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

Date of latest version of Annex I against which the assessment will be made: 15 February 2015 (Amendment 2)

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

Period covered: from 13 to 30 mo

Name, title and organisation of the scientific representative of the project's coordinator[1]:

Dr Constantinos Mylonas, Research Director, Hellenic Center for Marine Research

Tel: +30 2810 337878

Fax: +30 2810 337875

E-mail: Mylonas@hcmr.gr

Project website[2] address: www.diversifyfish.eu

[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

Table of Contents

2.1 Project objectives for the period

Reproduction & Genetics

Five objectives were completed during the 2nd Reporting Period as programmed in the DOW. The completed objectives were, to develop a protocol for paired spawning of meagre, compare genetic variability of pikeperch for breeding programs, develop a protocol for mullet egg transport and develop CASA evaluation for grey mullet and wreckfish sperm and cryopreservation protocols of wreckfish sperm. Work on a total of 18 programmed objectives was started or continued from the first reporting period:

- Experiments to improve spawning of greater amberjack (tanks and cages), Atlantic halibut, wreckfish and grey mullet,
- Sampling of tissues and blood to describe the reproductive cycle of greater amberjack (wild and captive) and wreckfish,
- Development of techniques to aid description of the reproductive cycle for greater amberjack and wreckfish
- Development of techniques to assess reproductive and nutritional state, and sperm quality for meagre and greater amberjack,
- Development of techniques to improve hormone induced spawning protocols for grey mullet,
- Development of SNP markers for fast and slow growing meagre,
- Experiments on *in vitro* fertilization for meagre and 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 second year have focused in first feeding regimes (enrichment products and weaning diets), growth-out diets and broodstock diets. In those species with problems to obtain the reproduction, information has been obtained to formulate the first broodstock diets to improve spawning quality. Therefore the objectives of this year have been focused in: 8.1 Improve current larval weaning feeds for meagre, 9.1 Improve larval enrichment products to enhance production of amberjack larvae, 9.2. Develop diets for grow-out of greater amberjack in order to maximize growth potential, 9.3. Development of an appropriate broodstock diet to improve unreliable 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, 11.1. Development of a protocol for early weaning for Atlantic halibut, 11.2. Develop a production strategy for on-grown Artemia and 11. 3. Improve growth in late Atlantic halibut larval stages, and juvenile quality, through feeding with on-grown Artemia, 12.2. Determine the influence of broodstock feeds on fecundity and spawning quality of wreckfish and 13.1. Improve enrichment products, weaning, grow out and broodstock diets for grey mullet.

Larval husbandry

In meagre the objective was to reduce costs by early weaning in meagre larvae and improve growth, survival and larval quality.

In greater amberjack the objectives were to study the effects of different feeding regimes in intensive systems, and compare the performance of semi-intensive and intensive rearing methods, looking also at the effect of environmental parameters such as light and current. These studies were also associated with an evaluation of the growth endocrine axis.

In pikeperch, the objective was to repeat an experiment carried out during the 1st Reporting Period, in which we studied the effects of four environmental factors (light intensity, water renewal rate, water flow direction, tank cleaning time) on the effectiveness of rearing of pikeperch larvae. In addition, a second experiment aimed at the determination of the effect of four feeding-related factors on the effectiveness of pikeperch larviculture. The studied factors were feeding frequency, co-feeding or not, weaning timing and weaning duration.

In Atlantic halibut, the objective was to improve larval survival and quality during early development, during yolk sac and first feeding stages and the evaluation of the effects on larval survival, quality and growth. To improve larval performance, the effect of probiotics on larval microbiota was also examined. Finally, we developed a method for producing on-grown *Artemia* as a means to provide older Atlantic halibut larvae with a larger prey of a higher nutrient content.

In wreckfish, the objective was to develop a larval rearing protocol based on the most effective prey density, succession of prey type, temperature and culture system.

Finally in the grey mullet, the objectives were to investigate environmental (turbidity, phytoplankton type) and nutritional factors that affect larval rearing,

Grow out husbandry

The tasks related to meagre were 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 the development of appropriate feeding methods. Also improvements related to the size dispersion that is frequently observed in juveniles will be provided.

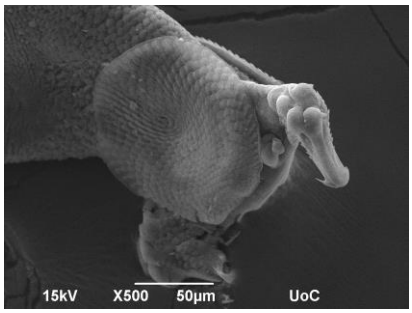
For the greater amberjack, the objectives of the work during the second periodic report were: (1) the development of feeding methods for fry and juveniles by identifying daily rhythms and feeding frequency, (2) to define optimal ranges of temperature and stocking density.

For pike perch the objectives were (1) to characterize the effects of multiple variables on stress, immune response and growth performances by a multifactorial approach and (2) to establish recommendations of optimal husbandry and environmental conditions for improving the welfare and yield of ongrowing of pikeperch juveniles in farm conditions.

Finally for grey mullet the objectives for the period were to evaluate the geographic range for grow-out of grey mullet in the Mediterranean basin, and to determine the cost-benefit of different weaning diets on the performance and health status of juvenile grey mullet.

Fish Health

There were several objectives during this period, to enable future deliverables to be met. For meagre these included:



1. Continuation of the study of different nutritional parameters, in order to dentify the causes of systemic granulomatosis (SG), and chronic ulcerative dermatopathy (CUD),
2. Investigate further anti-parasite treatments in juvenile meagre,
3. Complete the characterisation of immune genes,
4. Contrinue the effort to isolate and evaluate the occurrence of *Nocardia* infections in meagre and develop an autogenous vaccine,
5. Monitor the occurrence of different diseases and infections in the maintained stocks.

In greater amberjack, the objectives in this scientific discipline included the following:

1. Further work on mesocoms larval rearing in order to isolate and provide early diagnosis tools for Epitheliocystis,
2. Morphological study of the incidence of monogenean parasites including a determination of environmental conditions that can modulate greater amberjack resistance to parasitic infection, and formulation of a diet supplemented with mucus stimulation products,
3. Continue the characterisation of the immune system, with a focus on mucosal (skin/gill) defences,
4. Develop anti-monogenean parasites infection rearing protocols,
5. Monitor the occurrence of different diseases and infections in the maintained stocks

Finally in Atlantic halibut, the objective for this period was to complete the work to assess the two eukaryotic systems -microalgae and a protozoan (*L. tarentolae*) for the production of nodavirus (Viral Neural Necrosis, VNN) capsid protein, to be used for the development of an oral vaccine for Atlantic halibut.

Socioeconomics

The main objectives of this period were to finish the organizational and institutional context analysis by:

1. giving 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. assessing the obstacles for growth in the current aquaculture production chains and for these selected species, and
3. identifying market opportunities for future growth of the European aquaculture sector for the selected species.

Also planned for this period were the tasks to (a) develop new product concepts from selected species, by incorporating consumer and expert input, and (b) elect product ideas and develop physical new products from the selected species.

Finally, we focused in work planned to (a) 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, and (b) evaluate consumer sensory perceptions towards the newly developed DIVERSIFY species' products.

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 continued to develop tools for meagre breeding programs. Task 2.2 provided a protocol for the paired spawning of meagre. The efficacy of spawning pairs with male rotation was 76% and a total of 61 families were produced. Work in Task 2.3 described meagre sperm characteristics (motility initiated at 60% and lasted 60 s) and provided protocols for sperm storage and cryopreservation. The protocols were used in Task 2.4, to develop *in vitro* fertilization methods, and eggs stripped 39 h after treatment with GnRHa were fertilized successfully. In Task 2.5, the DNA was extracted from 400 meagre that had varying growth rates and analysis is underway to associate SNP markers with growth.

In Task 3.1, the effects of confinement on greater amberjack reproduction were demonstrated. The study of reproductive and nutritional state of wild and captive-reared fish showed that a severe impairment of gametogenesis occurred in captive-reared greater amberjack that exhibited low pituitary gonadotropin (GtH) expression, low GtH and sex steroid plasma concentrations, extensive atresia of vitellogenic follicles, high levels of male germ cell apoptosis and lower gonad content of specific lipid classes and fatty acids. Major advances were made with the development of spawning protocols (Tasks 3.2, 3.3 3.4 and 3.5). Large volumes of high quality eggs sufficient to stock a hatchery were produced using GnRHa treatments on fish maintained in cages in the Mediterranean Sea and moved to tanks after the hormonal therapy (Task 3.2), from spontaneous spawning of broodstock in tanks in the eastern Atlantic (Task 3.3), and from hatchery bred (F1) greater amberjack held in tanks in the eastern Atlantic (Task 3.4). In Task 3.5 only small amounts of eggs were collected from induced spawning in cages, however, this was shown to be a problem with egg collection and not spawning. Experiments with egg collector re-design will be made to retain more eggs.

Work on pikeperch in Tasks 4.1 and 4.1 has been completed and all deliverables have been submitted. The captive broodstock populations presented different levels of genetic variability that ranged from wide variability that is greater than observed in wild populations, to broodstocks that had reduced genetic variability due to inbreeding. Hatcheries with such broodstocks were recommended to take measures to introduce greater variation into the base population for future breeding programs.

Work with Atlantic halibut in Task 5.1 did not find significant differences in fecundity, fertilisation, hatching, egg size and hormone content between eggs from wild-caught and cultured females. However, wild-caught females were more predictable spawners that gave fewer, but larger batches of high quality eggs (>85% fertilization). In Task 5.2, a GnRHa implant therapy induced and synchronised ovulations without any effect on egg quality or quantity, and provides an approach to ensure predictable ovulations.

