for the degustation

Task 31.7 Dissemination to the food industry and consumers (led by APROMAR and EUFIC, Javier Ojeda and Laura Fernández).

In continuation of the collaboration established with the Association of International Seafood Professionals, AISP (seafoodprofessionals.org/), the Dissemination Leader , Rocio Robles, prepared an article with the summary of the 1st Promotional Workshop held in Bremen (Germany) to be included in the Blog of their web. Two AISP members attended the meeting and had a very active participation in the debate. Below is the article that was produced:

DIVERSIFY 1st Promotional Workshop on May 24, 2017 in Bremen

The past 24th of May, it was held in the Business Lounge (Hall 7, 1st floor), Theodor-Heuss-Strasse in Bremen (Germany) the 1st Promotional Workshop of the project DIVERSIFY. The objective of the workshop was to promote the DIVERSIFY activities mainly in the Socioeconomic Research Area and with focus on specific audience, such as fish processors (very important sector in Germany), retailers, consumers organizations etc. Thanks to our DIVERSIFY local partner, Matthias Keller from BVFi, relevant speakers from professional associations and consumer's organizations were invited to the workshop. A morning session with six presentations provided a perfect ground for an intense and constructive debate on marketing, commercialization and consumer related issues concerning seafood products.

The presentations started with the talk of the **Dissemination leader of the project Rocio Robles, CTAQUA, Spain**, that summarized the objectives, partner composition, research areas and achievements of the project with special emphasis on the new product development from the DIVERSIFY fish species (meagre, (*Argyrosomus regius),* greater amberjack (*Seriola dumerili*), wreckfish (*Polyprion americanus*), Atlantic halibut (*Hippoglossus hippoglossus*), grey mullet (*Mugil cephalus*), and pikeperch (*Sander lucioperca*).

Matthias Keller, from BVFi, **Germany**, gave the second talk, entitled "The German fish market in figures: Update on valuable data!" in which the market performance in Germany for fish and fishery products including products generated by aquaculture was highlighted. After presenting a supply balance for fish and seafood, Matthias Keller gave insight information on import and export figures and their development over the last years. On the basis of live weight equivalent, he summarized the importance of species and of product categories, which are important in Germany. With respect to in-house consumption he refers to data of GfK and highlighted the development of increased consumption of fresh and chilled fish production in the German market. He finished his presentation with a comparison of household activities with respect to sales of fresh and chilled fish products in Germany with other EU countries.

Next talk was from **Jürgen Pauly, Category Manager Fresh, Globus SB-Warenhaus**, a well renown German supermarket chain with more than 46 hypermarkets, 90 DIY-superstores, 8 electronic stores in Germany, 15 hypermarkets with DIY-superstores in Czech Republic ,12 hypermarkets in Russia and 2 in Luxemburg. Globus has a turnover of 7 billion euros per year. Jürgen Pauly presented the case of three new fish products introduced in their supermarkets: fresh fillet of tilapia skin on, fresh fillet of cobia skin on and fresh fillet of Scottish salmon. The three products count with GlobalGAP certification. In the case of the tilapia fresh fillet, the consumers did not accept it and in fact preferred, frozen tilapia fillet. Production and sales of tilapia fresh fillet of stopped in January 2016. With the fresh fillet of cobia, even being the first and only offer of fresh cobia fillet in German retail, consumer acceptance was low, probably due to the fact that the species was unfamiliar. On the contrast, with the fresh fillet of Scottish salmon, without any special effort in the promotion of the product, it had a high customer acceptance and sales were quite good. The most probably explanations is that salmon is a well-known species and that salmon farmed in Scotland is associated with pure nature more than the salmon farmed in Norway. Pauly's conclusions pointed out that new species need time to get into the market and that the people tend to consume what is familiar for them. Moreover, it is essential to have a stable and continuous supply with the necessary logistics covered. Overall, it is necessary an appropriate investment from all the partners in the chain.

After the coffee break, **Maria Banovic, from the MAPP Center, Department of Management, Aarhus University, Denmark** presented "Aquaculture products for the long run: Consumer-driven product idea development from Diversify". Maria Banovic described in her talk how, within the Diversify project, they have introduced new market concepts. Diversify project embodies "fish for the future" – a development of the European aquaculture industry through a greater diversity of fish species and new value-added products that could be deployed to face the expected increase in consumer demand. To successfully introduce new market concepts at the Diversify project we have involved consumers at an early stage of product transformation into marketable products to uncover product ideas for the next-generation aquaculture products with the power to grow. The Diversify path of collaboration with consumers to develop new aquaculture products that add value to consumers' lives' was taken in several steps.

First of all, consumers' were seen as a critical factor in better understanding of the most promising features of the new aquaculture products. We were working with the early adopters from five European countries (i.e. France, Germany, Italy, Spain and the UK) who are familiar with the concept of fishery products in general, as this understanding impacts the use of aquaculture products and their choice directly. Further we saw these consumers as a "co-creators" and as the starting point for identifying specific product variables likely to affect new aquaculture products as linkages between diverse ideas and perspectives on aquaculture production. The design and development of the Diversify products was further monitored and controlled by assessing their technical and commercial feasibility.

Finally, consumers were involved in product testing to identify possible product flaws, but also to uncover different contexts of product usage to enhance an overall value of the product. We have found through our research journey on the Diversify project that it is possible to develop new aquaculture products targeting early adopters, across big European markets, as we found more homogeneous converging fish-related culture. We found similar pattern in consumer choice-drivers. That is, country-of-origin and price come first in terms of purchase drivers, followed by quality certification (i.e. ASC logo), while nutrition and health claims appear to have varying impact and are highly depend on the type of product (i.e. level of processing) and EU country, showing a need for a certain degree of customization.

Further, information around aquaculture products should be more specific and visible so consumers could distinguish these products from their market counterparts. This is especially true as aquaculture is often overshadowed by legitimate doubts projected from less sustainable practices and cheap imports. Thus, less clutter and too intensive information and more emphasis on storytelling and benefits are important for new aquaculture products. Further, consumers romantic notions and associations to responsible consumption, fish welfare issues and healthy diet should be exploited, as this awareness is today at an all-time high.

However, a better link between artisanal traditions and large-scale industrial production and processing needs to be established. The more we provide solutions for these consumers' concerns, the more positive value perceptions they will have towards aquaculture products, and the more likely it is that they will trust and buy these products. Ultimately, impact of functional value and sensory aspects on consumers' perceptions and final acceptance of aquaculture products should not be neglected as these aspects are still the driving force of repeated purchases.

Following presentation was given by **Javier Ojeda, Manager of APROMAR, Spain**, and it was entitled "Traceability, labelling and certification of fish products". He gave an overview of the actual regulation on food safety principles. He pointed out that the EU has one of the most stringent regulations on food safety principles and the protection of consumer's interests. Our food law is aimed at the reduction, elimination or avoidance of any risk to health. The free movement of safe and wholesome food is an essential aspect of the internal market and contributes significantly to the health and well-being of citizens, and to their social and economic interests. He commented on the different regulations applicable to fisheries and aquaculture products. Namely, the regulations addressed were: Regulation 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety; Regulation 853/2004laying down specific hygiene rules for on the hygiene of foodstuffs; Regulation 1169/2011 provision of food information to consumers; Regulation 852/2004 on the hygiene of foodstuffs; Regulation 853/2004 laying down specific hygiene rules on the hygiene of foodstuffs and Regulation 1379/2013 common organization of the markets in fishery and aquaculture products. Ojeda described in detail the labelling information that should be included in products, namely (a) the commercial designation of the species and its scientific name; (b) the production method, in particular by the following words "… caught …" or "… caught in freshwater …" or "… farmed …"; (c) the area where the product was caught or farmed, and the category of fishing gear used in capture of fisheries; (d) whether the product has been defrosted and (e) the date of minimum durability, where appropriate. He finished his talk addressing the different certification schemes and their classification.

The last talk, before the debate panel, dealt with the "Cross-cultural consumer perception of new fish products", given by **Luis Guerrero, from IRTA-Food Technology Centre, Girona, Spain.** He described

how in the framework of the DIVERSIFY project, twelve products from new aquaculture fish species have been developed and tested from a technological, physical/chemical, microbiological and sensory perspective. These products were selected from a pool of 41 concepts based on their different degree of technological complexity and processing and taking into account the appropriateness for each of the species under study.

Intrinsic (sensory properties) and extrinsic characteristics (information provided) of the selected products/concepts were assessed by consumers in five countries (France, Germany, Italy, Spain and UK), thus focussing in both experiential and credence quality attributes.

Products with a lower degree of processing were those who generated higher expected acceptance. The most important parameter affecting liking expectations was the expected taste of the product. Health, nutritional and well-being related issues were relevant as well in order to increase individuals' expectations, but to a lower extent. These findings seem to indicate that, in general, consumers are unwilling to sacrifice taste by an improvement in health or functional properties. In a general sense, the perception of these products was similar across countries.

Once the product was blind tested, the most preferred product was the grilled fillet and the least appreciated the fish pâté in agreement with the previously reported expected liking. Even though the different products were perceived similarly in the different locations regarding the acceptability ratings, they were described in a clearly different way when dealing with the main intangible dimensions that might define them (taste, convenience, environmental impact, etc.). Generally speaking, the sensory dimension seems to have an important contribution to the overall acceptance of the product and to its purchase probability.

These results open a new framework of research aimed to understand the rationale behind the observed differences between countries and how they can be exploited to better design and commercialise the new products already developed. This information will be essential in order to build different business models aimed to develop launching strategies for the different tested new products in different markets.

Once the presentations were finished, all the speakers joined together at the podium to start the **Debate "*Consumer attitude to diversification in aquaculture fish products: trust of consumers in aquaculture products, sustainability and health-related behaviour*".** The debate was moderated by Marija Banovic from MAPP. In addition to the 6 speakers, two more attendees were included in the debate panel: Birgit Schmidt-Puckhaber, Aquaculture Responsible of the Fachzentrum Landwirtschaft, (German Agricultural Society (DLG)) and Machiel Reinders from Wageningen University.

The debate was very stimulating with a great participation of all the members of the audience. After a short introduction of Maria Banovic, Paul van der Hejden from Mature Development (WTC The Hague, The Netherlands) asked to the panelists their opinion with regard to the inclusion of the high digestibility of fish products in the product label, as a promotion characteristic of the product. He found very important to include specific information about all the "goodies" of fish consumption. Luis Guerrero answered that although it is an attractive characteristic, the high digestibility could be associated to a short satiation time so a hungriness feeling will appear shortly and this may not be such a great characteristic to be highlighted. On the other hand, Maria Banovic pointed out that this could be an attractive issue for the consumer sector concerned about their healthy feeding habits.

Florian Bauman, from Frozen Fish International (Iglo) started a discussion on the price stability and competition between wild and farmed fish. Yvonne Feucht from Thünen-Institute for Market Research and Birgitte Schmidt-Puckhaber commented on the relative importance of the fish origin for most of the consumers and the importance of having a story, an argument to support the buy of new fish products. This is especially important for the big buyers. Matthias Keller, commented on the examples of "nice stories" such as tilapia and urban farming but this niche markets are not pointing to most of the consumers; for this, fish products need to start in the Horeca sector and from there, big buyers (hypermarkets, supermarkets …) will follow. Some important aspects such as the relevance of an appropriate marketing campaign were highlighted and specially commented was the salmon success story. Some statements regarding the overload of information and choice that consumers have in the supermarkets were also discussed and some of the attendees agreed that to bring a new fish product to the market, the starting point should be the consumer, who will create the demand and the production technology will follow. Guus Pastor from the Dutch Fish Processors and Traders Federation (Rijswijk, The Netherlands) pointed out the need to have big volumes

available for the big players, which could be a limitation for the new aquaculture fish species to reach the market. Javier Ojeda added that the amount of environmental and administrative regulations existing in the EU also make difficult the expansion of the aquaculture industry within the EU. Some further comments on the lower price of internet sales, the probable decrease of fish availability from third countries and the impact of television cooking shows on consumer's attitude promoted quite lively discussions with special intervention of Jürgen Pauly pointing out how the salmon success has been achieved by mass production to decrease production costs.

After the debate, all the attendees to the meeting were invited to a degustation of two fish products developed within DIVERSIFY work packages: grey mullet in olive oil, elaborated by CTAQUA and fish pate (pikeperch), elaborated by IRTA.

All the presentations are available at the website of the project www.diversifyfish.eu.

Deviations from Annex I and their impact:

There were no deviations from the Annex I.

2.3 Project management during the period

Please use this section to summarise management of the consortium activities during the period. Management tasks are indicated in Articles II.2.3 and Article II.16.5 of the Grant Agreement.

Amongst others, this section should include the following:

- *Consortium management tasks and achievements;*
- *Problems which have occurred and how they were solved or envisaged solutions;*
- *Changes in the consortium, if any;*
- *List of project meetings, dates and venues;*
- *Project planning and status;*
- *Impact of possible deviations from the planned milestones and deliverables, if any;*
- *Any changes to the legal status of any of the beneficiaries, in particular non-profit public bodies, secondary and higher education establishments, research organisations and SMEs;*
- *Development of the Project website, if applicable;*

The section should also provide short comments and information on co-ordination activities during the period in question, such as communication between beneficiaries, possible co-operation with other projects/programmes etc.

Objectives

- Coordinate and implement the Technical Annex and Grant Agreement in a timely, efficient and successful manner,
- Provide the periodic reporting to the EU for the evaluation of the implementation of the programme, ensuring that correct and consistent financial and technical progress reports are submitted by participants and presented to the coordinator and submitted to the European Commission on time and in accordance with relevant guidelines,
- Organize and coordinate the work and exchange of information, samples and protocols among Partners involved in the same or different WPs,
- Organize and coordinate the work and exchange of information among Partners involved in work with the same species, but different work packages.

Modifications of management bodies

Some modifications have been made in the composition of the management bodies, which are explained below.