Despite the scarcity of wild wreckfish, two juvenile wreckfish were captured in Task 6.1. In Task 6.2, four broodstocks are being sampled to describe the reproductive cycle. Males exhibited good sperm quality (Task 6.4) with large amounts of expressible sperm during the reproductive period and a proportion of males had sperm throughout the year. Cryopreservation of wreckfish sperm offered a good solution for the management of sperm for *in vitro* fertilization (Task 6.4). In Task 6.3, tank spawning of wreckfish continued to be unpredictable, but fertilisation is improving and work towards *in vitro* fertilisation found that GnRHa induced oocyte maturation and ovulation reliably.

Grey mullet recombinant follicle stimulating hormone (r-FSH) was produced with the *Pichia* expression system and the bioactivity of the hormone was demonstrated. The r-FSH was used in hormone-based treatments to enhance and synchronize gametogenesis (Task 7.1). In Task 7.2, treatments were developed for inducing grey mullet spawning. Millions of fertilized eggs were produced during natural and shifted reproductive periods. Nevertheless, work continues on two problems: (i) failure of some females to ovulate and (ii) episodic fertilization rates ranging between 0 to 98%. A protocol was developed for the shipping of grey mullet eggs (Task 7.5).

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,
3. Development of tools that facilitate the implementation of genetic selection programs,

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

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

All tasks planned for this 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 progress towards objectives and details for each task (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.

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

This task 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 (**Fig. 2.1.1**). 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).

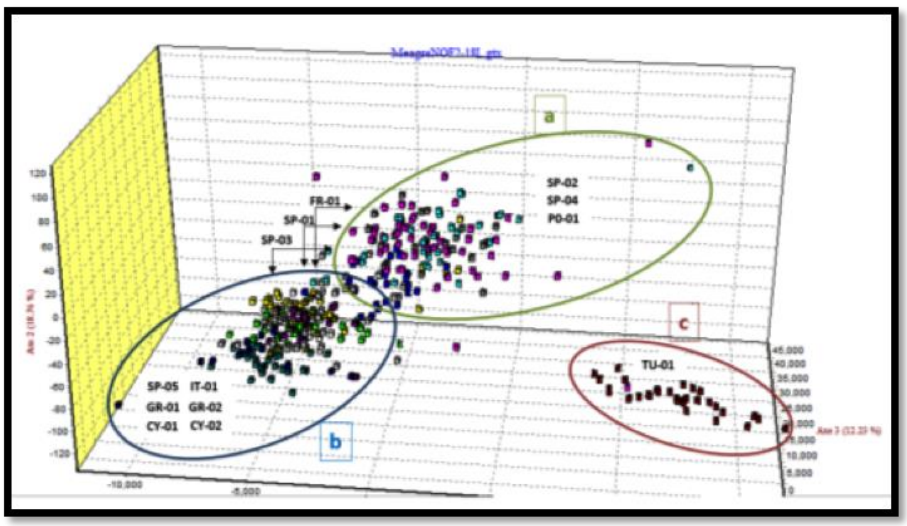


Figure 2.1.1. Graph of Factorial Correspondence Analysis from 18 loci and 376 fish distributed in 13 Mediterranean populations of meagre.

The full description of the work and results of this Task was 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 with the associated deliverable D2.3. Five paired spawning experiments were completed to determine the potential of paired spawning inductions with male rotation to perform a dialled cross mating design as the basis of a breeding program. Two experiments were completed during the first reporting period. In one experiment the same pairs were induced repeatedly to spawn on a weekly basis. In the second experiment one 6 x 6 (experiment II) dialled crosses of pairs with weekly male rotation and induced spawning was completed. During the second reporting period three further experiments were completed, each with 4 x 4 (experiments I y IIIa and IIIb) dialled crosses of pairs with weekly male rotation and induced spawning. Fecundity and percentage fertilization were determined when eggs were collected and percentage hatching and 5-d (day) larval survival were determined by incubating eggs from each spawn in replicated 96-well microtiter plates. In the same-pair experiment, the 4 pairs spawned up to 17 weeks in succession with high fecundity (>400,000 eggs kg^{-1} $spawn^{-1}$) and egg quality (> 80% fertilization). The efficacy of spawning pairs with male rotation was high, 76% (Experiment 1, 14 pairs spawned out of 16 (87%); experiment II, 22 pairs out of 37 (59%); experiments IIIa and IIIb, 25 pairs out of 27 (93%)) and across the three experiments a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success (**Fig 2.2.1**).

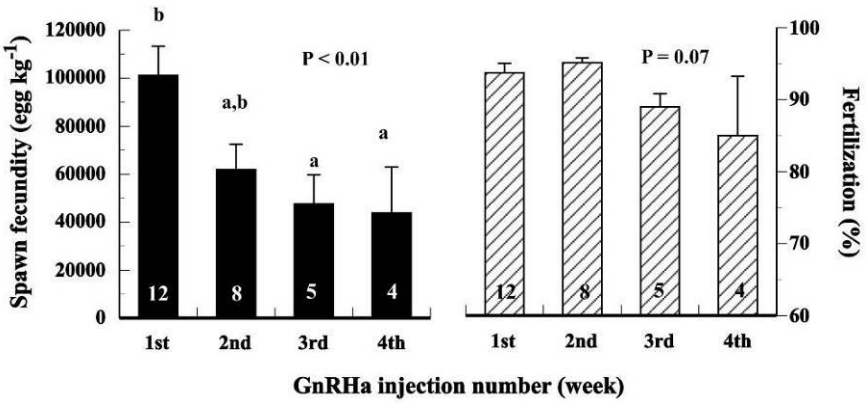


Figure 2.2.1. Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=4) after each GnRHa injection (n=4, once every week) during 2015. The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics. Different letter superscripts indicate significant differences between means. Data from experiment 2 completed in P1. HCMR.

However, not all paired crosses with male rotation were successful and a number of females after consecutive successful spawning inductions either failed to spawn or did not present vitellogenic oocytes and could not be induced as planned in the dialled cross design (**Fig. 2.2.2**). This failure to spawn or maintain maturity status after successive successful spawning inductions appeared to represent a change in spawning kinetics from the prolonged (up to 17 weeks) induced spawning period observed in the same-pair experiment and previous studies. This change in kinetics may be attributed to the stress of male rotation and consideration should be made that as the number of rotations increases, spawning pairs may fail or induced spawning may not be possible.

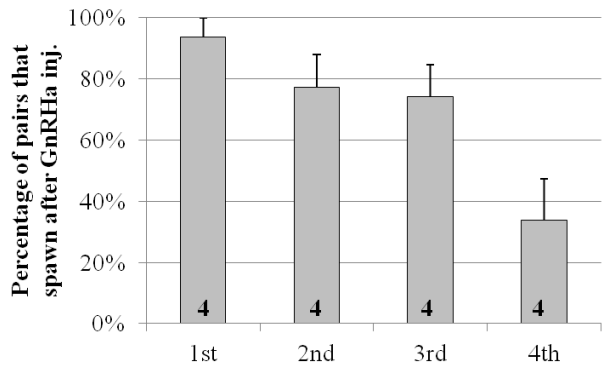


Figure 2.2.2. Mean (±SEM) percentage of pairs that spawned after GnRHa injection in the four experiments (experiment, I, II, IIIa and IIIb) completed in P3 IRTA and P1 HCMR.

However, together these experiments have shown that paired spawning of meagre is possible for the production of known families from parents with known phenotypes. Obtaining a large number of families with adequate fecundities that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite for a breeding program. The present studies were a successful "proof of concept" for this approach, highlighting both the positive potential of the approach and possible drawbacks.

The full description of the work and results of this Task has 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 second period was dedicated to the definition of settings to characterize sperm of meagre through the development of computer assisted sperm analysis (CASA) and the test of media for the conservation of gametes in order to establish the routine methods which can be applied to productive broodstocks. Due to the utilization of broodstocks of DIVERSIFY participating institutes for genetic and husbandry purposes during this period, we took profit of the geographic proximity and good relations between P14. IFREMER and a private company that does not participate in DIVERSIFY, Les Poissons Du Soleil (LPDS) to get adequate samples without any seasonal consideration.

Sperm characterization

Sperm of 8 males was sampled in LPDS and brought back to P14. IFREMER facilities in Palavas to be studied immediately and after 24 h under different types of conservation protocols. For this, the fish of one production tank of 25 m^3 were anaesthesized, 8 males were chosen at random and sperm was extracted without contamination (water or urine) after a thorough cleaning of the genital area. Volumes of 2 to 4 ml of clean semen were collected using 5 ml syringes. The semen was divided into subsamples that were either stored dry on ice or diluted in conservation media before being stored on ice. Finally, after transportation to P14. IFREMER, the diluted sperm was either stored at 4°C or cryopreserved according to the protocol used for European seabass (Fauvel et al 1999).

The concentration of spermatozoa was assessed after dilution to 1/500 of semen from each male in tap water. After dilution each sample was placed on a Thoma counting cell of 0.1 µl (with 400 squares of 0.025 mm^2, 0.1 mm depth). After 10 min sedimentation, a picture of each sample was taken using a photomicroscope and the pictures were analyzed using the Image J software after cropping a precise area of 24 squares and binarization using automatic thresholding (**Fig. 2.3.1**). In May 2015, the mean concentration of male semen in LPDS was 4.11 10^{10} (sd=0.7 10^{10}) spz ml^{-1}. The mean concentration of spermatozoa and the associated variability are usual in marine fish during the reproductive season. The variations of sperm concentration along the season will be studied within May and July 2016 in the facilities of P3.IRTA.

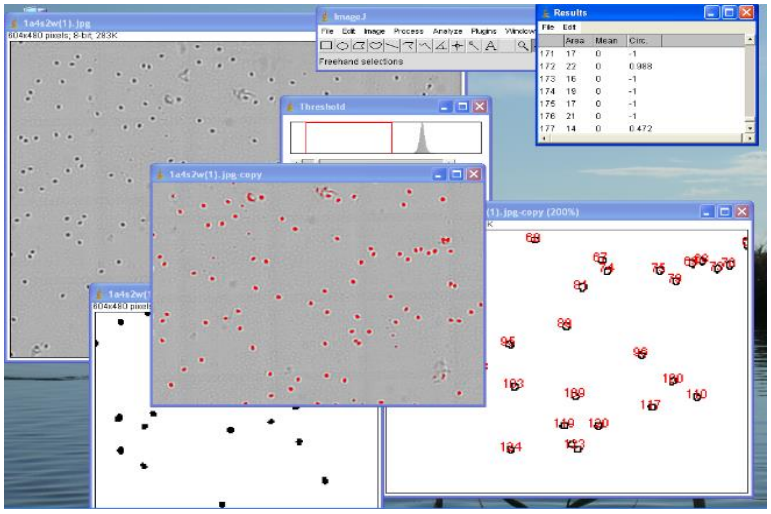


Figure 2.3.1. Screenshot summarizing automated sperm counting using the ImageJ software (NIH, USA).

The motility of meagre sperm was assessed by CASA with an adapted macro developed in ImageJ system, which takes into account the quality of the recorded movies. In order to record motility, sperm samples were pre-diluted in a non-activating medium, video record was launched just when pre-diluted sperm was mixed with seawater for activation. Finally activated sperm was deposited into a special cell (Leja, 10 µm depth) already settled and focused on the video-microscope. This procedure allows to assess first motility parameters within 10 seconds after sperm activation. The general pattern of motility features did not reveal any noticeable difference when compared to other marine fish with a maximum value for the velocity and the percentage of mobile spermatozoa just after activation and with a duration of movement lasting 50-60 sec (**Fig. 2.3.2.**). As expected, a progressive decrease of the mean speed of spermatozoa (Average path velocity) from around 150 µm s^{-1} down to 0 was observed within 1 minute (**Fig. 2.3.2**).

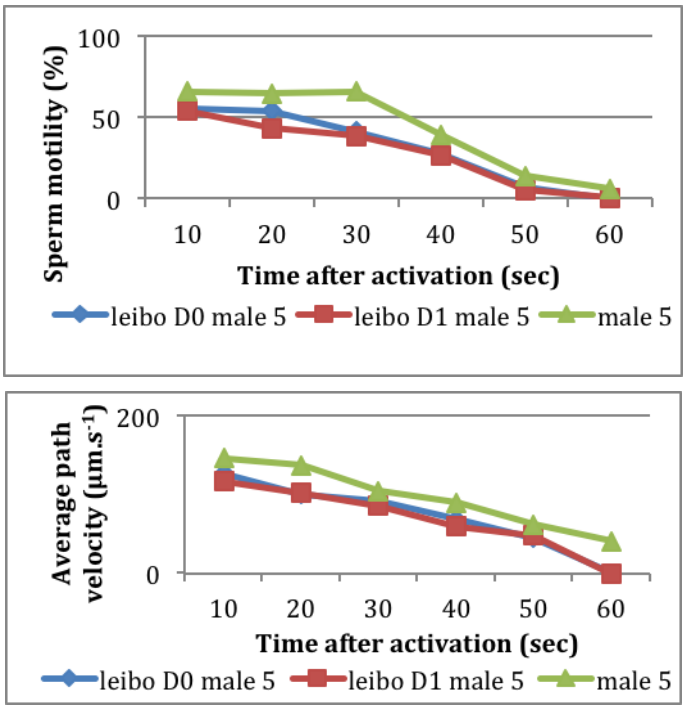


Figure 2.3.2. Motility of meagre sperm illustrated by the percentage of mobile spermatozoa (upper graph) and the speed on a smoothed track (Average Path Velocity) both parameters varying after sperm activation. This figure also compares the motility parameters between fresh sperm (green), diluted sperm chilled stored for 1 hour (Blue) and 24 hours (red).

Sperm storage protocol

Previous work showed that spermatozoa were able to survive in an oxygenated atmosphere in very small volumes. This was not the case when sperm had to be stored in large volumes for delayed fertilization protocols. According to the experience of sperm storage of P14. IFREMER in European seabass (French program Cryoaqua 2009-2010), but also on the results acquired by DIVERSIFY team on wreckfish Deliverable D2.5.2 and amberjack Deliverable D2.2.4 (to be delivered by the end of July 2016), meagre sperm was directly diluted to 1/3, (V/V) in different media for storage improvement. The different media tested were 2 commercial media with patented formulations (Storefish and Cryofish from IMV) and a medium designed by P14. IFREMER both for short term storage and cryopreservation, and based on modifications of Leibovitz L15 culture medium, the formula of which is publically available.

As an interesting result, modified Leibovitz L15 medium was more efficient for chilled sperm survival at 24 h than the 2 other media. The initial motility was decreased by 60% in both commercial media compared to

the initial motility of sperm stored in modified Leibovitz after 24h. Finally, when sperm had been stored for 1 day, its motility decreased much faster than that of fresh sperm whatever the medium (**Fig. 2.3.3**), however, this quick decrease of motility may not be deleterious for fertilization, something that must be tested.

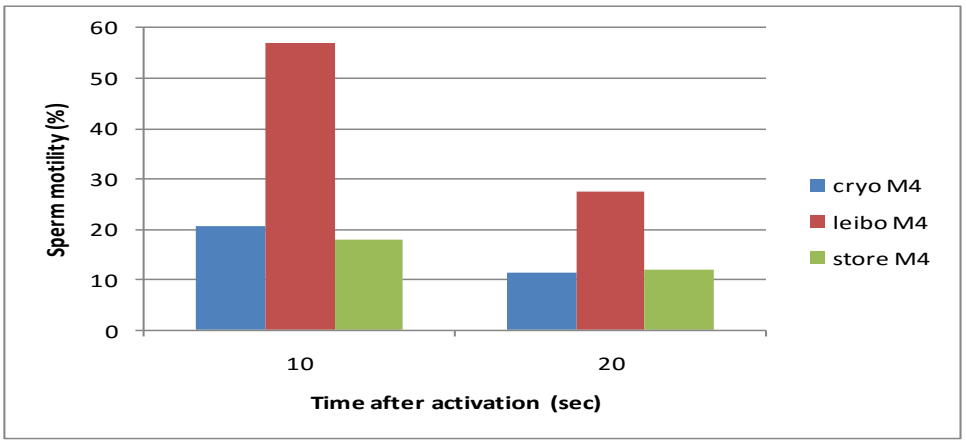


Figure 2.3.3. Comparison of motility of sperm subject to different commercial or experimental storage media for 24h showing the significant advantage of modified Leibovitz either at activation or after 20 seconds.

Cryopreservation trials and fertilization success assessment.

Since the modified Leibovitz seemed to yield a sufficient motility for fertilization, a large scale experiment was undertaken to evaluate the quality of either chilled stored or cryopreserved sperm. For this, sperm samples of 33 males were individually frozen after dilution to 1/3 in modified Leibovitz and final addition of 10% dimethyl sulfoxide, and sperm of 6 males was stored only chilled. A total of 6 meagre females were stimulated by heterologous GnRH analog, of which 5 responded with ovulation.

The *in vitro* fertilization of the females was performed individually as follows:

Each spawn was divided in aliquots of 20 ml of eggs, which were fertilized by chilled or cryopreserved sperm from one male. For each cross, the adequate number of straws for 400,000 spz egg^{-1} was thawed and immediately deposited on the eggs. The treatment of each spawn took around 1.5 h and followed the same sequence of males. After fertilization, the different aliquots were mixed for larval rearing, but a sample of several hundreds of eggs was kept separately for fertilization assessment.

Fertilization success was assessed at 4.5 h (necessary time to do the complete cross with 3 females) after fertilization and revealed a very interesting conclusion. The mean fertilization rate was quite similar for the 2 first females, but then it decreased in the third one (**Table 2.3.1**). Moreover, in each female the fertilization rate decreased along the sequence of insemination (**Fig 2.3.4**). The decrease of fertilization with time may be due the progressive overripening of the eggs after ovulation. The lack of precision about ovulation time may explain the hierarchy of fertilization with female 4 ovulating much later than female 3.

Transfer of knowhow between partners

Due to the large number of experiments for a large but limited broodstock in DIVERSIFY, it was decided to setup the analytical methods of sperm studies independently from the programmed husbandry purposes. After a regular transfer of information by mail, a session of experience exchange and particularly the practice of CASA was organised within 2 full days in P14. IFREMER facilities from 14 to 16 March 2016 joining 2 researchers from P3. IRTA and 1 researcher of P14. IFREMER.

Table 2.3.1. Fertilization performances of 33 cryopreserved (Cryo) and 6 chilled stored (Fresh) meagre sperm obtained in a full factorial cross with 3 females

		female 1	female 2	female3
Cryo	**mean**	**65,3**	**70,6**	**37,2**
	std	9,0	13,3	18,6
	cv	13,8%	18,9%	50,1%
	mini	38	33	9
	maxi	78	89	65
Fresh	**mean**	**49,4**	**79,3**	**26,9**
	std	11,1	11,0	11,9
	cv	22,6%	13,9%	44,2%
	mini	30	65	15
	maxi	61	96	43

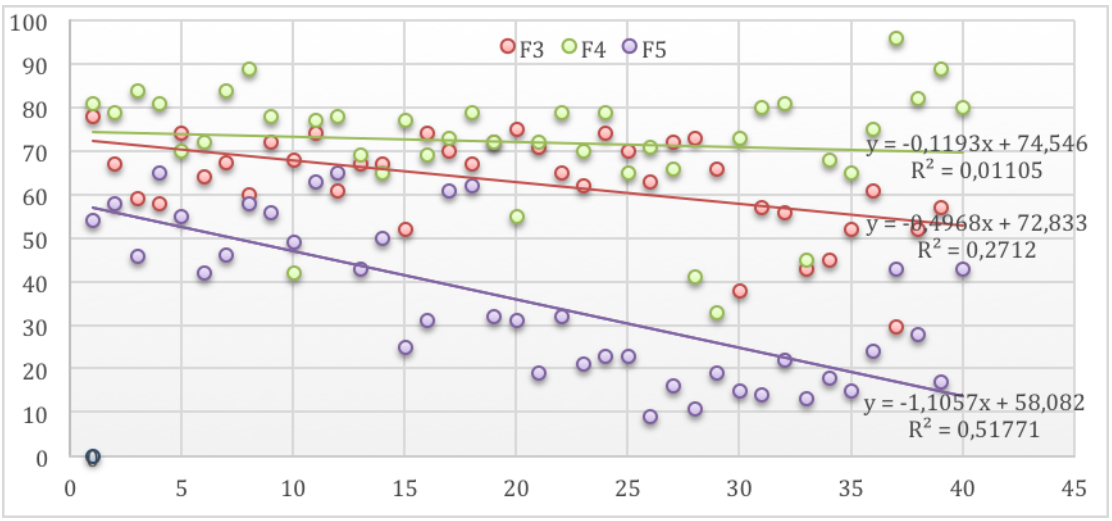


Figure 2.3.4. Individual fertilization success of cryopreserved sperm (males 1-33) and chilled stored sperm (males 34-40) showing the decrease with time of fertilization within females with a higher slope in female 5.