Steering Committee (SC): The SC consists of the PC, the GWPLs, three SME representatives (originaly P30. CULMAREX, P23. ARGO and P29. ASIALOR) and one representative from the professional associations (P12. APROMAR). During the second year of the project, P30. CULMAREX exited the consortium, so the SC was operating with one less member. Also, around the same time Mr. Kevin Debes left his position at P29. ASIALOR, and Mrs Tu-Linh Ly was appointed as the new member of the SC. Since P29. ASIALOR has also exited the consortium during the 3rd RP (see Amendement 3 later), Mrs Tu-Linh Ly has been substituted with Mr Jiri Bossuyt from the company that took the place of P29. ASIALOR (P39. F2B, **Fig. 2.3.1**)

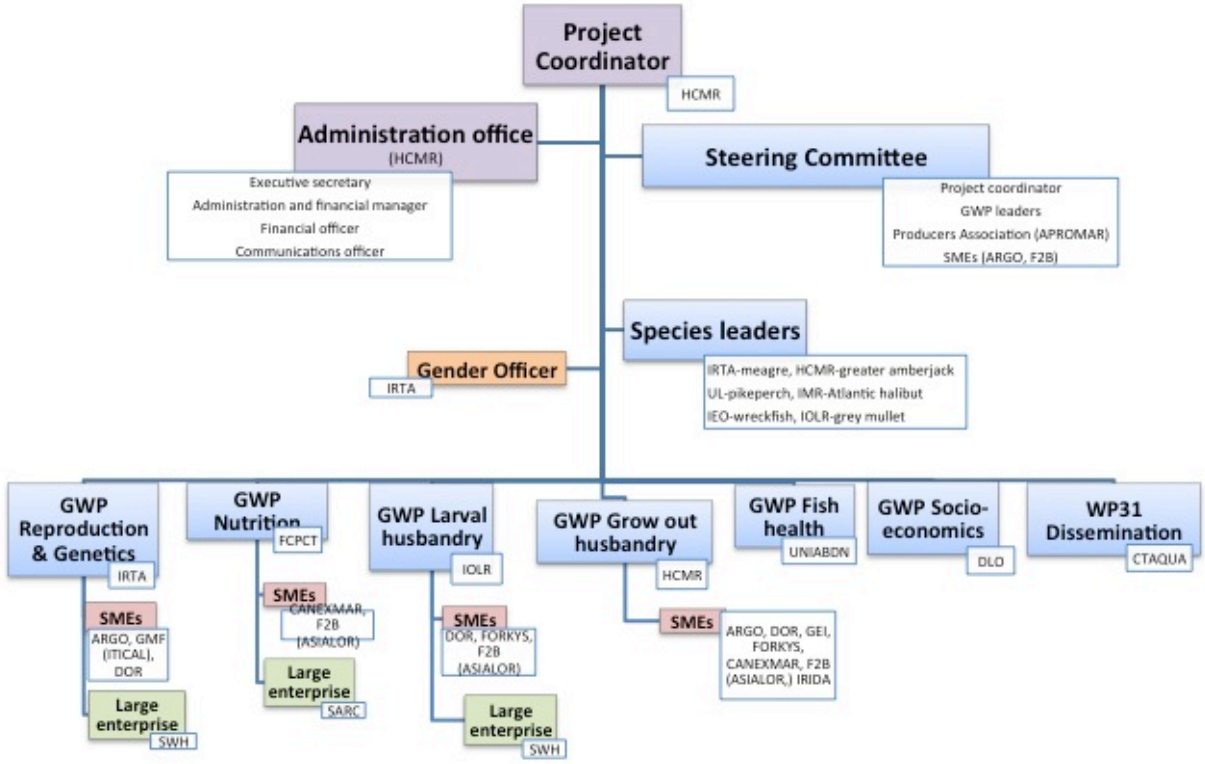

Figure 2.3.1 The management structures of the consortium DIVERSIFY, as modified due to the exit of P29 ASIALOR and the entrance of P39. F2B.

The ACM 2017 was hosted by Dr. Alicia Estevez of the Instituto de Recerca y Tecnologia Agronomica (P3. IRTA) and was held at two venues between 17-19 January 2017. The task-specific presentations during Days 1 and 2 took place at Palau Macaya. The Group Work Package (GWP) workshops took place at the Campus Del Mar of the University Pompeu Fabra. In addition, a half day meeting took place at the Hotel Ayre Rosellon on Friday 20 January 2017, for the participants of WP 30 Business model and marketing strategy development. The 3-day meeting was attended by 85 persons: 78 coming from the DIVERSIFY consortium and 8 invited guests from outside the consortium. No representative attended from three Beneficiaries (P26. GEI, P28. CANEXMAR and P37. EUFIC).

As for all previous ACMs, information regarding the meeting was uploaded continually on the project's web site (http://www.diversifyfish.eu/2017-annual-coordination-meeting-jan.html) to ensure that all participants had access to the most updated information. The Agenda (**Tables 2.3.1, 2 and 3**) was developed with assistance from GWP leaders and consisted of:

(e) DAY 1 and 2: a common session for all participants (including invited guests) presenting Task-specific presentations from various WPs, and presentations from invited guests,
(f) DAY 2: a presentation of the WP 31 Dissemination presenting the dissemination activities of the consortium, and organizing the preparation of Deliverables as well as of manuscripts for scientific articles, and
(g) DAY 3: a common session dealing with Dissemination, Scientific and Financial Reporting, and Management.
(h) In addition a brief meeting of WP 30 meeting was held on Friday 20 January

Table 2.3.1. Agenda of DAY 1 of the Annual Coordination Meeting 2017, which took place on the 16-19 January 2017, at the Palau Macaya, Barcelona, Spain.

DAY 1		17-Ιαν	Tuesday (Open Day presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration		Pick up badges
9.00	9.30		Welcome-Logistics	Alicia Estevez & CC Mylonas	
9.30	9.50		Welcome	Dr Sergi Tudela Casanovas	Director of Fisheries, Cataluna
9.50	10.10	1	Induced spawning of paired meagre with male rotation	Duncan, Neil	IRTA
10.10	10.30	2	Wreckfish reproduction status in Spain	Alvarez, Blanca	IEO
10.30	10.50	3	Some approaches to improve the nutrition and husbandry of DIVERSIFY´s target species. A U La Laguna collaborative contribution	Rodriquez, Covadonga	ULL
10.50	11.30		Coffee		
11.30	11.50	4	Effect of background color and photophase on performance of larval greater amberjack and expression of genes related to the GH/IGF axis	Tsalafouta, Aleka	HCMR
11.50	12.10	5	Prospects for probiotics with Atlantic halibut larve	Berg, Øivind	IMR
12.10	12.30	6	The effect of algal turbidity on larval performance and the ontogeny of digestive tract functionality in grey mullet	Koven, Bill	IOLR
12.30	12.50	7	Wreckfish ontogeny of the major organs related to feeding and digestion	Papadakis, Ioannis	HCMR
12.50	13.10	8	COLUMBUS Project – Knowledge Transfer for Blue Growth: Aquaculture knowledge outputs and case studie	Christofilogiannis, Panos	AQUARK (Invited)
13.10	13.30	9	Physical prototypes of new producs from the selected DIVERSIFY species	Bou, Ricard and Robles, Rocio	IRTA/CTAQUA
13.30	15.00		Lunch		
15.00	15.20	10	Epigenetics in aquaculture	Piferrer, Francesc	ICM (Invited)
15.20	15.40	11	How can CFeed copepods help bring new marine species to the table	Remman, Tore	C-Feed (Invited)
15.40	16.00	12	Results on mullet grow out in farm conditions: a multi-partner trial	Robles, Rocio	CTAQUA
16.00	16.20	13	Parasitic infections in greater amberjack in Greece	Katharios, Pantelis	HCMR
16.20	16.40	14	Construction of a genetic linkage map in meagre and identification of genetic markers related to growth for use in marker-assisted breeding programs through QTL mapping	Tsigenopoulos, Costas	HCMR
16.40	17.00	15	Consumer sensory perceptions of the selected new products from DIVERSIFY species	Guerrero, Lluis	IRTA
17.00	17.30		Coffee		
17.30	17.50	16	What do we know about the immune system of meagre and amberjack?	Milne, Douglas	UNIABD
17.50	18.10	17	Behavioral analysis of intra-cohort cannibalism in young pikeperch	Colchen, Tatiana	UL
18.10	18.30	18	Wreckfish larval rearing trials	Vilar, Antonio	MC2
18.30	18.50	19	Feeding pattern for greater amberjack: effects on growth, feed utilization and welfare	Montero, Daniel	FCPCT
20.00			Dinner at Ayre Rosellon Hotel (consortium dinner)		

Table 2.3.2. Agenda of DAY 2 of the Annual Coordination Meeting 2017, which took place on the 16-19 January 2017, at the Palau Macaya, Barcelona, Spain.

DIVERSIFY					
7FP-KBBE-2013-603121					
Meeting Agenda			2017 Annual Coordination Meeting	Barcelona 17-19 January 2017	Palau Macaya
DAY 2		18-Ιαν	Wednesday (Open Day presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration		Pick up badges
9.00	9.20	1	Protocol for the strip spawning of meagre females and in vitro fertilization	Ramos, Sandra	IRTA
9.20	9.40	2	Spawning kinetics of greater amberjack in response to multiple GnRHa injections or implants	Fakriadis, Ioannis	HCMR
9.40	10.00	3	Effects of phosphoglycerides and HUFA levels on ontogenetic development and performance of pikeperch larvae	Lund, Ivar	DTU
10.00	10.20	4	Sensory characterization of DIVERSIFY species	Grigorakis, Kriton	HCMR
10.20	10.40	5	Influence of dietary combinations of vitamin e, c and k in the development of systemic granulomatosis in meagre	Montero, Daniel	FCPCT
10.40	11.00	6	Systemic granulomatosis in meagre	Katharios, Pantelis	HCMR
11.00	11.30		Coffee		
11.30	11.50	7	Meagre behaviour and response to feeding training stimuli	Papadakis, Ioannis	HCMR
11.50	12.10	8	The effect of cage depth in the performance of meagre	Tsalafouta, Aleka	HCMR
12.10	12.30	9	Experimental consumer test of the new products from DIVERSIFY	Krystallis, Thanassis	HRH/AU
12.30	12.50	10	Spermatogenesis and sperm characteristics in captive greater amberjack	Zupa, Rosa & Fauvel, Christian	UNIBA/IFREMER
12.50	13.10	11	Why I have come to hate meagre and why amberjack is a jinxed species: 25 years of feelings & experiences from health diagnostics	Padros, Sito	Uni Autonoma Barcelona (invited guest)
13.10	15.00		Lunch		
15.00	15.20	12	Launching the new DIVERSIFY products: business models, market tests and market diffusion	Nijssen, Ed and vd Borgh, Michel	TU/e
15.20	15.40	13	Comparison of programmed and auto-demand type feeding in tanks	Duncan, Neil	IRTA
15.40	16.00	14	Multifactorial nutrition experiment in pikeperch	Kestemont, Patrick	FUNDP
16.00	16.20	15	Maturation and spawning induction of grey mullet	Rosenfeld, Hanna	IOLR
16.20	16.40	16	Atlantic halibut larval nutrition and drivers of asymmetric pigmentation and eye migration in flounders	Hamre, Kristin	NIFES
16.40	17.30		Coffee		
17.30	17.50	17	Induction of gonadal maturation in teleosts by recombinant gonadotropins	Gimenes, Ignacio	Rara Avis Biotech (invited guest)
17.50	18.10	18	Nodavirus in Atlantic halibut and possible vaccine strategies	Patel, Sonal	IMR
18.10	18.30		Dissemination activities, articles and uploading on ECAS system - Rocio Robles		
18.30	18.50		Dissemination activities, articles and uploading on ECAS system - Rocio Robles		

DAY 1 and 2 – Task-specific presentations of implemented work and invited guests

The morning session started with a welcoming presentation (**Fig. 2.3.2**) by the Project Coordinator (PC), Dr. C.C. Mylonas, presenting the Agenda for the meeting, welcoming the invited guests from outside the consortium and explaining the intentions of the consortium (as presented in the DOW, WP1 Project Management) for including other scientists and stakeholders in these ACMs. Also, Dr Sergi Tudela,

Director of Fisheries for the Catalonia government offered a welcoming. Dr. Tudela underlined the importance of DIVERSIFY for Spain and Catalonia, as the need for species diversification in the Mediterranean aquaculture has been recognized here as well.

The invited guests included Dr. Francesc Piferrer (Institute of Marine Sciences, CSIC, Barcelona, Spain), Dr. Francesc Padros (Autonomic University of Barcelona, Spain), Dr. Ignacio Gimenes (Rara Avis Biotech), Torre Remman (C-Feed S.A.), Dr Panos Christofilogiannis (AQUARK, S.A.), Mr Nigel Balmforth (5N Publishing), Mrs Rhiannon White (International Aqua Feed Magazine) and Mr. Javier Villa from a commercial aquaculture company (Andromeda SA from Greece/Spain).

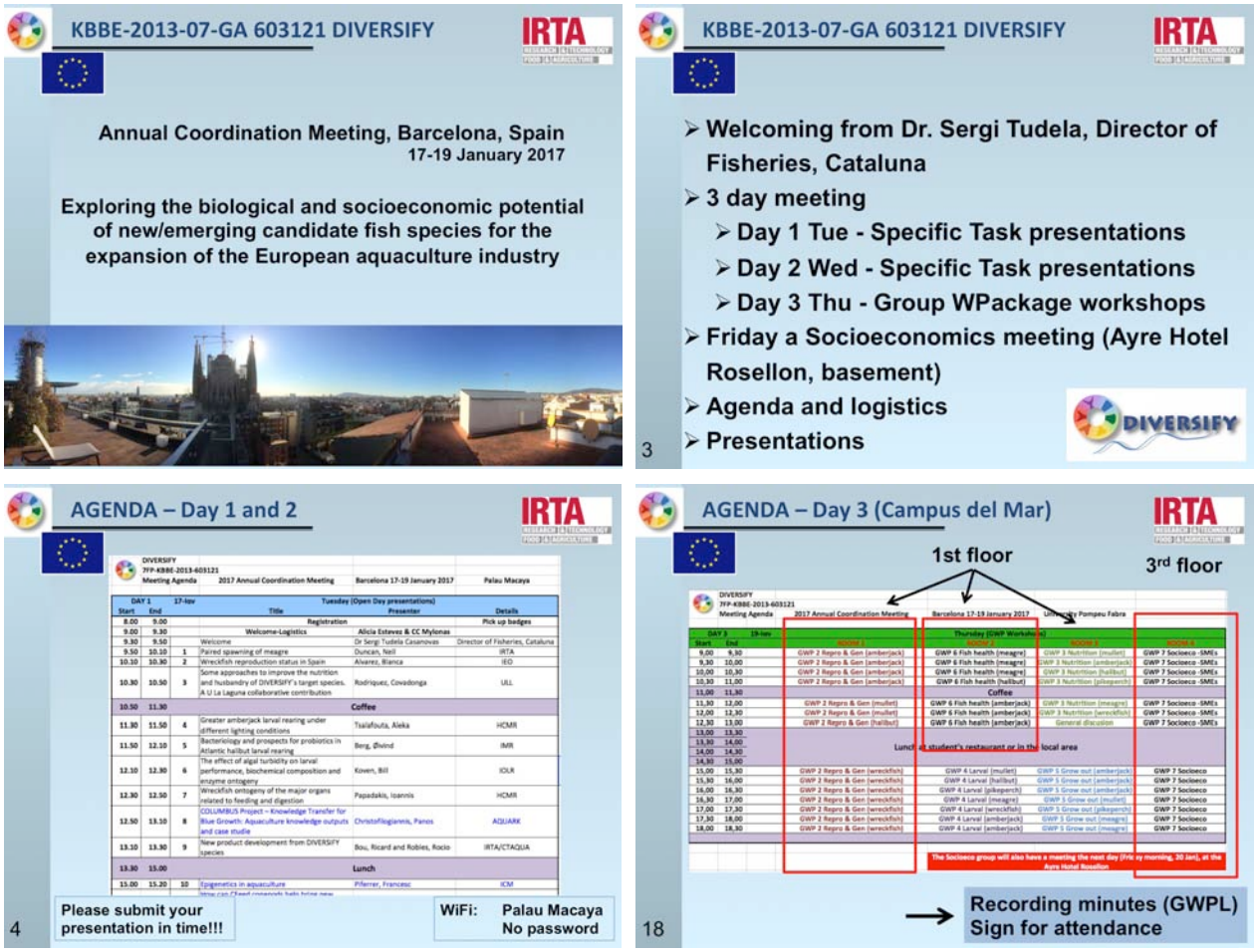

Figure 2.3.2. The opening slides for the Annual Coordination Meeting 2017, held by P3. IRTA in Barcelona, Spain, explaining the Agenda of the meeting (upper right slide) and the slides explaining the organization of the DAY 1 & 2 presentations (lower left slide) and the DAY 3 GWP workshop with the four parallel sessions (lower right slide).

The extended format of task-specific presentations for DAY 1 & 2 allowed a large number of the RTD partners to present their work –which in many cases was done in collaboration with the SMEs and Large companies participating in the project, as well as work to be presented from all Scientific Disciplines. In total, 18 RTD partners presented their work, representing collaboration with the two large companies and six SMEs from the DIVERSIFY consortium (**Fig. 2.3.3**).

Figure 2.3.3. The opening slides from some of the task-specific presentations of some of the RTD partners of the consortium during DAY 1 & 2.

The presentations from the invited guests (**Fig 2.3.4**), which followed the presentations from consortium GWP leaders and Partners, demonstrated both the interest of other organizations to participate in our ACMs and the interactions DIVERSIFY is trying to encourage with relevant researchers. Of great interest were the presentations of Dr. Francesc Piferrer (reproductive endocrinologist) on the recent knowledge of the epigenetic modification of gene expression in aquaculture, and the effects early rearing may have on sex differentiation. Also of specific interest to the DIVERSIFY consortium where the presentations of Dr Francesc Padros (fish pathologist) on his extensive experience with meagre and greater amberjack diagnostics, and of Dr Ignacio Gimenes (reproductive medicine physician) on the production of recombinant gonadotropins and their use in inducing gametogenesis in captive fishes exhibiting reproductive dysfunctions in captivity. Also, of great interest to the larval rearing scientists in the consortium was the presentation of Mr Torre Remman from C-Feed S.A., a commercial company specialing in the production of marine copepods for use as live food items for marine fish larvae. The participation of commercial aquaculture companies is also a clear indication of the relevance of DIVERSIFY to the EU industry, and the interest of their technical management to be updated with the current developments in the project. The connection with these companies also provides a means for DIVERSIFY to obtain relevant feedback from the sector, as well as having the potential to try some of the developed methodologies before the completion of the project and the release of the results. Some of these companies, such as Andromeda SA who attended the meeting for

the third year, continue to provide access to their facilities and fish stocks, and collaborate with DIVERSIFY as non-partners at no cost to the project. This ensures that expensive infrastructures and resources from outside the consortium are available to DIVERSIFY at no extra charge.

Figure 2.3.4. The opening slides from some of the presentations of some of the invited speakers on DAY 1 & 2.

Dissemination

At the end of Day 2, there was a presentation by the WP 31 Dissemination leader, Dr Rocio Robles. The presentation begun with a brief reiteration of the WP's many objectives, emphasizing the need for all Partners to participate actively in the preparation of dissemination materials and activities (**Fig. 2.3.5**). Then there was a presentation of the various dissemination activities carried out in the last 2 years (2014-2015), which included the publication of four semester Newsletters that are uploaded at the website of the project and three species-focused articles published at the quarterly magazine of the European Aquaculture Society (for greater amberjack, meagre and pikeperch). A special "DIVERSIFY" session was held at the annual conference of the European Aquaculture Society (Deliverable 31.10). The Special Session was titled "New/emerging finfish species (EU Diversify project)" and was organized in the order of the species' work in the DOW. The session opened with a summary presentation for DIVERSIFY, given by the PC of the project -see ***Deliverable 31.9 Annual presentation of DIVERSIFY (Y2) at a relevant conference***.

Figure 2.3.5. Photos from the presentation of WP31 leader Rocio Robles on Day 2.

As regards the DIVERSIFY website, the partners were informed that the website of the project (www.diversifyfish.eu) is being modified in order to make it easier for the visitors to find recent findings of the project, as well as the scientific articles that are now being produced and published (**Fig. 2.3.6**). In order to facilitate the production of short reports on implemented work and acquired results to be uploaded in our site, the Dissemination leader prepared in 2014 a format file to be used by all scientists to prepare dissemination materials, in a way that would be easy for the partners to fill. The format file is available in the INTRA page of the DIVERSIFY website. Unfortunately, not many such reports have been produced so far, and more effort must be dedicated to encourage DIVERSIFY scientists to start preparing these short dissemination material from their activities.

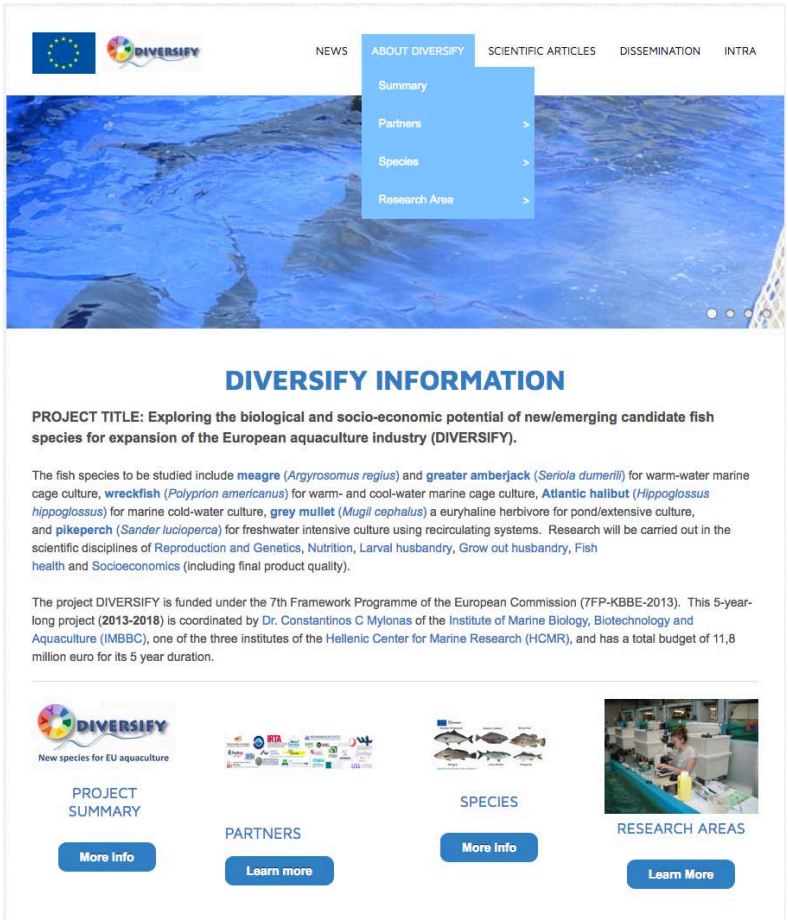

Figure 2.3.6. The new version of the project's website, modified to give more emphasis on recent activities and news, as well as the easy dissemination of the scientific articles that are now being produced at a fast pace.

The Dissemination WP leader then discussed again the issue of uploading dissemination activities ion the ECAS portal, as well as preparing the work done in DIVERSIFY for submission to scientific magazines. See also the report of WP 31 Dissemination, earlier.

In agreement with the intentions of the consortium to be as open as possible and to disseminate the results as promptly as possible, all the presentations of the ACM 2017 were uploaded on the website of the project within 2 weeks after the end of the meeting (end of January 2017), to be available to all interested stakeholders. In addition, it was agreed that all GWP leaders will submit a paragraph with the major highlights of the work implemented so far in their Scientific Disciplines, in order to prepare a 1-2 page flyer, which will then be translated to various languages by our Professional Association partners and disseminated to their members (*e.g.* in Greece, Spain, Hungary and Germany).

The next ACM was planned for 23-26 January 2018 in Tenerife, Spain. In the DOW, it was proposed that one of these meetings would be held in Norway, and would be organized by P7. IMR. However, due to the fact the time coincides with the mid of winter in this partner it was proposed by the PC, after communication with P8. IEO and P15. ULL to hold the next meeting in Tenerife, Canary Islands, Spain. This was received with enthusiasm by the Partners, therefore the next meeting will be hosted by the latter partners.

DAY 3 – Group Work Package workshops

During Day 3 of the meeting, six Workshop Sessions were organized according to Scientific Disciplines with the objective of (a) reviewing and evaluating the work carried out and (b) planning the work to be implemented in the various scientific WPs during the fourth year (2017) of the project (**Table 2.3.3**).

Table 2.3.3. Agenda of DAY 3 of the Annual Coordination Meeting 2017, which took place on the 17-19 January, at the Campus del Mar of the University Pompeu Fabra, Barcelona, Spain.

DIVERSIFY					
7FP-KBBE-2013-603121					
Meeting Agenda		2017 Annual Coordination Meeting	Barcelona 17-19 January 2017	University Pompeu Fabra	
DAY 3	19-Ιαν	Thursday (GWP Workshops)			
Start	End	ROOM 1	ROOM 2	ROOM 3	ROOM 4
9,00	9,30	GWP 2 Repro & Gen (amberjack)	GWP 6 Fish health (meagre)	GWP 3 Nutrition (mullet)	GWP 7 Socioeco -SMEs
9,30	10,00	GWP 2 Repro & Gen (amberjack)	GWP 6 Fish health (meagre)	GWP 3 Nutrition (amberjack)	GWP 7 Socioeco -SMEs
10,00	10,30	GWP 2 Repro & Gen (amberjack)	GWP 6 Fish health (meagre)	GWP 3 Nutrition (halibut)	GWP 7 Socioeco -SMEs
10,30	11,00	GWP 2 Repro & Gen (amberjack)	GWP 6 Fish health (halibut)	GWP 3 Nutrition (pikeperch)	GWP 7 Socioeco -SMEs
11,00	11,30	Coffee			
11,30	12,00	GWP 2 Repro & Gen (mullet)	GWP 6 Fish health (amberjack)	GWP 3 Nutrition (meagre)	GWP 7 Socioeco -SMEs
12,00	12,30	GWP 2 Repro & Gen (mullet)	GWP 6 Fish health (amberjack)	GWP 3 Nutrition (wreckfish)	GWP 7 Socioeco -SMEs
12,30	13,00	GWP 2 Repro & Gen (halibut)	GWP 6 Fish health (amberjack)	General discusion	GWP 7 Socioeco -SMEs
13,00	13,30	Lunch at student's restaurant or in the local area			
13,30	14,00				
14,00	14,30				
14,30	15,00				
15,00	15,30	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (mullet)	GWP 5 Grow out (amberjack)	GWP 7 Socioeco
15,30	16,00	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (halibut)	GWP 5 Grow out (amberjack)	GWP 7 Socioeco
16,00	16,30	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (pikeperch)	GWP 5 Grow out (amberjack)	GWP 7 Socioeco
16,30	17,00	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (meagre)	GWP 5 Grow out (mullet)	GWP 7 Socioeco
17,00	17,30	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (wreckfish)	GWP 5 Grow out (pikeperch)	GWP 7 Socioeco
17,30	18,00	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (amberjack)	GWP 5 Grow out (meagre)	GWP 7 Socioeco
18,00	18,30	GWP 2 Repro & Gen (wreckfish)	GWP 4 Larval (amberjack)	GWP 5 Grow out (meagre)	GWP 7 Socioeco
			The Socioeco group will also have a meeting the next day (Friday morning, 20 Jan), at the Ayre Hotel Rosellon		

The workshops of DAY 3 were running in parallel (4 Scientific Disciplines at a given time) in an attempt to minimize the potential time conflict for most Beneficiaries. The duration of each session was decided by the

GWP leader based on the number of WP included in the Scientific Discipline, as well as the amount of work that needed to be presented and discussed, and the workload expected for the upcoming year. Therefore, GWP Reproduction & Genetics and GWP Socioeconomics requested full-day Workshops, so a room was dedicated to their work. In addition, the Workshops were organized in a way that the WPs dealing with the same species were planned at different times during the Workshops, to allow all scientists attending all the WPs of the same species. This was also achieved, to a degree, by the participation to the ACM 2016 of more than one scientist from some of the beneficiaries that are involved in many GWPs. For example, P3. IRTA was represented by eight researchers and P1. HCMR by nine researchers. Unfortunately, P2. FCPCT that has the third largest budget in the project was represented only by a single scientist (Dr Daniel Montero, the GWP leader for Nutrition), while the PI of the organization was not present at this ACM also.