Conclusion

This second reporting period allowed characterizing meagre sperm, which presents expected characteristics on the basis of common knowledge about marine fish semen. Moreover, the use of analytical tools provided good means to assess the effect of sperm storage protocols both in chilled and frozen condition, which proved useful for further gamete management in industrial production purposes and particularly for genetic improvement of stocks. A last integrative experiment was undertaken in P3. IRTA facilities in June 2016 (Mo 31) with two scientists from P14.IFREMER to complete the experience and share results between partners. These results will be reported in the 3rd Periodic Report.

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

The task was initiated during the 2nd Reporting Period and is progressing towards the development of *in vitro* fertilization methods for planned crosses. A total of seven female breeders have been successfully induced to ovulate and the ova stripped and fertilised with sperm that was previously obtained from males. All selected females had ovaries with vitellogenic oocytes >500 µm and males had spermiation index of 2 or 3

(where 0 = no sperm, 1 = sperm present but not flowing, 2 = sperm flows and 3 = sperm flows easily and abundantly). Breeders were selected from a stock of wild and captivity-bred fish, and females had a mean weight of 21.24±3.69 kg and males 16.12 ±2.61 kg. To induce ovulation and enhance sperm production, GnRHa (des-Gly10, [D-Ala6]-gonadotropin releasing hormone, Sigma, España), was administered to the selected breeders at doses of 15 µg kg^{-1} to females and males. The breeders were placed in 10-16 m^3 tanks with >400% water exchange. Males were placed together in one tank and females were placed isolated into a tank (1 female per tank) or in pairs of two females per tank. Temperature was maintained constant at 18ºC. A surface water egg collector was used to determine if any floating eggs were liberated. Females were held in complete darkness during the period from when the GnRHa was applied until stripping of ova was finished. Outside of this period females received a natural photoperiod. The GnRHa was applied to females at approximately 21:00 and 36.5 hours after hormone application (≈ 08:30) the females were checked for ovulated ova by applying abnominal pressure. The procedure of checking for and stripping ova from the females was repeated every 2.5 hours (≈ 11:00, 13:30, 16:00 and 18:30) until three-four batches of ova had been obtained (**Photo 2.4.1**).

Photo 2.4.1 Stripping ovulated ova from a meagre (*Argyrosomus regius*) breeder.

Males were held under a natural photoperiod. The GnRHa was applied to males at approximately 24 hours before female ovulation was anticipated (≈ 08:30 the day before ovulation). Sperm quality in terms of motility and density was checked when GnRHa was applied, and before the collected sperm was being used for fertilisation. Sperm quality was checked using the ImageJ CASA system, and during the day when females were stripped, sperm was stripped just once at ≈ 08:30 and held in a non-activating modified Leibovitz medium (see Task 2.3).

The task was in progress when this report was prepared (Mo 30 and 31) and the majority of the work described has been executed during Mo30 and the task will be completed during Mo31-32. The results and data that have been collected are being processed and no firm conclusions towards the development of methods should be drawn until the work is completed. However, some general observations can be made. The highest percentage fertilisation of ova has been obtained with ova stripped approximately 39 hours after the application of GnRHa (**Fig. 2.4.1**).

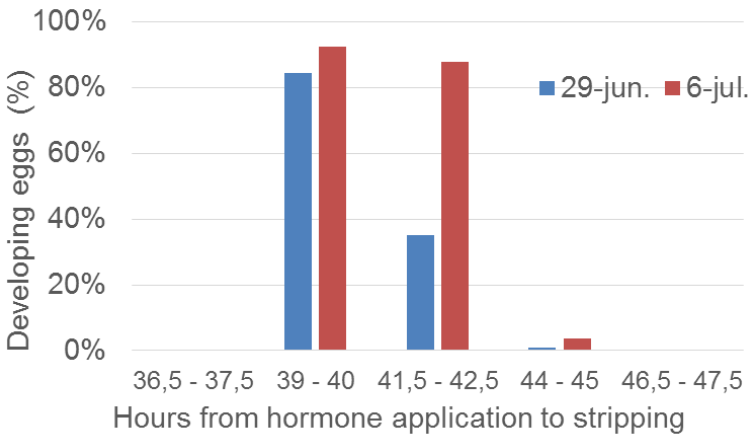


Figure 2.4.1. Percentage of developing eggs from batches of ova stripped at different times (hours) after GnRHa was applied to induce ovulation. All egg batches were fertilised with sperm that maintained constant quality during the period.

Fertilisation capacity of fresh sperm and sperm in modified Leibovitz medium was similar. Sperm quality before and 24 hours after GnRHa application was similar. The application of GnRHa maintained or increased the spermiation index. Work planned for the 3rd Reporting Period (M31-32) aims to determine the minimum number of sperm required for fertilisation, and to make a small scale *in vitro* fertilisation trial in collaboration with P14. IFREMER to make a dialled cross between males and females to produce a maximum number of families. The work is progressing as anticipated and no deviations or delays are expected for the associated deliverable.

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).

The first objective of this task was to identify SNPs in the genome of meagre using RNASeq, which could be then used to genetically characterize individuals or to implement QTL analysis and marker assisted selective breeding programs; results have been reported in Deliverable 2.1. In this deliverable, the muscle and liver transcriptome of meagre was sequenced and characterized. The outcome from meagre transcriptome sequencing is two-fold. First, it provides information on meagre gene content and sequence -on a global scale- allowing the further study of any gene family or genetic pathway of the species. Second, it allowed a transcriptome-wide scan for genetic marker discovery. A thorough marker discovery pipeline was implemented that led to thousands of SNP and STR markers that can be useful in future marker-assisted selection or other analyses. Our current work, in combination with the forth-coming SNP information that will be produced from the planned linkage analysis using full- and half-sib families in the next months will establish a new standard in meagre genetics setting the groundwork for deeper studies on growth and other traits of the species.

One of the principal bottlenecks to meagre production is the occurrence of variable growth rates, causing uncertainty in the prediction of total yield from each on-growing cycle. Fast and predictable growth is an important and highly desired trait, which affects the profitability of food animal production, since feed costs account for the largest proportion of production costs. The SNPs explain the greatest part of the genetic differences between individuals and are suitable for genetic evaluation and strategies that employ molecular genetics for selective breeding. Therefore, this task aims at using SNPs to potentially identify markers and genes associated with genetic variation in growth through Next Generation Sequencing (NGS) of the whole transcriptome of 16 fish from different families and phenotypic size (of the same age) that will provide a data-set of thousands of markers. The SNP and Short Tandem Repeats (STR or microsatellite) markers identified by RNA-Seq that will presumably be associated with growth traits in future studies are reported and catalogued.

Biological material

Sixteen meagre individuals were selected from four groups of fish (Lset1, Lset2, Mset1 and Mset2, see **Table 2.1.1**). The groups were from two spawning dates that were one week apart; set 1 consisted of two families spawned on the 24/04/2014 and set2 of three families spawned on the 01/05/2014. The two sets were graded and fish for RNA extraction were selected from the largest (groups “L”) and the medium grades (groups “M”) in order to have the highest chances to sample from 4 families (1 & 4 plus 2 & 5, respectively; **Table 2.1.1**). Selection was based on the expected kinship of individuals targeting on fish that are theoretically not closely related. For this purpose, fish were selected from the four groups that contained five different meagre crosses (families) that resulted mostly from wild outbred parents (**Table 2.1.2**). Muscle and liver tissues were dissected and preserved in RNAlater (IRTA, Spain).

Table 2.1.1 Details for the meagre used for RNA extraction and transcriptome sequencing from the six families initially formed and stocked in two sets according to spawning date (set 1 on 24/04/2014 and set 2 on 01/05/2014) after size-grading for small (S), medium (M) and large (L) fish.

Rearing Tank	Number of fish	Proportion of each family at start				
		1	2	4	5	6
(L-set1)	12	38%		62%		
(M-set1)	46	61%		39%		
(S-set1)	224	51%	14%	33%		3%
(L-set2)	19		26%		74%	
(M-set2)	49		83%			17%
(S-set2)	66		83%			17%

Table 2.1.2 Characteristics of the six families formed in meagre, spawning dates, ID number of breeder and origin (wild or cultured) of the breeders used.

Family	Tank (Spawning Date)	Female	Male
1	V8-1 (24/04/2014)	5-wild	19-wild
2	V8-1 (01/05/2014)	5-wild	20-wild
3	V8-2 (01/05/2014)	1-wild	19-wild
4	C2 (24/04/2014)	16-cultured	21-wild
5	C1 (01/05/2014)	2-wild	22-wild
6	V6 (01/05/2014)	13-cultured	17-wild

RNA extraction, Library preparation and Sequencing

Muscle and liver samples were further processed in P1. HCMR, Greece. The RNA extraction protocols have been completed for the liver and muscle tissues of 16 sampled individuals (four fish/family). For both tissue types, RNA was extracted after grinding the tissue with liquid nitrogen using pestle and mortar. In the case of liver tissues, total RNA was extracted with Qiagen's RNeasy Plus extraction kit, while muscle tissues were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.) and RNA was isolated according to the manufacturer's instructions. The quantity of the isolated total RNA was measured spectrophotometrically with NanoDrop® ND-1000 (Thermo Scientific), and quality was tested on an agarose gel (electrophoresis in 1.5% w/v) (**Figure 2.1.1**).

Following extraction, RNA from different individuals was pooled in equal quantities for each of the two tissue types. Then, an RNASeq library was constructed for each tissue following standard Illumina TruSeq protocols. The two libraries were loaded into one lane of an Illumina HiSeq2500 instrument (2x100bp).

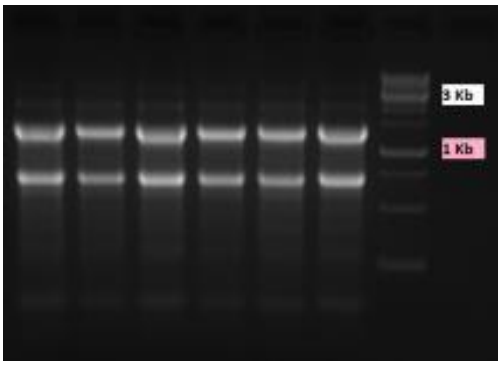


Figure 2.1.1 Total RNA extraction profile from meagre liver tissues. The size marker on the right side of the gel is the 1Kb DNA ladder RTU from Nippon Genetics GmbH.

Raw read pre-processing

Raw read quality was assessed with FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw data quality control took place in four steps. First, sequence contamination was removed from Illumina adapter sequences with Scythe (https://github.com/vsbuffalo/scythe). Scythe recognizes adapter sequences by taking into account quality information and thus increasing the efficiency of removing them especially at the 3' end of the reads where read quality drops. Second, Sickle (https://github.com/najoshi/sickle), a tool that uses sliding windows to identify reads with low-quality regions especially for 5' and 3' regions of the reads, was used to trim the low-quality ends of the reads. The next step was to use the general quality control software Trimmomatic (Bolger et al. 2014) that removes low quality reads applying various filters, including cutting adapter and other Illumina-specific sequences from the read, performing a sliding window trimming, cutting once the average quality within the window falls below a threshold and cutting bases off the start/end of a read if below a threshold quality. Finally, Prinseq (Schmieder and Edwards 2011) was used to remove any remaining sequences that are the result of adapter contamination. Each of the applied software tools applies alternative methodologies to remove errors. The combination of all four pieces of software has led to an efficient quality control.

Transcriptome assembly and annotation

To build the transcriptome assembly, quality-filtered reads were input in the software Trinity (version trinityrnaseq_r20140717; Grabherr et al. 2011) and ran with default settings. Trinity is specialized in assembling Illumina reads and is considered one of the most efficient assembly software (Haas et al. 2013). The assembly process required ~ 100Gb of RAM memory and 10 CPUs and was run successfully at the computer cluster of IMBBC, HCMR. To annotate the transcripts and get an idea of their potential role, a similarity search was conducted through *blastx* against Swiss-Prot protein database (*e*-value threshold 10^{-12}). *blastx* was run in parallel using NOblast (Lagnel et al. 2009) and the best hit was kept for each transcript. Blast output was summarized with custom shell commands/scripts.

Genetic marker discovery

The assembled sequences were scanned for STRs with Phobos (Mayer 2006-2010). Non-exact STRs were detected with a 2–10 repeat unit length and a minimum length of 20 nucleotides. A custom Perl script was used to parse the output. The SNP discovery took place using SAMTOOLS (Li et al. 2009), one of the most efficient SNP discovery tools for next generation data. First, quality-filtered reads from both muscle and liver samples were mapped onto the transcriptome assembly using the mapping software bowtie2 (Langmead and Salzberg 2012). Then, the alignment files (.sam file) were analyzed with samtools *mpileup* function. The SNP calling was conducted with *bcftools call* command and then quality filtered with *bcftools filter* command keeping only SNPs with the above a strict quality threshold (Q>19). Finally, SNPs were further filtered based on the number of high quality read coverage (DP>9).

Raw data quality control

Illumina sequencing led to the production of 523,137,020 raw reads. Filtering steps reduced this to 341,439,304 (65%) high quality paired reads (182,802,502 for muscle and 158,636,802 for liver sample) (**Table 2.1.3**). Application of Sickle and Trimmomatic resulted in the greatest filtering. Prinseq removed relatively few more sequences, while Scythe trimmed adapter sequences from the reads' ends. Overall, read quality was significantly improved after the application of filtering criteria, especially at the 5' and 3' prime ends of the reads (**Fig. 2.1.2**).

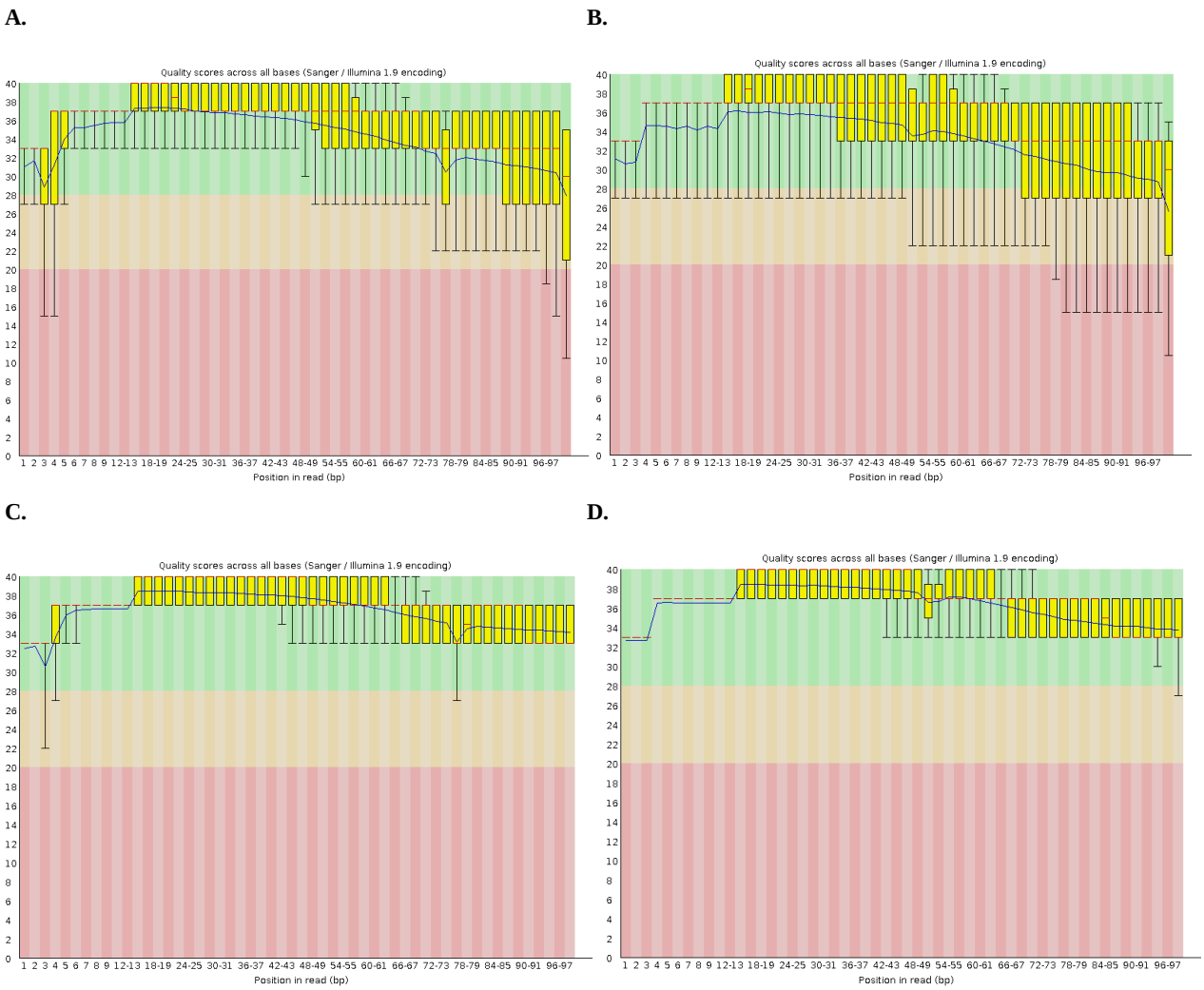


Figure 2.1.2 Quality filtering for the muscle sample. On top, the "per base" quality of read1 (A) and read2 (B) of the raw data is shown; on the bottom, the "per base" quality of read1 (C) and read2 (D) of the quality-filtered data are presented. Quality is measured in Illumina 1.9 Phred score. Plots were made with FASTQC toolkit.

Table 2.1.3 Illumina reads surviving in pairs (excluding orphans) after each quality-filtering step.

Filtering step	Software	Muscle tissue	Liver tissue	Total
1	Scythe	280,804,390	242,332,630	523,137,020
2	Sickle	250,526,202	216,487,756	467,013,958
3	Trimmomatic	183,041,946	158,882,592	341,924,538
4	Prinseq	182,802,502	158,636,802	341,439,304

Meagre Transcriptome

The transcriptome of meagre was reconstructed with the 341,439,304 paired filtered reads that passed through all quality control filters applied. The assembly consisted of 95,964 transcripts belonging to 80,824 loci or genes (**Table 2.1.4**). It has an average length of 1,058.83 bp, and N50 statistic (i.e. the length N for which half of all bases in the sequences are in a sequence of length L < N) of 2,183 bp. To understand the

basic function of the assembled transcripts a *blastx* similarity search was made against Swiss-Prot. Out of 95,964 transcripts, approximately 37% (35,888) had a significant hit against Swiss-Prot (*e*-value 10^{-12}) and in particular against Nile tilapia (*Oreochromis niloticus*) proteins. The produced assembly provides an excellent reference for future needs in terms of meagre coding sequences.

Table 2.1.4 Meagre transcriptome assembly statistics.

Assembly Statistic	Value
Number of Sequences	95,964
Total Length	101,609,879
Average Length	1,058.83
Median	472
Min	201
Max	25,456
% GC	46.19
N25	3,771
N50	2,183
bps at N50	50,805,786
N75	932
N90	365

*All statistics length is in bp.