The minutes prepared by the GWP leader of each scientific discipline from the different GWP workshops (**Fig. 2.3.7**) were provided to the EU Scientific Officer (Dr. Marta Iglesias), together with the minutes of the whole meeting, which are presented below.

Figure 2.3.7. Photos from the DAY 3 Workshops in the various scientific discipline GWP.

Steering Committee meeting

There was no steering committed meeting at this ACM, since a number of issues were discussed via email during the previous months, in preparation of the 3rd Amendment to the DOW (see later), which was submitted initially on 30 November 2016, and then resubmitted after a minor correction at the request of the Legal officer (Mrs Patricia Oprea),on 12 January 2017.

Special meeting of WP 30. Socioeconomics

A special workshop was also planned among some of the Socioeconomics partners during the morning of Friday 20 January 2017, in order to discuss the market testing proposed in the DoW. Present were Gemma Tacken (GWP-leader), Hellas Saltavarea, Kostas Larentzakis, Lluis Guerrero, Marija Banovic, Thanasis Krystallis, Ed Nijssen and Machiel Reinders. Based on the discussion, the indication was that it is not realistic to perform the test as stated in the text of the DoW. However we first must make sure if products can be delivered and when products can be delivered. The group agreed on the following procedure:

- First check whether we can perform the market test in the original way as stated in the DoW: experimental design/ checklist for the SMEs with what we need for a real market test.
    - Ed already has this design (Taguchi-experiment)
    - We need an answer of the SMEs to have a go/ no go:
        - Availability of products
        - Availability of partners
        - Whether things can be settled in time
    - Decision should be made by the 1st of May.

The minutes prepared by the GWP leader of each Scientific Discipline from the different Workshops were provided to the EU Scientific Officer (Dr. Marta Iglesias), together with the minutes of the whole meeting. Also, the full report of the ACM 2017 has been submitted as Deliverable ***D1.9 Annual Coordination Meeting for Y4 (2017).***

Communication with the European Commission

As planned in the DOW, the EC's project Scientific Officer (Dr Marta Iglesias) has been invited to all the project meetings and was sent the detailed minutes of the Annual Coordination Meeting 2016, within a month of the meeting (Jan 2017). Deliverable ***D1.9 Annual Coordination Meeting for Y4 (2017)*** reporting on the meetings have also been uploaded on the Participants Portal.

As in the past, the PC has made every possible effort to keep the EU Scientific Officer informed in a timely manner, about any important developments, problems and major dissemination activities. We believe that an excellent communication channel exists between DIVERSIFY and the EU Scientific, Financial and Legal Officer who have been very responsive (with some exceptions) to all requests for information, in a prompt and very constructive and effective way.

Mid-term Evaluation of Progress

An external review was undertaken on the 14 September 2016, after the submission of the 2nd Period Report. The results of the review were sent to the Project Coordinator (PC) at the beginning of October 2016. Based on the overall assessment of the external reviewer "Significant progress and achievement has been by DIVERSIFY in key areas of Aquaculture …" and "…the project has achieved most of its objectives and technical goals for the period with relatively minor deviations" (**Fig. 2.3.8**). As can be seen, no major problems with the implementation of the project were identified by the external referee necessitating any modification by the consortium, while some recommendations for the enhancement of the project were proposed (**Fig. 2.3.9**).

The external review was forwarded to all Steering Committee (SC) members, who were asked to consider its conclusions and then based on their experience with the progress of the project and any difficulties phased, to propose any modifications that need to be implemented for the remaining period of the project.

1. Overall Assessment

a. Executive summary: Comments, in particular highlighting the scientific/technical achievements of the project, its contribution to the State of the Art and its impact:	
Significant progress and achievement has been made by DIVERSIFY on the key area for Aquaculture, of larval hatchery production. By studying a range of species at different stages of current knowledge and development they have gained significant knowledge in the fields of a firmer control over reproduction, better nutrition and larval husbandry issues. Much of DIVERSIFY's work in this Review Period is seeking to develop a predictable process to obtain good quality eggs (and thus a more predictable supply of juveniles). The high cost of hatchery produced fish is always a drawback for Aquaculture. Progress is being achieved in this area, technical achievements have been made in reducing the more expensive stage for meagre, this could have a significant reduction in the cost of production of meagre juveniles. Different species are at different stages of development, and knowledge. A new species like wreckfish with no Aquaculture specimens available is going to be continually breaking new ground in terms of our understanding of this deep-water finfish. Greater knowledge on this area could have commercial Aquaculture benefits or conservation Aquaculture knowledge that could be applied to other deep-water species. This species in particular could be of interest to engage the interest of the general public in the work of DIVERSIFY. Good progress is also being made in areas that are difficult to carry out such as experiments with grow out husbandry. In grey mullet, fishmeal substitution at the weaning diet stage has still been able to produce good growth performance. This could have significance in terms of reducing costs and for environmental sustainability and therefore its attractiveness as a selling point to the market. Fish Health is a key bottleneck in the development of meagre, new data and understanding is being established that could lead to better knowledge of how to approach reducing mortality losses from SG and CUD. Halibut which is more established in its development as a commercial species progress has been brought further onto the next stage to produce an antigen for the development of a vaccine against nodavirus. The Socio-economics of the projects fish species will also be making new ground. Some of the DIVERSIFY species of the study are relatively unknown to the broader European market place with little current market presence. This is a bottleneck as it can be difficult, costly but still possible (e.g. river cobbler), to develop a market demand. The segmentation study of DIVERSIFY has identified a clear group of consumers that could be 'early adapters' of the study species. This could be a 'gateway' for fish species into the market leading to other consumers to try and buy the product and develop sales in that way.	
Progress	Good progress (the project has achieved most of its objectives and technical goals for the period with relatively minor deviations)

Figure 2.3.8 The evaluation of the external review regarding the progress achieved by the consortium.

b. Overall recommendations (e.g. on overall modifications, corrective actions at WP level, or re-tuning the objectives to optimise the impact or keep up with the State of the Art, or for other reasons, like best use of resources, re-focusing...).

At this stage of the project month 30 out of a total of 60 months, there are no crucial recommendations to be made, as the work is in full progress and obviously running well with good co-operation between beneficiaries. The scientific and technical achievements that can be made in hatchery larval production are the forte of RTD science networks. The purpose of this stage of work is to come up with a predictable and affordable source of juveniles to be available in order for juveniles to be sold on and grown.

As a rule, the Aquaculture of any species, is made up of a small number of specialist technical hatcheries – supplying a larger number of on-grower units. Investment in the 6 DIVERSIFY species could bring them to a commercial reality and expand Aquaculture. On-grower interest in the future will be focussed on the price of juvenile and the logistics of the Grow out stage in particular. Creating knowledge on the Grow out stage by DIVERSIFY is an area that can optimise the project's impact to the Aquaculture sector. Any further encouragement of close co-operation with the Aquaculture grow out sector would be beneficial at this forthcoming point of the project.

However, as the project goes into the second half there is a greater emphasis planned on Socio-economic work. It could be noted that the wild fisheries has an effect on supply and demand, and thus in setting the price of all the species in the market place. This could be taken into account during the Socio-economic studies as it affects the feasibility of species for Aquaculture. Furthermore, the closer that the WPs of DIVERSIFY are to commercial retail propositions (e.g. prototypes of fish products for the market), the more the project would benefit from the experience and input from processor / retail SMEs. To always try and make sure that DIVERSIFY is market led and giving the market the type of product it wants to buy.

Figure 2.3.9. The recommendations of the external review regarding the remaining duration of the project.

Maintenance of project website

The project website continued to play an important role in the dissemination of the knowledge generated by the consortium, as well as in the communication within the consortium and management of the consortium (See Section WP 31 Dissemination in this report). Some major modifications to the appearance and organization of the website have been made, in order to better accommodate and exhibit the gathering number of scientific articles that are being produced, as well as to promote the Species-specific Knowledge Transfer workshops that are being organized for the last year of the project.

Consortium modifications

The following partners have exited the consortium

P24. ITTICAL. On 11 January 2015 Dr Fulvio Cepollaro (P.I.) informed us of his leaving the company, and that a new person (Mr. Stefano Carbonara) is taking over his duties in DIVERSIFY. On 19 July 2016 (Mo 31) during the final compilation of the 2nd Period Report, the PC received an email message from Mr Andrea Novelli, the CEO of P24. ITTICAL (SME), notifying the consortium of the intention of this partner to exit the consortium. The reasons presented were problems with the implementation of the assigned tasks, due to inadequate environmental conditions at the land-based facilities of the company. This has already been reported and approved in the 2nd Periodic Report. We have already been in contact with the EU financial (Mrs Paula Wahlman Dakhiland) and legal officers (Mrs Maria-Valeria Iliadou) about this consortium modification, and have obtained all the necessary documents and have taken the required actions.

P29. ASIALOR. In May 2016 (Mo 31) we were informed that the company has filed for bankruptcy and they are going to exit the consortium. The company has fulfilled so far their obligations and has been a valuable partner. Efforts have already been initiated to find another SME that could take on their remaining tasks. The pikeperch Species Leader Dr. Pascal Fontaine (P9. UL) has made some contacts and we have identified a potential company that is able and willing to participate in the project. The company is called

Fish 2 Be NV, and has contacted the PC with their interest to join. If we agree, we will transfer the remaining budget of P29. ASIALOR to this new partner. The negotiations will continues and as soon as we complete the 2nd Periodic Report will try to finalize them. We have already been in contact with the EU financial (Mrs Paula Wahlman Dakhiland) and legal officers (Mrs Maria-Valeria Iliadou) and have obtained all the necessary documents and have taken the required actions.

The following partners have entered the consortium

P39. F2B. The company Fish 2 Be NV, is taking over the remaining responsibilities of P29. ASIALOR, which exited the consortium at the end of the 2nd Period. It will take most of the remaining available budget from this partner.

P40. GMF. The company Galaxidi Marine Farms (GMF) is taking over the responsibilities of P24. ITTICAL related to WP 3 Reproduction & Genetics – greater amberjack, which exited the consortium at the end of the 2nd Period. The company will implement the spawning induction protocols developed by HCMR. It will take some of the remaining available budget from this partner. In addition, the company will take over the larval rearing activities from P27. FORKYS, who apparently it is not capable of completing its obligations. A budget transfer will be done to cover the costs of GMF.

3rd Amendment

An amendment was requested in November 2016 and was approved in February 2017. In addition to the exit and entry of partners, there were some changes to the DOW as described below. The modifications were discussed with and were approved by the scientific office of the project (Dr Marta Iglesias) prior to the application for the amendment.

A. Modification in the implemented work

1. **WP 2 Reproduction and genetics – meagre**. Due to recent technological and research advances concerning molecular methods to genotype and genetically characterize fish (or organisms in general) and some constraints that are related to the biology of the meagre (*Argyrosomus regius*), we propose to modify the DOW for Task 2.5, in terms of (a) the method to be used and (a) the time-schedule. No change in budget allocation, staff effort or the number of deliverables will result form this modification. This has already been reported in the 2 Periodic Report, and discussed and approved by the EU Scientific Officer (Dr Marta Iglesias, 14 April 2015).
2. **WP 3 Reproduction and genetics – greater amberjack**. Task 3.2. Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (led by HCMR) will not be implemented as planned in the DOW. Originally, it was proposed that a number of spawning performance experiments were going to be undertaken in Y2-4 with 2 broodstocks maintained in tanks by P1. HCMR, in order to determine (a) the best time of administration, (b) the method and (c) the dose of the GnRHa treatment. The main deviation from the DOW regards the failure to implement the spawning induction experiment comparing GnRHa injections and implants in the tank-reared tank of P1. HCMR, as planned for Y2 of the project. This was not completed due to failure of the breeders to reach the appropriate reproductive stage to be treated with the hormonal therapy. An attempt was made again during this Reporting Period (Mo 30-31), considering that after 3 years of acclimation to the tank system, the fish may perform better. However, again the fish at the P1. HCMR tank facilities, as well as at the P27. FORKYS facilities failed to undergo gametogenesis to the point that they could be induced to spawn with exogenous hormones. A contingency plan included the implementation of the same experiment using the sea cage-reared fish maintained in P23. ARGO, which last year matured well and responded adequately to the hormonal therapy. So, this experiment was indeed implemented during Mo 31-32 with great success, and was reported briefly in the 2nd Periodic Report. A full report will be included in the 3rd Periodic Report. No negative impact on the success of the project is foreseen from this deviation. With the addition of P40. GMF, some spawning induction experiments will also be

implemented there. This has already been reported in the 2 Periodic Report, which has been approved by the EU Scientific Officer.

Also, Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic (led by IEO) is not implemented as planned in the DOW, due to a significant loss of a large part of the original broodstock during Y1. In the DOW, it was proposed to study the reproductive performance of an F1 broodstock treated with different doses of GnRHa in a controlled – release delivery system (implants) in a dose-response experiment during Y2. Due to the loss of a large number of breeders, this is no longer possible due to a small number of available fish. Instead, one GnRHa dose was tested in Y2 (50 μg kg^{-1}) and another is currently being tested in Y3 (75 μg kg^{-1}) during Mo 30 – 33. A third dose will be examined in Y4, (25 μg kg^{-1}) in the same broodstock, under similar rearing (*e.g.* tank, treatment method and sampling) and environmental conditions (*e.g.* treatment time, water quality, etc.). We believe this approach is also valid, albeit not the optimal, to address the objectives proposed in the DOW. Already, the second dose used in Y3 (2016) has been producing very good results, as reported in the 2nd Periodic Report. This has already been reported in the 2 Periodic Report, which has been approved by the EU Scientific Officer.

3. **WP 24 Fish Health – meagre**. In Task 24.4 we had anticipated that we would isolate *Nocardia* from cultured meagre. *Nocardia* has been considered a possible threat since it was connected with SG. However, the pathogen has not been found or isolated from any of the fish examined. The isolation of *Nocardia* is a prerequisite for the development of the autogenous vaccine in the same task and for the vaccine to be tested in subsequent trials in P3. IRTA. Since we have been unable to culture *Nocardia* from infected fish (in subtask 24.4.1), we now plan to go forwards with optimisation and evaluation of a Vibrio (*V. anguillarum*) vaccine for meagre. Fish will be vaccinated and 8 weeks later challenged. Immune tissue samples will be collected post-challenge for gene expression analysis and histology. We will also determine the pathogen load in the samples by PCR, in addition to evaluating the degree of protection seen in parallel tanks of fish. This work will result in the modification of ***Deliverable 24.12***, which will now be "Determination of the efficacy of vaccination of meagre against Vibriosis", and ***Deliverable 24.13***, which will now be "Description of immune gene expression post-immunisation and challenge of meagre with a *Vibrio* vaccine". This has already been reported in the 2 Periodic Report, which has been approved by the EU Scientific Officer.

B. Modifications related to specific partners

4. **P1. HCMR.** An increase of the budget of HCMR will result from a shift from the budget allocated to one of the exiting partners (P24. ITTICAL), as more experiments in Task 3.2 Spawning induction of greater amberjack in the Mediterranean, have been planned for the remaining two years, to be done at the facilities of P23. ARGO. This has already been reported in the 2nd Periodic Report, which has been approved by the EU Scientific Officer.

5. **P6. SRW (previous DLO).** For embedding the project results in marketing plans and feasibility output, more sector knowledge is necessary. Therefore SRW needs more money to support TU/e in this task. A small increase in budget has been requested and agreed by the PC.

6. **P8. IEO.** On 26 May 2016, we were informed that there was a change in the P.I. of the partner, from Dr. Jose Benito (Tito) Peleteiro who retired on 30 June 2016, to Dr (Mrs) Montserrat Perez. The official letters required for the change have been forwarded to the EU Legal and Scientific Officers, with an email from the PC on 2 June 2016. Also, the position of Dr. Peleteiro as the Species Leader for wreckfish will be taken by Dr (Mrs) Blanca Alvarez from IEO, who is also going to be the WP Leader of WP 6 Reproduction and Genetics –wreckfish and WP 18 larval husdandry - wreckfish. This has already been reported in the 2nd Periodic Report, which has been approved by the EU Scientific Officer.

7. **P11. AU**. Due to the fact that Prof. A. Krystallis, the PI of P11. AU will relinquish his position at AU, the partner has requested a change in the PI to Professor Klaus Grunert that some of its tasks in WP 29 (Sub-tasks 29.3.2 and Task 29.4) be transferred to P38. HRH, along with the appropriate budget of 28,335 plus overheads (EU contribution). This has already been reported and approved in the 2nd

Periodic Report. We include official letters from both P11. AU, P38. HRH, Prof. A. Krystallis and Prof. K. Grunert, indicated their approval of the above changes. This has already been reported in the 2nd Periodic Report, which has been approved by the EU Scientific Officer.

8. **P18. CTAQUA**. This partner requests a budget shift among WPs. At the middle of the project, most of the work within WP28 Socioeconomics-New Product development (R&D activity), is finalized. However, there are other WPs responsibilities of this partner that will need further economic support. One of these cases is Task 23.4 "Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild juveniles at two different densities in ponds in Spain", which is still running and will last for a while longer. It is foreseen that more support will be needed to finalize the task properly, since there is quite some analytical work involved from the final samples to be taken at the end of the grow-out period. Likewise in sub-task 13.3.5 "Comparison of vegetable oil-no fish meal grow-out diet with an n-3 HUFA rich fish meal finishing diet on the nutritional and organoleptic values of fish flesh and bottarga quality", the budget will not be sufficient to correctly cover the obligations of the partner within this task. So the partner requests a shift from WP28 to WPs 23 and 13.

   Also, in the case of WP 31 Dissemination, there are important activities to come in the next reporting periods. Tasks 31.5 and 31.6 will require a considerable amount of travels and staff dedication to the correct organizations of these events, which we find out will not be covered sufficiently with the budget allocated in the DOW for WP31. So the partner requests a transfer from WP28 to WP31. The total EU contribution for P18. CTAQUA will not be changed with the proposed activities, but the allocation of the budget between RTD and DISSEMINATION activities, and among WPs will be modified. This has already been reported in the 2nd Periodic Report, which has been approved by the EU Scientific Officer.

   Another aspect to be commented about this partner, is the fact that the PI Rocio Robles will work for the partner as consultant from November 2016 onwards. Her role in the project will remain as described in the DOW, but her contractual relation with the partner will be established on a consultant basis, in accordance to the conditions established in the Financial Guidelines for FP7 projects (p 61).

9. **P19. CMRM**. Due to the fact that the wreckfish stock of this partner did not mature the first year of the study (although they were of the right size, so we did not expect this development), they had to put much more staff effort to this Task (WP 6). As a result, they had to dedicate more staff effort (from existing personnel), but they will not need any additional budget (they will use lower cost personnel). So, in essence they will not require any more money, but they will claim more staff effort (from 13.6 in the original DOW to 18.8 PM now). This has already been reported in the 2nd Periodic Report, and discussed and approved by the EU Scientific Officer (email of 21 Dec 2015).

10. **P23. ARGO.** To address some of the problems that we faced in Task 3.2. Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (described above in **Item 2. WP 3**) we have planned to be implemented a number of spawning performance experiments using the sea cage-reared fish maintained in P23. ARGO, which last year matured well and responded adequately to the hormonal therapy. A slight increase in the budget of this parnter has been made to allow them to contribute further to this work. This has already been reported in the 2nd Periodic Report, which has been approved by the EU Scientific Officer.

11. **P24. ITTICAL.** On 11 January 2015 Dr Fulvio Cepollaro (P.I.) informed us of his leaving the company, and that a new person (Mr. Stefano Carbonara) is taking over his duties in DIVERSIFY. On 19 July 2016 (Mo 31) during the final compilation of the 2nd Period Report, the PC received an email message from Mr Andrea Novelli, the CEO of P24. ITTICAL (SME), notifying the consortium of the intention of this partner to exit the consortium. The reasons presented were problems with the implementation of the assigned tasks, due to inadequate environmental conditions at the land-based facilities of the company. This has already been reported and approved in the 2nd Periodic Report. We have already been in contact with the EU financial (Mrs Paula Wahlman Dakhiland) and legal officers (Mrs Maria-Valeria Iliadou) about this consortium modification, and have obtained all the necessary documents and have taken the required actions.

A contingency plan for the exit of P24. ITTICAL includes the transfer of this partner's activities to a combincation of existing and new partners. Regarding WP 3 Reproduction and Genetics – greater amberjack, the spawning induction tasks (Task 3.2) will be allocated to a new partner SME (Galaxidi Marine Farms, GMF), which is not a member of the consortium, but has so far dedicated their greater amberjack broodstock and facilities to the experiments of WP 3 for no charge to the Consortium, and has also performed exceptionally so far. This company also runs a marine hatchery and sea-cage grow out sites, and if needed it will be able to participate also in the implementation and the industrial trials on the larval rearing and grow out.

Regarding WP 7 Reproduction and Genetics – grey mullet, the Task 7.4 of rearing wild grey mullet fingerlings to reproductive maturation for the evaluation of "bottarga" production under complete captivity can be implemented by P4. IOLR and P26. GEI, who have already acquired wild fingerlings during Y1 of the project, in order to implement other tasks. They have already been contacted and they are willing to undertake this responsibility.

12. **P26. GEI.** This partner is involved in WP 23 Grow out husbandry – grey mullet, and with a small increase in budget has accepted to take the responsibility of maintaining a number of fish until reproductive maturation for the evaluation of "bottarga" production under complete captivity.
13. **P29. ASIALOR.** In May 2016 (Mo 31) we were informed that the company has filed for bankruptcy and they are going to exit the consortium. The company has fulfilled so far their obligations and has been a valuable partner. Efforts have already been initiated to find another SME that could take on their remaining tasks. The pikeperch Species Leader Dr.Pascal Fontaine (P9. UL) has made some contacts and we have identified a potential company that is able and willing to participate in the project. The company is called Fish 2 Be NV, and has contacted the PC with their interest to join. If we agree, we will transfer the remaining budget of P29. ASIALOR to this new partner. The negotiations will continues and as soon as we complete the 2nd Periodic Report will try to finalize them. We have already been in contact with the EU financial (Mrs Paula Wahlman Dakhiland) and legal officers (Mrs Maria-Valeria Iliadou) and have obtained all the necessary documents and have taken the required actions.
14. **P34. BVFi.** This partner has been involved in the dissemination activities of the consortium and is very interested in contributing more effort towards this direction. We have increased slightly its budget to allow the partner to contribute more to the project.
15. **P38. HRH.** Due to the fact that Prof. A. Krystallis, the PI of P11. AU will relinquish his position at this Partner (see above at P11. AU), it has been requested that some of its activities in WP 29 (Sub-tasks 29.3.2 and Task 29.4) be transferred to P38. HRH, along with the appropriate budget. HRH has accepted this transfer and this has already been reported and approved in the 2 Periodic Report.

    In addition, P38. HRH made a formal request to the PC to increase its EU budget in order to address some higher than expected costs, involved in the implementation of Tasks 28.1 and 29.2 in the five selected countries. The higher costs are due the following reasons:

    a. It was decided by the GWP Socioeconomics participants in the 1st ACM (Bari, Italy) to conduct 2 Focus Groups (per country) instead of 1, and 3 Expert Interviews (per country) instead of 5 for Task 28.1. This decision was based on budget constrains and it was also decided that the local partners would help in Germany and Spain, in order to reduce the cost of subcontracting. However, the additional expenses for 1 more Focus Group were much higher than the savings from the 2 less Expert Interviews,

    b. Regarding the hedonic sensory tests of Task 29.2, it was decided by the GWP Socioeconomics participants in the 2nd ACM (Nancy, France), to increase the sample of consumers to 100, instead of 80 that is mentioned in DOW, in order to improve the validity of the results. This change created higher recruiting and lab rental expenses, as well as hosting and processing higher, at the level of 25% more than budgeted,

    c. Regarding the experimentation with product mock-ups in the five countries investigated (Tasks 29.6 and WP29.7) and the identification of the optimal intrinsic-extrinsic product quality profiles, based on the sensory testing results across the 5 countries, there was a

decision to increase the sample size to 300 per country instead of the original 200. This increase will give the opportunity to accommodate for three products deemed as promising in the previous tasks in the currently running of Task 29.3 (same 3 across all five countries). This is a different product number from the initially planned number of two products per country, which has been the plan on which the initial budget was estimated upon. WP-29 partners (i.e. AU, HRH, IRTA, CTAQUA) strongly believe that testing 3 products would be ideal for the project, since it would be a sub-optimal practice to waste useful findings from the very expensive sensory part against a request for a rather small budget. In particular, assuming that the decision is that three products are going to be developed in Task 29.3, each product should be evaluated by 100 consumers, so three products would command 300 participants in total (per country). So, to cover the extra costs of using more consumers and more mock-ups, a budget increase was allocated to this Partner.

16. **P39. F2B.** The company Fish 2 Be NV, is taking over the remaining responsibilities of P29. ASIALOR, which exited the consortium at the end of the 2nd Period. It will take most of the remaining available budget from this partner.
17. **P40. GMF.** The company Galaxidi Marine Farms (GMF) is taking over the responsibilities of P24. ITTICAL related to WP 3 Reproduction & Genetics – greater amberjack, which exited the consortium at the end of the 2nd Period. The company will implement the spawning induction protocols developed by HCMR. It will take some of the remaining available budget from this partner.

4th Amendment

Another amendment (the last one!) will be requested in the Spring of 2017, to address some modifications in the implemented work (transfer from one partner to another; modification of the planned work, etc.), as well as some budgetary issues. As always, the modifications have already or will be discussed with and approved by the scientific office of the project (Dr Marta Iglesias) prior to the application for the amendment.

3.3 Deliverables and milestones tables

Deliverables

The project has a total of 202 Delieverbales. So far, 111 Deliverables have been submitted and 38 are delayed (19% of Total). We do not expect that any deliverables will not be completed and submitted by the completion of the project. To enable the Scientific Officer to identify the delays in the Deliverables and Milestones, those are shown in yellow highlight.

TABLE 1. DELIVERABLE

Del. no.	Deliverable name	Version	WP no.	Lead beneficiary	*Nature*	Dissemination level[3]	Delivery date from Annex I (proj month)	Actual / Forecast delivery date Dd/mm/yyyy	Status Not submitted/ Submitted	Comments
1.1	Kick-off meeting and Annual coordination meeting for Y1	1	1	1	Other	RE	2	10/02/2014	Submitted	Due to the project starting in December, it was not possible to have the meeting during month 1 of the project.