Genetic markers discovery

Genetic marker discovery was two-fold. First, the transcripts were searched for non-exact short tandem repeats (STRs) of 2- up to 10-nucleotides at the assembled transcriptome of meagre. The search revealed 20,582 total STRs located in 16,517 transcripts belonging to 12,565 genes (summarized in **Table 2.1.5**).

Table 2.1.5 Short Tandem Repeats (STRs or microsatellites) discovered in meagre transcriptome.

STR type	Number of STRs found
2-nucleotide	3,856
3-nucleotide	4,439
4-nucleotide	1,444
5-nucleotide	1,222
6-nucleotide	2,838
7-nucleotide	1,242
8-nucleotide	1,129
9-nucleotide	2,229
10-nucleotide	2,183
Total	20,582

The next step included a search for SNPs in the transcripts of meagre. A total of 133,613 SNPs were discovered. Quality filtering led to elimination of 46,259 SNPs and resulted to 87,354 high quality SNPs. Finally, further depth filtering resulted in 71,736 SNPs located in 20,309 transcripts belonging to 18,657 loci.

The SNP and STR loci discovered comprise a valuable source of genetic markers widely distributed in the transcriptome of meagre. To be able in the future to use markers not linked for genetic applications (e.g. SNP-chip construction, parentage analysis, QTL identification, etc.), indirect linkage information was extracted based on sequence similarity against Nile tilapia. The gene with the highest similarity to a tilapia gene (top hit) was tracked to the genomic scaffold that this hit is located in tilapia genome (see ANNEX 2.1). The rationale behind indirect linkage information is that in cases where the top tilapia hits of two meagre genes are located on the same scaffold, it can be assumed that there is high chance that those two genes are linked in meagre genome too.

The use of individuals from multiple families increases the chance of including in our data multiple polymorphisms including those involved in growth. The implemented experimental design allowed the identification of both SNP and STR markers in unprecedented magnitude. It is anticipated that future analyses based on those markers will lead to a better understanding of meagre genetics. Finally, in combination with the QTL mapping approach, which is in progress and expected to be delivered by Month 36, this will greatly expand the SNP catalogue for meagre. The full description of the above work and results has been provided in Deliverable ***D2.1. Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers.***

On January 20th 2016, 400 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. Total length and weight was measured for all fish. The cage contained approximately 80.000 fish (data to be confirmed with collaborating fish farm), which were stocked into the cage as juveniles (**Fig. 2.1.3**). 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 GnRH to induce spawning.

A. **B.**

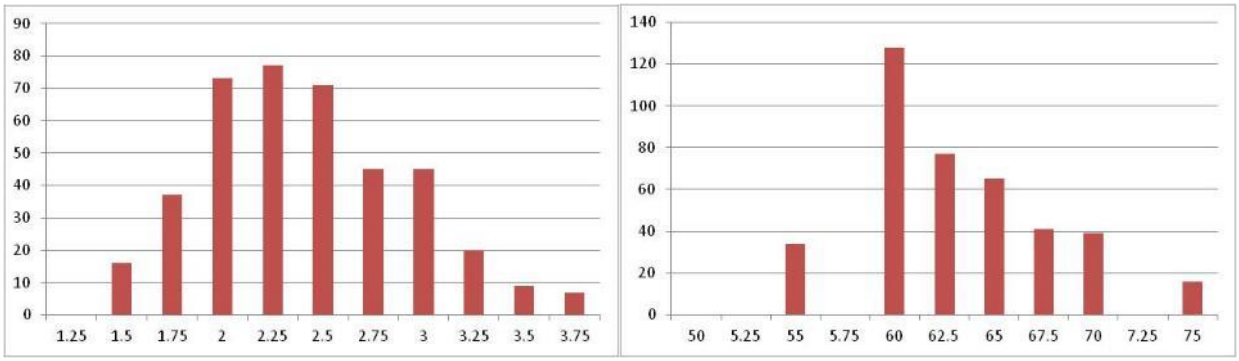


Figure 2.1.3 A. Weight (in kg), and B. total length (in cm) of the 400 meagre fish sampled.

DNA was extracted from all fish using standard protocols and genotyped for the 10 locus multiplex used in Task 2.1 (Loci: Cacmic14, UBA054, UBA050, UBA053, Soc431, UBA042, UBA853, UBA005, Soc405, and UBA006) using the Qiagen multiplex PCR kit. Results were evaluated with FAP software (Taggart, J. B. 2007. FAP: an exclusion-based parental assignment program with enhanced predictive functions. Molecular Ecology Notes 7:412-415) to infer parentage of those 400 fish based on the parental genotypes of 19 breeders. Parentage was based on nine loci since locus UBA053 was excluded from the analysis.

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; females 403 and 404 have participated the most and to a smaller extend females 391 and 406. Likewise, six out of the eleven males were identified as probable parents of the offspring; Male 405 seems to be responsible for nearly half of them followed by males 397, 388 and 402 (see **Table 2.1.6**).

Table 2.1.6 Parentage assignment in the meagre stock.

		Males						
		388M	**397M**	**398M**	**401M**	**402M**	**405M**	**Total**
Females	**391F**	10	3	3	7	10	25	**58**
	394F						1	**1**
	403F		77			3	24	**104**
	404F	29				6	95	**130**
	406F	1	8			3	40	**52**
	Total	**40**	**88**	**3**	**7**	**22**	**185**	

The fifteen families were ranked according to their median weight and body length (**Table 2.1.7**); the two families with only one offspring were excluded.

Table 2.1.7 Ranking of the 15 meagre families for body weight (kg) and total length (cm).

Code	Dam	Sire	bwt	len	bwt_rank	len_rank	Nb of Fish
A	BR391F	BR405M	0.236017	22.88583	2	1	25
B	BR404F	BR388M	0.179912	20.58313	5	2	29
C	BR404F	BR405M	0.226776	19.63287	3	3	95
D	BR403F	BR405M	0.176233	19.55027	7	4	24
E	BR404F	BR402M	0.26567	18	1	5	6
F	BR403F	BR397M	0.21635	15.92225	4	6	77
G	BR406F	BR405M	0.136498	12.95112	10	7	40
H	BR391F	BR398M	0.1519	12.33333	9	8	3
I	BR406F	BR397M	0.177244	11.875	6	9	8
J	BR406F	BR402M	0.1008	9.083333	11	10	3
K	BR391F	BR401M	0.075707	7.602679	12	11	7
L	BR391F	BR388M	0.075143	6.1	13	12	10
M	BR391F	BR402M	0.155067	5.525	8	13	10
N	BR391F	BR397M	0.021233	1.75	15	14	3
O	BR403F	BR402M	0.033233	1.583333	14	15	6

From the above fish, 260 fish from families A, B, C, D, F and M and their seven breeders (three females: 391, 403 and 404 and four males: 388, 397, 402 and 405) were chosen for the construction of two ddRAD libraries according to the protocol described and successfully applied in a published study from our group (Manousaki, T., A. Tsakogiannis, J. B. Taggart, C. Palaiokostas, D. Tsaparis, J. Lagnel, D. Chatziplis, A. Magoulas, N. Papandroulakis, C. C. Mylonas, and C. S. Tsigenopoulos. 2016. Exploring a Nonmodel Teleost Genome Through RAD Sequencing—Linkage Mapping in Common Pandora, *Pagellus erythrinus* and Comparative Genomic Analysis. G3: Genes|Genomes|Genetics **6**:509-519). This work is currently in progress and will be reported in the 3rd Reporting Period.

Deviations from Annex I and their impact:

There were no deviations from the Annex I in this WP.

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	P24. ITTICAL	
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), Christian Fauvel (P14), Covadonga Rodriguez (P15), Tasos Raftopoulos (P23), Stefano Carbonara (P24)			

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):

- A 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).
- Preliminary experiments in the Mediterranean stock 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.
- 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.

- 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.
- 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 progress towards objectives and details for each task (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.

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

According to the DOW, during Y2 a comparative study on reproductive and nutritional state between wild and captive-reared greater amberjack was carried out by UNIBA, HCMR, ULL, IORL and ARGO. Part of the work carried out within the present task has been submitted in Deliverables D3.1 and D3.3, and part of it will be included in D3.2 (originally due on month 18, postponed to month 33), D3.4 (month 32), D3.5 and D3.6 (both on month 46).

Sub task 3.1.1 Wild and captive-reared greater amberjack sampling

According to the DOW, it was scheduled to undertake samplings of wild and captive-reared greater amberjack in three different periods of the reproductive cycle. Based on the available data on the reproductive biology of the species in the Mediterranean Sea, the following phases of the reproductive cycle were identified: early gametogenesis (EARLY; late April-early May), advanced gametogenesis (ADVANCED; late May-early June) and spawning (SPAWNING ; late June-July).

The sample collection of wild greater amberjack started in Y1 and was finalised during Y2. On 1 May and 29 June 2015, a total of 16 (8 males and 8 females) wild adult greater amberjack were caught around the Pelagic Islands (Sicily, Italy). Soon after capture, blood was collected, centrifuged and stored in dry ice. Subsequently, the following samples were taken: gonads, liver, muscle, brain, pituitary, first spiniform ray of the first dorsal fin, scales, otoliths and caudal vertebrae. From each fish, the following biometric data were recorded: fork length, FL, in cm; body mass, BM, in kg; gonad mass, GM, in g (**Table. 3.1.1**). Once the sampling was finalised, different shipments were arranged in order to provide the samples to the relevant Partners (HCMR, IOLR and ULL).

Due to the loss of the broodstock in ITTICAL in Y1 and the following vain attempts to reconstitute a new one, a wild-caught broodstock was made available for the present task by ARGO (Salamina Island, Greece). Greater amberjack juveniles were captured in 2011 in the area of Astakos (Ionian Sea, Greece), and then transferred to ARGO in September 2014. The fish were initially fed raw fish and, once transferred to ARGO, they were reared in sea cages and fed on a commercial diet (Vitalis Cal, Skretting).