[3] **PU** = Public
PP = Restricted to other programme participants (including the Commission Services).
RE = Restricted to a group specified by the consortium (including the Commission Services).
CO = Confidential, only for members of the consortium (including the Commission Services).
Make sure that you are using the correct following label when your project has classified deliverables.
EU restricted = Classified with the mention of the classification level restricted "EU Restricted"
EU confidential = Classified with the mention of the classification level confidential " EU Confidential "
EU secret = Classified with the mention of the classification level secret "EU Secret "

1.2	Consortium Agreement	1	1	1	Other	CO	3	20/03/2014	Submitted	A delay was due to one Partner not being able to sign the CA (P32. MC2)
1.3	Annual Coordination Meeting for Y2	1	1	1	Other	RE	12	28/11/2014	Submitted	
1.4	Periodic Report, including financial and administrative reports for Mo 1-12	1	1	1	Report	RE	14	30/12/2014 and 20/1/2015	Submitted	The Financial Report was submitted a month later than the Scientific Report
1.5	Interactions with other projects	1	1	1	Report	PU	24	29/05/2015	Submitted	
1.6	Annual Coordination Meeting for Y3	1	1	1	Report	PU	24	07/03/2016	Submitted	
1.7	Midterm evaluation of progress	1	1	1	Report	PU	30	22/11/2016	Submitted	
1.8	Periodic Report, including financial and administrative reports for Mo 13-30	1	1	1	Report	PU	32	01/08/2016	Submitted	
1.9	Annual Coordination Meeting for Y4	1	1	1	Report	PU	37	31/01/2017	Submitted	
2.1	SNP library and chip to genetically characterise meagre or to use in marker assisted breeding programs.	1	2	1	Report	PU	18	01/06/ 2015	Submitted	
2.2	Genetic characterization of different meager captive broodstocks and evaluation of available variability	1	2	2	Report	PU	12	15/12/2014	Submitted	

2.3	Protocol for paired spontaneous tank spawning of meagre.	1	2	3	Report	PU	21	22/09/2015	Submitted	
2.4	Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre	1	2	1	Report	PU	36	28/11/2016	Submitted	
2.5	Genetic characterisation of fast and slow growing meagre	1	2	1	Report	PU	36	30/11/2016	Submitted	
2.6	Description of sperm characteristics and cryopreservation protocol of meagre sperm	1	2	14	Report	PU	36	08/11/2016	Submitted	
2.7	Protocol for the strip spawning of meagre females and in vitro fertilization	1	2	3	Report	PU	36	17/11/2016	Submitted	
3.1	Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor).	1	3	4	Report	PU	12	17/03/2015	Submitted	
3.2	Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack	1	3	4	Report	PU	18	19/12/2017	Submitted	
3.3	Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in	1	3	13	Report	PU	24	26/01/2016	Submitted	

	the wild, in terms of proliferating									
3.4	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of greater amberjack sperm	1	3	14	Report	PU	32	22/07/2016	Submitted	
3.5	Description of the process of oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH	1	3	13	Report	PU	46	06/12/2017	Submitted	
3.6	Description of the process of spermatogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of testicular development, (c) pituitary level	1	3	13	Report	PU	46	06/12/2017	Submitted	
3.7	Comparative effectiveness of a GnRHa injection vs GnRHa implant treatment for the induction of spawning of greater amberjack in the eastern Atlantic	1	3	2	Report	PU	48	31/10/2017	Submitted	
4.1	Genetic analysis of domesticated pikeperch broodstocks	1	4	9	Report	PU	12	19/11/2014	Submitted	
4.2	Population genetic analysis of	1	4	1	Report	PU	16	19/03/2015	Submitted	

	wild and comparison with domesticated pikeperch populations to be applied in future breeding programs of the species									
5.1	Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut	1	5	7	Report	PU	30	27/9/2016	Submitted	
5.2	An optimised GnRHa therapy protocol to improve spawning performance of F1/F2 Atlantic halibut, and to increase availability of eggs of stable and predictable quality	1	5	7	Report	PU	30	12/05/2016	Submitted	
5.3	Identification of potential disturbances in reproductive development in F1/F2 Atlantic halibut females	1	6	14	Report	PU	24	01/06/2018	Delayed	A postponement was agreed at the ACM 2016 (D1.6)
6.1	Computer Assisted Sperm Analysis (CASA) for wreckfish sperm	1	6	14	Report	PU	24	27/11/2015	Submitted	
6.2	Cryopreservation method for wreckfish	1	7	14	Report	PU	12	12/12/2014	Submitted	
6.3	Spawning induction methods with in vitro fertilization of wreckfish	1	7	8	Report	PU	24	1/10/2018	Delayed	Due to an accident of the lead beneficiary and an accompanied sick leave, this will be delayed to March 2018
6.4	Establish reliable collection methods and protocols to form new wreckfish broodstocks	1	7	19	Report	PU	24	01/10/2018	Delayed	More work needs to be done, in order to collect the

										necessary data
6.5	Description of the reproductive cycle of wreckfish	1	7	8	Report	PU	48	01/06/2018	Delayed	We are missing the data on FSH/LH content in the brain. Everything else is completed
6.6	An in vitro fertilization protocol to be employed by the industry to spawn wreckfish	1	7	8	Report	PU	54	01/08/2018	Delayed	More work needs to be done, in order to collect the necessary data
7.1	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of grey mullet sperm	1	7	4	Report	PU	18	12/06/2015	Submitted	
7.2	Production of recombinant bioactive LH and FSH assay for grey mullet	1	7	4	Report	PU	24	27/11/2015	Submitted	
7.3	Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet	1	7	4	Report	PU	24	30/11/2015	Submitted	
7.4	Protocol for shipping grey mullet eggs	1	3	4	Report	PU	24	10/1/2017	Submitted	
7.5	Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development	1	3	13	Report	PU	48	30/03/2018	Delayed	Delays from IOLR. Everything else ready to submit
8.1	Improvement of larval weaning diets	1	8	2	Report	PU	24	3/12/2015	Submitted	

8.2	Recommended essential fatty acids contents in diets to promote meagre growth, welfare and health	1	8	2	Report	PU	48	25/11/2017	Submitted	
9.1.	Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids	1	9	2	Report	PU	24	16/12/2015	Submitted	
9.2	Lys requirements of greater amberjack juveniles	1	9	1	Report	PU	36	20/02/2017	Submitted	
10.1	Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch	1	10	21	Report	PU	36	31/05/2018	Delayed	Part of the study has been postponed to 2018 UNAMUR)
10.2	Protocol for optimal early fatty acid enrichment to reduce stress sensitivity in pikeperch	1	10	21	Report	PU	36	21/12/2017	Submitted	
10.3	Formulation for a diet better adapted to pikeperch requirements	1	10	39	Report	PU	48	01/10/2018	Delayed	The exit of ASIALOR and entry of new SME caused some delay
11.1	Report on nutrient profile of Artemia nauplii and ongrown Artemia from IMR and SWH	1	11	7	Report	PU	24	28/11/2015	Submitted	
11.2	Report on optimal characteristics of feed particles and feeding environment for early weaning of Atlantic halibut larvae	1	11	7	Report	PU	36	28/11/2016	Submitted	
11.3	Report on the nutrient retention and digestive	1	11	17	Report	PU	36	28/11/2016	Submitted	

	physiology in Atlantic halibut larvae fed *Artemia nauplii* and on-grown *Artemia*									
11.4	Report on the nutrient retention and digestive physiology in Atlantic halibut larvae reared in RAS vs FTS	1	11	17	Report	PU	36	28/11/2016	Submitted	
11.5	Report on the effect of dietary phospholipids on Atlantic halibut juveniles	1	11		Report	PU	48	31/08/2018	Delayed	An error occurred on the diet preparation, making difficult the interpretation of the results. Some more analyses will be done, to overcome this problem.
13.1	Determine changes in the essential fatty acid requirement as a function of developmental stage and ambient salinity in grey mullet	1	13	4	Report	PU	18	30/11/2018	Delayed	The lead beneficiary is working on the preparation of the report
13.2	Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet	1	13	4	Report	PU	18	30/11/2018	Delayed	The lead beneficiary is working on the preparation of the report
13.3	Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression	1	13	4	Report	PU	36	30/11/2018	Delayed	The lead beneficiary is working on the preparation of the report.

	accumulation									
13.4	Determine the effects of essential fatty acids and Tau in non-fish meal feeds on flesh and bottarga quality in grey mullet	1	13	4	Report	PU	48	30/11/2018	Delayed	The lead beneficiary is working on the preparation of the report.
14.1	Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles		14	3	Report	PU	30	16/05/2016	Submitted	
15.1	Effective greater amberjack larval stocking densities	1	15	2	Report	PU	16	09/05/2016	Submitted	
15.2	Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing	1	15	8	Report	PU	27	16/02/2017	Submitted	
15.3	Optimum hydrodynamics and light conditions during greater amberjack larval rearing	1	15	2	Report	PU	27	16/03/2017	Submitted	
15.4	Ontogeny of greater amberjack larval visual and digestive system	1	15	1	Report	PU	27	23/05/2016	Submitted	
15.5	An industrial protocol for greater amberjack larval rearing	1	15	8	Report	PU	48	31/05/2018	Delay	Not enough eggs were available in 2016 to carry all planned experiments, so this was pushed to 2017. Data is currently under

										analysis
16.1	Determine effect of environmental factors on pikeperch larval rearing	1	16	9	Report	PU	12	28/7/2016	Submitted	Due to a catastrophic event in the new facilities of the partner (high ammonia, due to immature biological filter) the trials had to be repeated, and this caused problems in the planning of the associated analyses. The work is now completed and is being written up to prepare the deliverable.
16.2	Determine effect of nutritional factors on pikeperch larval rearing	1	16	9	Report	PU	24	27/11/2016	Submitted	
16.3	Determine effect of population factors on pikeperch larval rearing	1	16	9	Report	PU	36	31/10/2017	Submitted	
16.4	Identification of optimal combinations of factors for pikeperch larval rearing	1	16	9	Report	PU	48	31/05/2018	Delayed	Difficulties in obtaining larvae pushed the experiments for later.
17.1	Production protocol of on-grown Artemia	1	17	7	Report	PU	24	28/11/2015	Submitted	
17.2	Determine if RAS is a more	1	17	7	Report	PU	36	30/03/2018	Delayed	The lead

	effective protocol than FT for Atlantic halibut larvae									beneficiary is working on the data analysis
17.3	The effect of probiotics on Atlantic halibut larval microbiota and survival	1	17	7	Report	PU	36	31/03/2018	Delayed	The lead beneficiary is working on the data analysis
17.4	Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance	1	17	7	Report	PU	48	01/12/2016	Submitted	
17.5	Development of an industrial protocol for probiotic treatment of halibut larvae	1	17	7	Report	PU	48	30/04/2018	Delayed	
18.1	Development of the digestive system of wreckfish	1	18	1	Report	PU	36	31/10/2017	Submitted	
18.2	Determine optimum temperature conditions for rearing wreckfish larvae	1	18	8	Report	PU	36	31/03/2018	Delayed	It has been proven very difficult to culture wreckfish larvae so far, and we hope for better success in the last year of the project
18.3	Develop a feeding protocol for wreckfish larvae	1	18	1	Report	PU	36	30/11/2018	Delayed	It has been proven very difficult to culture wreckfish larvae so far, and we hope for better success in the last year of the project
18.4	Determine the most effective	1	18	8	Report	PU	36	31/03/2018	Delayed	It has been proven

	culture system (RAS vs flow-through) for wreckfish larvae									very difficult to culture wreckfish larvae so far, and we hope for better success in the last year of the project
19.1	Determine most effective type and concentration of algae used in grey mullet larval rearing	1	19	4	Report	PU	24	20/11/2016	Submitted	
19.2	Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production	1	19	4	Report	PU	36	31/10/2018	Delayed	The lead beneficiary is working on the data analysis
19.3	Determine weaning time and type of feed according to the shift from carnivorous to omnivorous feeding	1	19	4	Report	PU	36	14/11/2017	Submitted	The lead
19.4	Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing	1	19	4	Report	PU	48	31/10/2018	Delayed	The lead beneficiary is working on the data analysis
20.1	Methodology to avoid size variability in meagre juveniles	1	20	3	Report	PU	24	28/11/2015	Submitted	
20.2	Definition of the optimum conditions for cage culture of meagre	1	20	3	Report	PU	39	31/03/2018	Delayed	The exit of partner CULMAREX and the rearrangement of the proposed work in Amendment 2 caused some delays
20.3	Methodology for meagre	1	20	3	Report	PU	42	31/05/2018	Delayed	The exit of partner

	feeding									CULMAREX and the rearrangement of the proposed work in Amendment 2 caused some delays
22.1	Effects of multiple variables on stress, immune response and growth performances and recommendations of optimal conditions for pikeperch grow out	1	22	16	Report	PU	24	17/5/2016	Submitted	
22.2	Validation of optimal rearing variables under commercial farm conditions	1	22	39	Report	PU	42	31/05/2018	Delayed	The exit of partner ASIALOR and the rearrangement of the proposed work in Amendment 3 to Partner F2B caused some delays.
22.3	Effects of domestication level and geographical origin on stress, immune response and growth performances and strain recommendation	1	22	16	Report	PU	48	31/05/2018	Delayed	The exit of partner ASIALOR and the rearrangement of the proposed work in Amendment 3 to Partner F2B caused some delays.
23.1	Cost-effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status	1	23	3	Report	PU	18	1/10/2015	Submitted	