In Y2, the sampling program of captive-reared greater amberjack took place, and involved HCMR, UNIBA IFREMER and ARGO. On 24 April, 4 June and 2 July 2015, a total of 24 fish (4 males and 4 females per sampling) were sampled. The fish were confined in a small cage area using a PVC curtain and anesthetized lightly with about 0.01 ml l^{-1} clove oil. Then, they were gently directed into a PVC stretcher, brought on board of a service vessel, and anesthetized deeply with 0.03 ml l^{-1} clove oil. Subsequently, fish were sexed using a gonadal biopsy, and a blood sample was obtained from the caudal musculature. The fish were euthanized by severing the gills, placed in crushed ice and transferred to the farm facility where biometric data were recorded (**Table. 3.1.2**) and the same biological samples as for wild fish were taken. In addition, sperm was collected for quality evaluation (see Task 3.1.8 below). The shipment of captive-reared fish samples to the relevant Partners (IOLR and ULL) was managed by HCMR, whereas samples destined to UNIBA and IFREMER were taken by the relevant staff soon after each sampling. During each sampling, Sea Surface Temperature (SST, in C°) was recorded.

Table 3.1.1. Biometric data and estimated age of wild greater amberjack sampled around the Pelagic Islands (Italy).

Sampling Date	Sex	Fork length (FL, cm)	Body Mass (BM, kg)	Gonad Mass (GM, g)	Age (years)
Early gametogenesis period					
01/05/2015 (SST = 18.1 °C)	m	111	14	300	6
	m	112	20	450	6
	m	112	15	300	6
	m	117	19	550	6
	m	113	19	400	6
	f	103	14	100	5
	f	112	19	200	6
	f	116	20	300	6
	f	103	15	200	5
	f	106	13	100	5
Advanced gametogenesis period					
31/05/2014 (SST = 19.3 °C)	m	124	22	1900	7
	m	102	13	650	5
	m	115	19	2200	6
	m	99	14	1150	5
	f	117	22	1650	6
	f	114	21	1600	6
Spawning period					
29/06/2015 (SST = 23.8 °C)	m	100	12	650	5
	m	102	14	700	5
	m	104	16	950	5
	f	101	14	500	4
	f	114	19	1000	6
	f	109	16	700	6
30/06/2014 (SST = 23.4 °C)	m	100	11	400	5
	m	99	11	577	4
	f	99	11	500	5
	f	100	12	490	5
	f	97	12	450	5
	f	100	12	400	5
	f	98	12	500	4
	f	96	12	390	4
	f	102	13	600	5
	f	104	14	950	5
	f	95	12	450	5

Table 3.1.2. Biometric data and estimated age of adult captivity-reared greater amberjack from ARGO (Salamina Island, Greece).

Sampling Date	Sex	Fork length (FL, cm)	Body Mass (BM, kg)	Gonad Mass (GM, g)	Age (years)
Early gametogenesis period					
	m	101	15	95	4
	m	94	12	60	4
	m	92	12	65	4
24/04/2015	m	94	13	60	4
(SST = 17.5 °C)	f	87	10	85	4
	f	97	14	155	4
	f	96	14	125	4
	f	100	14	160	4
Advanced gametogenesis period					
	m	90	9	370	4
	m	97	14	295	4
	m	98	13	600	4
04/06/2015	m	103	15	690	4
(SST = 20.0 °C)	f	97	13	335	4
	f	97	13	920	4
	f	106	17	305	5
	f	101	12	660	4
Spawning period					
	m	96	13	140	4
	m	95	11	155	4
	m	91	10	70	4
02/07/2015	m	96	12	130	4
(SST = 25.5 °C)	f	92	8	95	4
	f	96	12	130	4
	f	95	11	135	4
	f	97	12	140	4

3.1.2 Age determination of wild and captive greater amberjack

Among the hard structures sampled, the scales (**Fig. 3.1.1**) proved to be the easiest to be processed and read, and were therefore used for age determination. The scales were rinsed in tap water and in 70% ethanol, placed between two microscope slides and observed with a binocular lens microscope under transmitted light, connected through a digital camera (DC 300, Leica, Wetzlar, Germany) to the image analyser (Quantiment 500 W, Leica, Wetzlar, Germany). The estimated ages are reported in **Tables 3.1.1 and 3.1.2**.

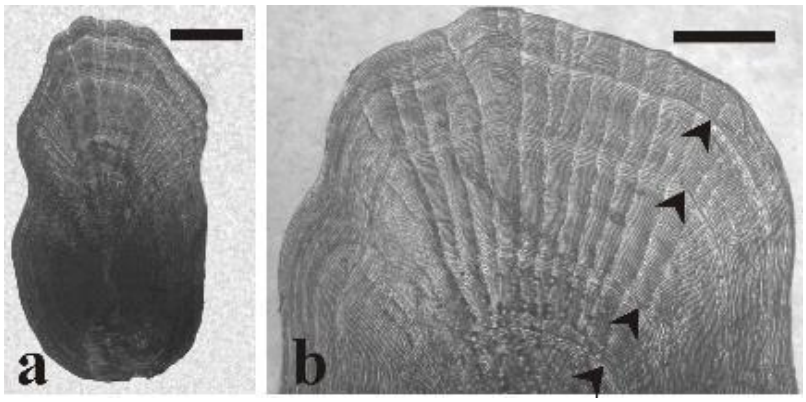


Figure 3.1.1. Images of greater amberjack scales. (a) Scale from a 95-cm FL specimen sampled on 24 April 2015 in ARGO (Salamina Island, Greece). (b) Particular of (a) showing growth marks (annuli) (arrowheads). Magnification bar = 2 mm in (a) and 1 mm in (b).

3.1.3 Reproductive state assessment

The assessment of the reproductive state was carried out through the calculation of the gonadosomatic index (GSI = 100 GM BM^{-1}) and the histological analysis of the gonads for both sexes. For histological analysis, one-cm thick gonad slices were cut and fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µm thick sections were stained with haematoxylin-eosin, and Mallory's trichrome.

Females

Significant seasonal changes occurred in GSI of both wild and captive-reared females, with an increase from EARLY to ADVANCED, followed by a decrease during SPAWNING. Wild fish showed a trend towards higher GSI than captive-reared individuals in all the examined periods, although the difference was statistically significant only in SPAWNING (**Fig. 3.1.2**).

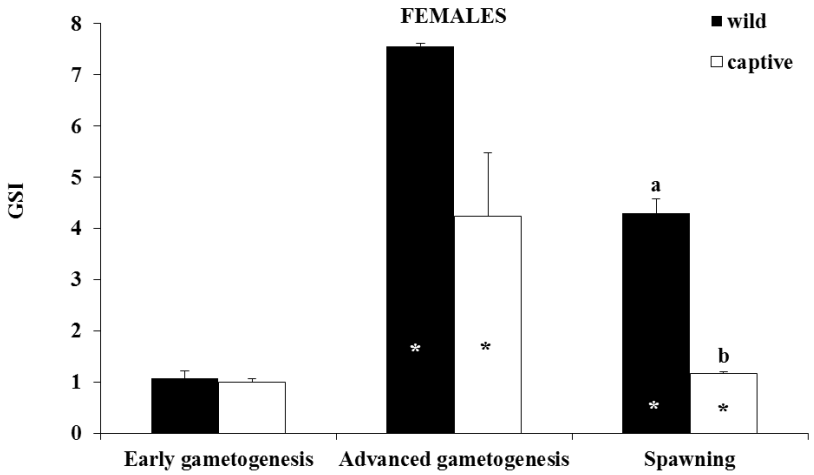


Figure 3.1.2. Mean (±SE) gonado-somatic index (GSI) of wild and captive-reared female greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period within the same group. Different letters indicate significant differences between wild and captive individuals in the same period of the reproductive cycle (ANOVA, $P < 0.05$).

Female reproductive state was assessed by recording the most advanced oocyte stage for each specimen. Moreover, the presence of postovulatory and atretic follicles was recorded. During EARLY, among the wild fish analysed one had perinucleolar oocytes (**Fig. 3.1.3a**), two showed oocytes at cortical alveoli stage (**Fig. 3.1.3b**) and two had early vitellogenic oocytes as the most advanced stage (**Fig. 3.1.3c**). The two females sampled during ADVANCED had late vitellogenic follicles along with post-ovulatory follicles (sign of recent spawning) in their ovaries (**Fig. 3.1.3d**). Among the 12 females sampled during the SPAWNING period, 10 showed late vitellogenic follicles along with post-ovulatory follicles and two had hydrated oocytes (**Fig. 3.1.3e**).

Among the captive-reared females sampled during EARLY, one showed ovaries with perinucleolar oocytes and three had few early vitellogenic oocytes. During ADVANCED, all the sampled individuals showed oocytes in late vitellogenesis stage, but three of them were affected by an extensive atresia (more than 50% of late vitellogenic follicles were atretic) (**Fig. 3.1.3f**). During SP, three females had ovaries with late vitellogenic follicles undergoing extensive atresia and one showed only perinucleolar oocytes. All these fish were considered to be in a regressing condition.