23.2	Stocking protocols for pond monoculture grow out of F1 and wild caught grey mullet	1	23	4	Report	PU	30	31/5/2018	Delayed	The lead beneficiary is working on the data analysis
23.3	Comparison of the project's improved grey mullet grow-out feed under the different environmental and water conditions in Israel, Greece and Spain	1	23	4	Report	PU	40	31/5/2018	Delayed	The lead beneficiary is working on the data analysis
24.1	The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre	1	24	1	Report	PU	20	7/1/2016	Submitted	
24.2	The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre	1	24	1	Report	PU	24	15/06/2016	Submitted	
24.3	Cloning of key marker genes of innate and adaptive immune responses in meagre	1	24	5	Report	PU	26	20/01/2016	Submitted	
24.4	Isolation and characterization of Nocardia from infected meagre	1	24	1	Report	PU	36	13/12/2016	Submitted	
24.5	The effect of high plant protein diets in the development of Systemic Granulomatosis in meagre	1	24	1	Report	PU	36	25/05/2017	Submitted	
24.6	Experimental vaccine for Nocardia for meagre	1	24	1	Report	PU	42	31/07/2018	Delayed	Complications in finding juveniles from the market that are not vaccinated have

										caused delays, as the experiments need to be repeated
24.7	Diagnostics protocol for Chronic Ulcerative Dermatopathy in meagre, aetiological factors and solutions	1	24	1	Report	PU	44	31/01/2018	Delayed	This delay is due to the fact that we have done more experiments to support our hypotheses.
24.8	Report on the prevention/treatment of Chronic Ulcerative Dermatopathy in meagre	1	24	1	Report	PU	44	31/01/2018	Delayed	This delay is due to the fact that we have done more experiments to support our hypotheses.
24.9	Determination of effective treatments for common monogenean parasites in meagre	1	24	3	Report	PU	48	31/03/2018	Delayed	The lead beneficiary is working on the data analysis.
25.1	Marker genes of mucosal immunity in greater amberjack cloned and ways to increase their expression level determined	1	25	5	Report	PU	39	16/03/2017	Submitted	
25.2	Mucus defences of greater amberjack analysed and immune potential characterised	1	25	2	Report	PU	39	26/05/2017	Submitted	
25.3	Impact of dietary regime on parasite resistance and mucosal defences of greater amberjack juveniles	1	25	5	Report	PU	42	27/07/2017	Submitted	
25.4	Protocol for early diagnosis of	1	25	1	Report	PU	44	31/01/2018	Delayed	This delay is due

	epitheliocystis during early stages of greater amberjack culture									to the fact that we have done more experiments to support our hypotheses.
26.1	Assess the use of two eukaryotic expression systems; microalgae and a protozoa (*Leishmania tarentolae*) for production of nodavirus capsid protein	1	26	7	Report	PU	24	13/11/2015	Submitted	
26.2	Testing of the delivery of vaccine candidates through Artemia to Atlantic halibut larvae	1	26	7	Report	PU	30	30/04/2018	Delayed	Need to obtain a different batch of more uniform larvae to perform the experiment delayed the vaccination. Data are currently being processed.
26.3	Determine immune response and effectiveness of orally delivered VNN capsid protein on protection of Atlantic halibut larvae	1	26	7	Report	PU	40	30/04/2018	Delayed	Need to obtain a different batch of more uniform larvae to perform the experiment delayed the vaccination. Data are currently being processed.
27.1	Report on external environmental factors that affect or will affect the production chains of meagre, greater amberjack, pikeperch,	1	27	6	Report	PP	14	22/05/2014	Submitted	The time required for this Deliverable was under-estimated

	Atlantic halibut, wreckfish and grey mullet									
27.2	Report on current certification schemes and standards and their business dynamics in the fish supply chain	1	27	6	Report	PP	14	7/03/2014	Submitted	
27.3	Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet	1	27	6	Report	PU	12	15/01/2015	Submitted	
27.4	Report on trend mapping for the European aquaculture, seafood sector and protein market in the (near) future	1	27	6	Report	PU	12	5/12/2014	Submitted	
27.5	Report with results of international survey on industrial buyers' attitudes and perceptions regarding cultured fish	1	27	6	Report	PU	12	28/11/2014	Submitted	
27.6	List of critical success factors for market acceptance	1	27	6	Report	PU	12	20/02/2015	Submitted	
27.7	Report on the analysis of the business models and supply chains of the participating SME's	1	27	6	Report	PU	12	28/11/2014	Submitted	
28.1	Report with results of focus groups with consumers and experts regarding ideas for new products	1	28	11	Report	PU	14	14/4/2015	Submitted	
28.2	List of ideas for new product development	1	28	1	Report	PU	16	21/7/2015	Submitted	
28.3	Report on product and process solutions for each species	1	28	1	Report	PU	18	5/10/2015	Submitted	

	based on technological, physical and sensory characteristics									
28.4	Physical prototypes of new products from the selected species meagre, greater amberjack, wreckfish, pikeperch and grey mullet	1	28	3	Report	PU	26	19/4/2016	Submitted	
29.1	Dataset of consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the selected species in the five	1	29	6	Report	PU	9	27/08/2014	Submitted	
29.2	Report on the segmentation analysis based on consumer value perceptions about the selected species in the five countries investigated (value-based segmentation task)	1	29	11	Report	PU	24	7/10/2015	Submitted	
29.3	Development of the actual product samples from the selected species for the sensory testing with consumers in the five countries investigated	1	29	3	Report	PU	28	4/4/2016	Submitted	
29.4	Report on the actual products' sensory profiling in the five countries investigated	1	29	3	Report	PU	29	27/7/2016	Submitted	
29.5	Development of the product mock-ups for use in the experimentation with consumers in the five countries investigated	1	20	11	Report	PU	30	27/7/2016	Submitted	

29.6	Report on the experimentation with product mock-ups in the five countries investigated and identification of the optimal intrinsic-extrinsic product quality profiles for targeted segments	1	20	11	Report	PU	30	14/03/2017	Submitted	
29.7	Development of the stimulus (i.e. written and broadcasted information material) that will be used in the communication experiments in the five countries investigated	1	20	11	Report	PU	30	28/07/2017	Submitted	
29.8	Report on the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers attitudes and behaviour towards the products coming from the selected	1	20	11	Report	PU	30	31/10/2017	Submitted	
30.1	Report on value propositions for the producers and Partners	1	30	10	Report	PU	46	31/10/2017	Submitted	
30.2	Report on indications of resources for creating customer value for the specific products	1	30	10	Report	PU	46	31/10/2017	Submitted	
30.3	Guidelines to cultivate buyer-supplier relationships per species	1	30	10	Report	PU	48	05/12/2017	Submitted	
30.4	Revenue (pricing & costs	1	30	10	Report	PU	48	01/03/2018	Delayed	The lead beneficiary is

	structures) model per species									working on the data analysis
31.1	Establishment of website (www.diversifyfish.eu)	1	31	18	Report	PU	4	02/04/2014	Submitted	
31.2	Project logo and brochure	1	31	18	Report	PU	6	24/06/2014	Submitted	
31.3	Publication of the first of two articles in Food Today	1	31	37	Report	PU	6	30/05/2014	Submitted	
31.4	Production and release of audio-visual material	1	31	18	Report	PU	6	24/06/2014	Submitted	
31.5	Collaboration agreement with food industry and consumer organization; linkage of websites	1	31	18	Report	PU	9	18/11/2014	Submitted	It proved to be much more difficult than expected to convince these organization to sign an agreement
31.6	Annual presentation of DIVERSIFY (Y1) at a relevant conference (mainly Aquaculture Europe, EU Forum (by the Project Coordinator)	1	31	1	Report	PU	9	27/10/2014	Submitted	
31.7	Production and release of audiovisual material	1	31	18	Report	PU	12	15/01/2015	Submitted	
31.8	Production and release of audiovisual material	1	31	18	Report	PU	18	31/08/2015	Submitted	Production and release of audiovisual

										material
31.9	Annual presentation of DIVERSIFY (Y2) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	21	29/10/2015	Submitted	
31.10	Presentations of DIVERSIFY at the Aqua Europe meetings (Diversification Sessions) by the Species leaders (Y2)	1	31	1	Report	PU	21	16/11/2015	Submitted	
31.11	Scientific publications in relevant journals	1	31	1	Report	PU	30/11/2018	30/11/2018	New dates approved	This Deliverable was erroneously planned for 24, and was corrected in the 2nd Amendment to Mo 60
31.12	Production and release of audiovisual material	1	31	18	Report	PU	24	30/12/2015	Submitted	Production and release of audiovisual material
31.13	Production and release of audiovisual material	1	31	18	Report	PU	30	25/6/2016	Submitted	Production and release of audiovisual material
31.14	Annual presentation of DIVERSIFY (Y3) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	33	15/10/2016	Submitted	
31.15	Production and release of audiovisual material	1	31	18	Report	PU	36	09/02/2016	Submitted	

31.16	Promotional workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) (1st workshop)	1	31	18	Report	PU	37	28/07/2017	Submitted	
31.17	Production and release of audiovisual material	1	31	18	Report	PU	42	31/10/2017	Submitted	
31.18	Promotional workshops (2nd) for specialized audience in fish market sector (Spain, UK, Italy or Greece)	1	31	18	Report	PU	43	08/11/2017	Submitted	
31.19	Annual presentation of DIVERSIFY (Y4) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	44	02/11/2017	Submitted	
31.20	Presentations of DIVERSIFY at the Aqua Europe meetings (Diversification Sessions) by the Species leaders (Y4)	1	31	1	Report	PU	44	03/11/2017	Submitted	
31.21	Presentation of DIVERSIFY at the European SEAFOOD Expo	1	31	1	Report	PU	44	25/10/2017	Submitted	
31.22	Production and release of audiovisual material	1	31	18	Report	PU	48	31/1/2018	Delayed	Some delays in obtaining the necessary material from the SLs

Milestones

TABLE 2. MILESTONES							
Milestone no.	**Milestone name**	**Work package no**	**Lead beneficiary**	**Delivery date from Annex I dd/mm/yyyy**	**Achieved Yes/No**	**Actual / Forecast achievement date dd/mm/yyyy**	**Comments**
1	Kickoff meeting and Annual coordination meeting for Y1	1	1	31/12/2013	Yes	30/01/2014	P1. HCMR, Crete, Greece
2	Consortium agreement	1	1	31/01/2014	Yes	20/03/2014	
3	Annual coordination meeting for Y2	1	1	31/01/2015	Yes	6/11/2014	P13. UNIBA, Bari, Italy
4	Periodic Report (Mo1-12) to DG RTD, including financial and administrative reports	1	1	31/01/2015	Yes	30/12/14	
5	Annual coordination meeting (for Y3)	1	1	31/01/2016	Yes	04/02/2016	P9. UL, Nancy, France
6	Periodic Report (Mo13-30) to DG RTD, including financial and administrative reports	1	1	31/5/2016	Yes	29/7/2016	
7	Annual coordination meeting (Y4)	1	1	30/11/2016	Yes	18/1/2017	
8	Annual coordination meeting (Y5)	1	1	30/1/208	Yes	23/1/2018	
16	SNIP library with candidate SNIPs potentially associated with growth in meagre	2	2	30/05/2015	Yes	30/05/2015	

17	Database of genetic variability of pikeperch	4	1	30/11/2014	Yes	30/11/2014	Excel database completed
18	Documentation of ovulatory cycles in wild and F1 halibut broodstock	5	7	31/05/2016	Yes	31/05/2016	
19	Basic diet formulation for meagre grow out studies	8	2	30/11/2014	Yes	30/11/2014	Established
20	Digestive utilization of experimental weaning diets for meagre	8	2	30/11/2015	Yes	30/11/2015	
21	Basic diet formulation for greater amberjack grow out studies	9	2	30/11/2014	Yes	30/11/2014	Established
22	Definition of reproductive quality parameters to be studied in amberjack	9	2	30/11/2014	Yes	30/11/2014	Literature search completed
23	Definition of parameters for skeleton study in pikeperch	10	21	30/11/2014	Yes	30/11/2014	Definitions and analytical parameters for skeleton studies has been included in the experimental protocols
24	Influence of salinity or temperature on LC-PUFAs synthesis in pike perch	10	21	30/11/2016	Yes	30/11/2016	
25	Ranges of digestive enzymes activities in Atlantic halibut	11	7	31/08/2016	Yes	31/08/2016	
26	Obtain viable gametes (oocytes and sperm) for larvae production in wreckfish	12	19	31/08/2016	Yes/Partly	31/08/2016	The egg production is still not optimal, and we have low fertilization and survival in the embryos, hindering some of the larval rearing experiments
27	Definition of methodology to study cost-benefit of grey mullet weaning studies	13	4	30/11/2014	Yes	30/11/2015	

28	Protocol for weaning meagre larvae	14	2	31/5/2015	Yes	16/5/2016	
29	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack producing good quality eggs	15	2	31/5/2014	Yes	30/6/2014	Provision of eggs for larval nutrition and rearing experiments in Greece and Spain.
30	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2015	Yes	30/6/2015	Egg production has been achieved in both Mediterranean and Atlantic broodstocks, as well as in F1 broodstocks in Y2
31	Protocol for tank design, lighting and probiotics of larval rearing of greater amberjack	15	2	31/5/2015	Yes	30/7/2016	
32	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2016	Yes	31/6/2016	
33	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2017	Yes	18/06/2017	
34	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2014	No	31/6/2014	Eggs were produced both in Greece and Spain, but their quality was poor and did not allow implementation of larval rearing experiments.
35	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2015	Yes, partly	31/6/2015	Eggs were produced both in Greece and Spain, and allowed a limited implementation of the larval rearing experiments.

36	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2016	Yes, partly	31/5/2016	Eggs were produced in Spain, and allowed a limited implementation of the larval rearing experiments
37	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/06/2017	Yes, partly	06/03/2017	Eggs were produced both in Spain and Greece, but allowed only a limited implementation of the larval rearing experiments
38	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2014	Yes	31/10/2015	Millions of eggs of high quality were produced, allowing the start of larval rearing experiments.
39	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2015	Yes	31/10/2015	
40	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2016	Yes	30/09/2016	
41	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2017	Yes	20/09/2017	
42	Results on feeding stimuli of meagre	20	3	01/06/2015	Yes	01/06/2015	
43	First cage trials (different volume and light conditions) with meagre implemented	20	3	30/11/2015	Yes	01/12/2015	
44	Results on feed distribution method in cages with meagre	20	3	30/11/2015	Yes	01/12/2016	
45	Feeding pattern of greater amberjack fry available	21	1	31/8/2015	Yes	31/8/2015	

46	First results on optimum husbandry practice (thermal ranges, stocking density) of greater amberjack	21	1	31/3/2016	Yes	31/3/2016	
47	First experiment on cage culture condition (net volume, cage type) of greater amberjack implemented	21	1	31/5/2016	Yes	01/09/2016	
48	Experiment on the definition of optimal conditions for pikeperch on growing implemented	22	16	31/5/2016	Yes	31/5/2016	
49	First trial with different strains of pikeperch implemented	22	16	30/11/2017	No	31/05/2018	It was not possible to have juveniles of different geographic origins and domestication levels due to a total loss of larvae by a Rhabdovirus occurrence in April 2016 in the URAFPA facilities. Therefore, the *in vivo* experiment for this task has just started in October 2017, and will last 3 months.
50	Experimental trials of grey mullet in the three locations implemented	23	4	28/2/2015	Yes	31/5/2016	
51	Design of primers for amplification of meagre target gene DNA sequences	24	5	30/11/2014	Yes	30/11/2014	
52	Grow-out of larvae and collection of samples from immune ontogeny time-line	24	5	30/11/2015	Yes	30/11/2015	
53	Amplification and sequencing of target gene sequences from stimulated tissues	24	5	31/5/2016	Yes	31/5/2016	
54	Completion of challenge and collection of samples for study of immune gene modulation	24	5	30/11/2016	Yes	30/11/2016	

55	Complete preparation of cDNA synthesis from all meagre samples	24	5	31/05/2017	Yes	31/05/2017	
56	Complete gene expression analysis of immune ontogeny	24	5	31/05/2017	Yes	31/05/2017	
57	Complete genes analysis for immune stimulus/response	24	5	31/10/2017	Yes	31/10/2017	
58	Design of primers for amplification of greater amberjack target gene DNA sequences	25	5	31/5/2015	Yes	31/5/2015	
59	Successful Chlamydia screening and sequencing	25	5	31/5/2016	Yes	1/5/2016	
60	Samples collected from stimulated primary cultures/explants, ready for immune gene expression analysis	25	5	31/5/2016	Yes	30/11/2015	PhD student Douglas Milne of P5. UNIABDN visited P1. FCPCT in November 2015 to undertake the work
61	Ideas for new products	28	1	31/5/2015	Yes	21/7/2015	
62	Optional physical new products	28	1	31/3/2016	Yes	19/4/2016	
63	Insights in the consumer and B2B market for cultured fish	29	1	30/11/2014	Yes	30/11/2014	
64	Selection of new products, with good sensory perception	29	1	31/5/2016	Yes	30/7/2016	
65	Intrinsic and extrinsic attributes related to the new products	29	11	30/11/2016	Yes	30/11/20116	
66	Communication concept for behavioral change to cultured fish	29	11	31/08/2017	Yes	11	31/08/2017
67	Business models to market the new products	30	10	30/11/2017	Yes/Partly	31/10/2017	The work is in progress, but with some delays

70	Agreement on project logo for website and publications, this will provide a recognizable image of DIVERSIFY	31	1	01/06/2014	Yes	01/06/2014	
71	Design and printing of project brochure (hard-copy) including the project logo, inserts with project	31	18	01/06/2014	Yes	24/06/2014	
72	Agreements with food industry and consumers associations for web linkage	31	18	31/08/2014	Yes	20/11/2014	Considerable difficulties have been faced in reaching an agreement with organizations proposed in the DOW.
73	Agreement on the Promotional workshop (1st) program	31	18	01/07/2016	Yes	01/07/2016	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events
74	Agreement on the Promotional workshop (2nd) program	31	18	01/07/2016	Yes	01/06/2016	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events

75	Agreement on the Promotional workshop (3rd) program	31	18	31/5/2017	Yes	1/11/2017	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events
76	Agreement on the Promotional workshop (4th) program	31	18	30/11/2017	No	01/02/2018	A few more weeks are needed to complete the planning for this workshop

4 Explanation of the use of the resources and financial statements (Staff effort only)

The financial statements have to be provided within the Forms C for each beneficiary (if Special Clause 10 applies to your Grant Agreement, a separate financial statement is provided for each third party as well) together with a summary financial report which consolidates the claimed Community contribution of all the beneficiaries in an aggregate form, based on the information provided in Form C (Annex VI of the Grant Agreement) by each beneficiary. The "Explanation of use of resources" requested in the Grant Agreement for personnel costs, subcontracting, any major costs (ex: purchase of important equipment, travel costs, large consumable items) and indirect costs, have now to be done within the Forms (user guides are accessible within the Participant Portal)[4].

When applicable, certificates on financial statements shall be submitted by the concerned beneficiaries according to Article II.4.4 of the Grant Agreement.

The use of the resources is explained in detail in the submitted Forms C from each Beneficiary. However, for the convenience of the potential reviewer we include the staff effort in the tables below. **Tables 4.1a-b** show the staff effort for the 3rd Reporting period.

[4] In the past, the explanation of use of resources requested in the Grant Agreement was done within a table in this section. The merge of this table within the Forms C was a measure of simplification aimed at avoiding duplication and/or potential discrepancies between the data provided in the table 'Explanation of use of resources' and the data provided in the Forms C.

Table 4.1a Staff effort per Partner (P1-22) and WP, during the 3rd Reporting Period (31-48 Mo).

DIVERSIFY	3rd Periodic Report (31-48 months)																							
KBBE 2013.1.2.09. Diversification of fish species and products in aquaculture																								
Calculation of staff effort per parter and sub WP																								
				Example				RTD partners							RTD partners							RTD partners			
Work Package (Proposal)	Title	DOW WP	Total	1. HCMR	2. FCPCT	3. IRTA	4. IOLR	5. UNIABDN	6.DLO	7. IMR	8. IEO	9. UL	10. TU/e	11. AU	12. APROMAR	13. UNIBA	14. IFREMER	15. ULL	16. FUNDP	17. NIFES	18. CTAQUA	19. CMRM	20. SARC	21. DTU	22. SWH
			545,87	137,22	88,82	19,76	14,30	19,09	8,41	23,71	10,16	11,62	9,18	19,00	3,38	12,40	2,69	22,84	8,25	4,55	11,57	3,62	3,35	9,51	3,40
WP1 Management	Management	1	29,66	18,00		0,41		0,60	0,73	0,02	0,16	0,67	0,37	0,10	0,18	0,10	0,59	0,08		0,54	0,13	0,20	0,06	0,53	0,10
WP2 Reproduction and Genetics			83,82	15,83	4,46	2,98	4,05	0,00	0,00	4,39	3,20	0,00	0,00	0,00	0,00	9,30	2,10	3,63	0,00	0,00	0,00	1,71	0,00	0,00	2,30
2.1	Reproduction - meagre	2	8,14	4,33	1,46	1,19											1,16								
2.2	Reproduction - amberjack	3	56,01	9,50	3,00		3,15				2,20					6,30	0,94	1,72							
2.3	Reproduction - pikeperch	4	0,00																						
2.4	Reproduction - halibut	5	6,69							4,39															2,30
2.5	Reproduction - wreckfish	6	6,41	2,00		0,95					1,00							0,08				1,71			
2.6	Reproduction - mullet	7	6,57			0,84	0,90									3,00		1,83							
WP3 Nutrition			80,06	2,50	29,94	0,08	8,60	0,00	0,00	2,37	1,40	0,00	0,00	0,00	0,00	2,00	0,00	8,54	2,00	3,63	0,54	1,28	0,00	8,78	0,00
3.1	Nutrition - meagre	8	6,53															3,71						2,82	
3.2	Nutrition - amberjack	9	19,53	2,50	7,20						0,90							0,53							
3.3	Nutrition - pikeperch	10	15,23		5,46													1,81	2,00					5,96	
3.4	Nutrition - halibut	11	8,50							2,37								2,50		3,63					
3.5	Nutrition - wreckfish	12	3,96		2,18						0,50											1,28			
3.6	Nutrition - mullet	13	26,32		15,10	0,08	8,60									2,00					0,54				
WP4 larval husbandry			48,45	2,88	15,00	0,93	0,87	0,00	0,00	9,45	3,25	7,56	0,00	0,00	0,00	0,00	0,00	6,39	0,00	0,38	0,00	0,43	0,00	0,00	1,00
4.1	Larval husbandry - meagre	14	1,13															1,13							
4.2	Larval husbandry - amberjack	15	24,49	2,38	15,00						1,85							5,26							
4.3	Larval husbandry - pikeperch	16	7,56									7,56													
4.4	Larval husbandry - halibut	17	10,83							9,45										0,38					1,00
4.5	Larval husbandry - wreckfish	18	2,64	0,50							1,40											0,43			
4.6	Larval husbandry - mullet	19	1,80			0,93	0,87																		
WP5 Grow out husbandry			131,99	38,75	30,18	3,12	0,78	0,00	0,00	0,00	1,50	3,01	0,00	0,00	0,00	0,00	0,00	0,35	6,25	0,00	2,75	0,00	0,00	0,20	0,00
5.1	Grow out husbandry - meagre	20	36,62	28,50		3,12																			
5.2	Grow out husbandry - amberjack	21	72,08	6,25	30,18						1,50							0,35							
5.3	Grow out husbandry - pike perch	22	9,46									3,01							6,25					0,20	
5.4	Grow out husbandry - mullet	23	13,83	4,00			0,78														2,75				
WP6 Fish health			82,85	38,20	9,24	3,10	0,00	18,49	0,00	7,48	0,40	0,00	0,00	0,00	0,00	0,00	0,00	2,65	0,00	0,00	0,00	0,00	3,29	0,00	0,00
6.1	Fish health - meagre	24	49,33	34,70		3,10		8,24															3,29		
6.2	Fish health - amberjack	25	26,04	3,50	9,24			10,25			0,40							2,65							
N/A	Fish health - halibut	26	7,48							7,48															
WP7 Socioeconomics			77,44	18,56	0,00	8,85	0,00	0,00	7,68	0,00	0,00	0,00	8,81	18,90	2,72	0,00	0,00	1,21	0,00	0,00	3,85	0,00	0,00	0,00	0,00
7.1	Institutional and organizational context	27	0,00																						
7.2	New Product Development	28	25,18	17,56		4,30			1,76									1,21			0,35				
7.3	Consumer value perceptions and behavioral change	29	32,55	1,00		3,40			0,48					18,90							3,50				
7.4	Business model and marketing strategy development	30	19,71			1,15			5,44				8,81		2,72										
WP8 Dissemination	Dissemination	31	11,61	2,50		0,29					0,25	0,38			0,48	1,00					4,30				

Table 4.1b Staff effort per Partner (P23-38) and WP, during the 3rd Reporting Period (13-48 Mo).

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DIVERSIFY	3rd Periodic Report (31-48 months)																		
KBBE 2013.1.2.09. Diversification of fish species and produ aquaculture																			
Calculation of staff effort per parter and sub WP																			
			SME partners									New Partners							
Work Package (Proposal)	Title	DOW WP	23. ARGO	24. ITICAL	25. DOR	26. GEI	27. FORKYS	28. CANEXMAR	29. ASIALOR	30. CULMAREX	31. IRIDA	32. MC2	33. FGM	34. BVFi	35. MASZ	36. ANF	37. EUFIC	38. HRH	39. F2B	40. GMF
			36,20	0,00	0,00	6,50	7,40	37,95	0,00	0,00	0,95	1,05	0,55	0,14	1,40	1,08	0,16	5,66	4,05	13,51
WP1 Management	Management	1				1,00	0,20	3,75			0,15	0,07	0,05	0,01	0,49	0,34	0,03		0,10	0,27
WP2 Reproduction and Genetics			29,20	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,67	0,00	0,00	0,00	0,00	0,00	0,00	0,00	5,48
2.1	Reproduction - meagre	2																		
2.2	Reproduction - amberjack	3	29,20																	5,48
2.3	Reproduction - pikeperch	4																		
2.4	Reproduction - halibut	5																		
2.5	Reproduction - wreckfish	6										0,67								
2.6	Reproduction - mullet	7																		
WP3 Nutrition			0,00	0,00	0,00	0,00	0,00	8,40	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
3.1	Nutrition - meagre	8																		
3.2	Nutrition - amberjack	9						8,40												
3.3	Nutrition - pikeperch	10																		
3.4	Nutrition - halibut	11																		
3.5	Nutrition - wreckfish	12																		
3.6	Nutrition - mullet	13																		
WP4 larval husbandry			0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	7,76
4.1	Larval husbandry - meagre	14																		
4.2	Larval husbandry - amberjack	15																		7,76
4.3	Larval husbandry - pikeperch	16																		
4.4	Larval husbandry - halibut	17																		
4.5	Larval husbandry - wreckfish	18										0,31								
4.6	Larval husbandry - mullet	19																		
WP5 Grow out husbandry			7,00	0,00	0,00	5,50	7,20	24,60	0,00	0,00	0,80	0,00	0,00	0,00	0,00	0,00	0,00	0,00	3,90	0,00
5.1	Grow out husbandry - meagre	20	5,00																	
5.2	Grow out husbandry - amberjack	21	2,00				7,20	24,60												
5.3	Grow out husbandry - pike perch	22																	3,90	
5.4	Grow out husbandry - mullet	23				5,50					0,80									
WP6 Fish health			0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
6.1	Fish health - meagre	24																		
6.2	Fish health - amberjack	25																		
N/A	Fish health - halibut	26																		
WP7 Socioeconomics			0,00	0,00	0,00	0,00	0,00	1,20	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	5,66	0,05	0,00
7.1	Institutional and organizational context	27																		
7.2	New Product Development	28																		
7.3	Consumer value perceptions and behavioral change	29																5,27		
7.4	Business model and marketing strategy development	30						1,20										0,39	0,05	
WP8 Dissemination	Dissemination	31											0,50	0,13	0,91	0,74	0,13			

This is the end of the 3rd Periodic Report.