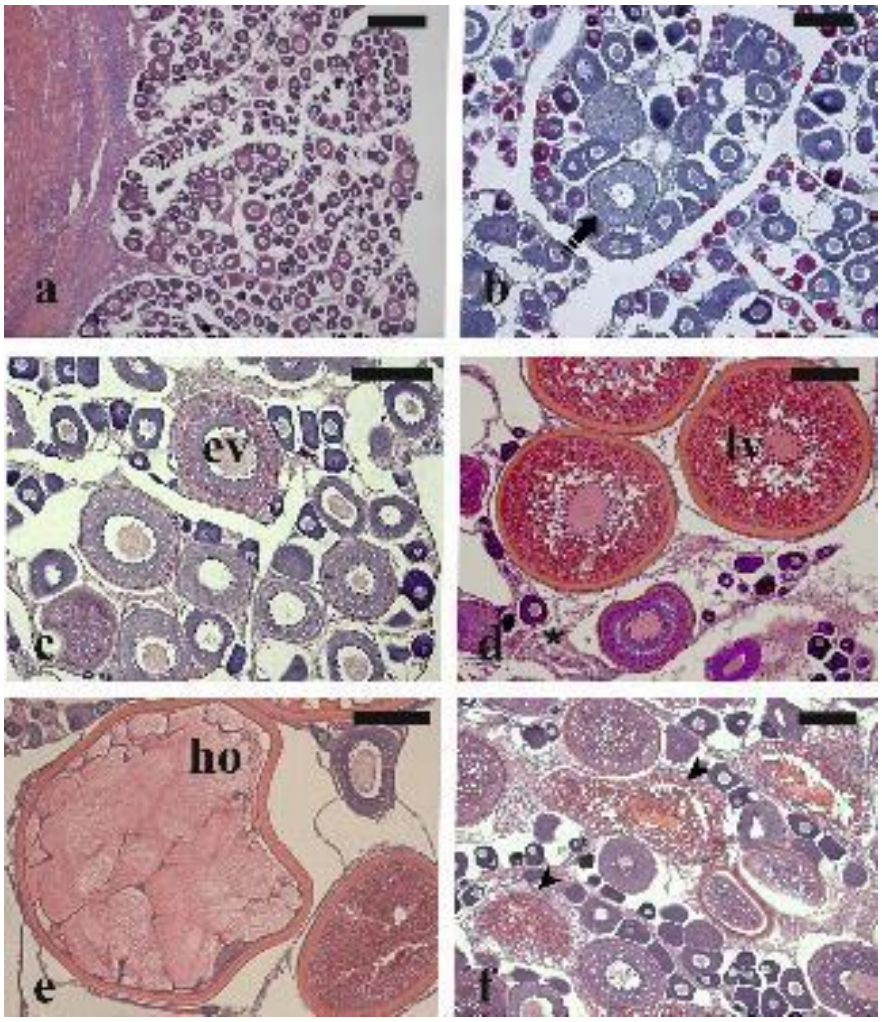


Figure 3.1.3. Micrographs of ovary sections from female greater amberjack sampled in three different periods of the reproductive season. (a) Wild individual sampled on 01 May 2015 showing perinucleolar oocytes as the most advanced stage in the ovary. (b) Cortical alveoli oocytes from the ovary of a wild specimen captured on 01 May 2015. (c) Early vitellogenic oocytes from the ovary of a wild individual sampled on 01 May 2015. (d) Late vitellogenic oocytes together with post-ovulatory follicles from a wild spawning fish caught on 31 May 2014. (e) Hydrated oocyte from a spawning wild fish sampled on 30 June 2014. (f) Extensive atresia of late vitellogenenic follicles in a captive-reared specimen sampled on 04 June

2015. Haematoxylin-eosin staining in (a), (c), (d), (e) and Mallory's trichrome staining in (b). Magnification bars = 300 µm in (a) and 150 µm in (b)-(f). Arrowhead: atretic late vitellogenic follicle; asterisk: post-ovulatory follicle; dashed arrow: cortical alveoli stage oocyte; ev: oocyte in early vitellogenesis stage; ho: hydrated oocyte; lv: oocyte in late vitellogenesis stage.

Oocyte yolk accumulation

In order to compare oocyte yolk accumulation in wild and captive-reared individuals, oocytes at early and late stage of vitellogenesis, having a large and centrally located nucleus were selected. Oocyte diameter (µm) and surface occupied by yolk granules (μm^2) were measured from microphotographs taken with a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK). No difference in oocyte yolk accumulation between wild and captive-reared specimens was found (ANOVA $P > 0.05$).

Males

The GSI of both wild and captive-reared male greater amberjack increased significantly from EARLY to ADVANCED, and then decreased during the SPAWNING. GSI was significantly higher in wild males in all the three considered periods (**Fig. 3.1.4**).

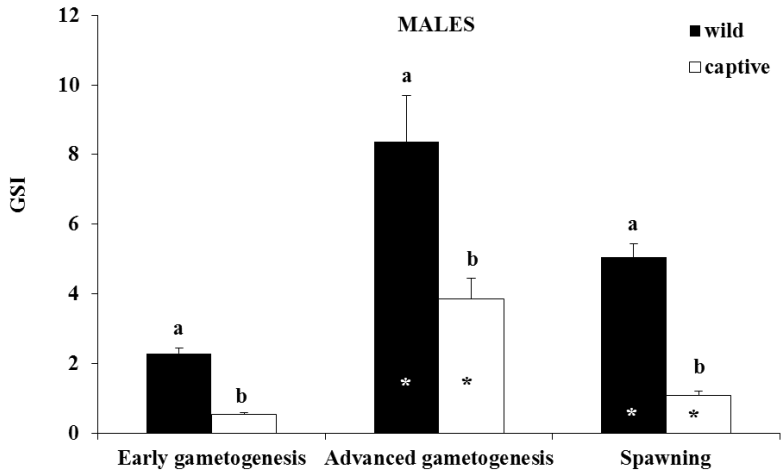


Figure 3.1.4. Mean (±SE) gonado-somatic index (GSI) of wild and captive adult male greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period within the same group. Different letters indicate significant differences between wild and captive individuals at the same period of the reproductive cycle (ANOVA, $P < 0.05$).

For the classification of the reproductive state of males, the type of spermatogenic cysts was recorded, and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively. During EARLY, the testes of the five wild males analysed contained germ cells in all the spermatogenic stages and spermatozoa in the lumen of seminiferous lobules (**Fig. 3.1.5a**). All the four wild males sampled during the ADVANCED period and four out of five fish sampled during the SPAWNING period showed all stages of spermatogenesis in the germinal epithelium as well as large amount of luminal spermatozoa (**Fig. 3.1.5b**). One of the fish sampled in the SPAWNING period was partially spent, showing seminiferous lobules with residual spermatozoa.

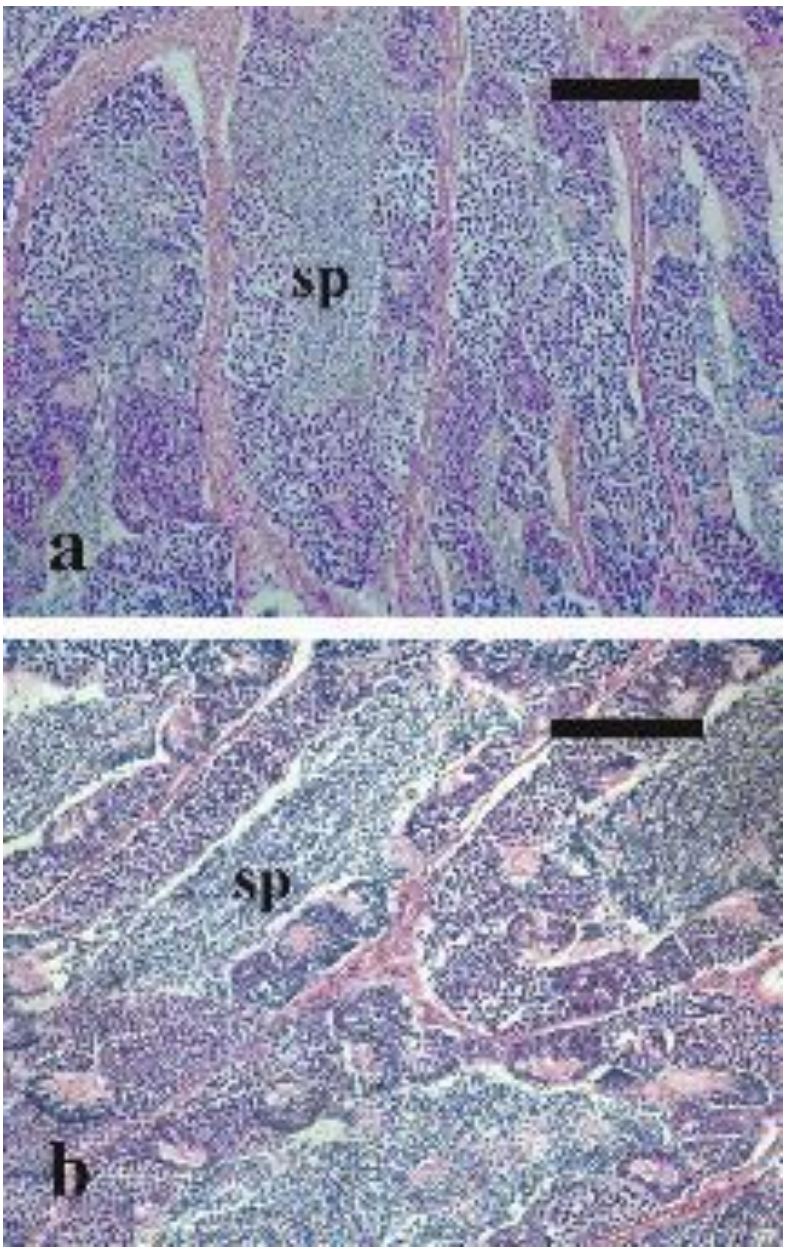


Figure 3.1.5. Micrographs of testis sections of wild greater amberjack sampled in two periods of the reproductive season. (a) Testis section from an individual sampled in early gametogenesis period showing the presence of all stages of spermatogenesis in the germinal epithelium and a limited amount of luminal spermatozoa. (b) Testis section from a fish caught during the advanced gametogenesis period, showing all stages of spermatogenesis as well as large amount of luminal spermatozoa. Haematoxylin-eosin staining. Magnification bars = 100 μm. sp: luminal spermatozoa.

The four captive-reared males sampled in EARLY had all stages of spermatogenesis in the germinal epithelium and only rare luminal spermatozoa (**Fig. 3.1.6a**). In ADVANCED, two fish had testes in active spermatogenesis and two had ceased their spermatogenic activity, showing only residual sperm cysts in the germinal epithelium and abundant spermatozoa in the lumen of seminiferous lobules (**Fig. 3.1.6b**). All four males sampled during SPAWNING period had ceased their spermatogenic activity still showing only a moderate amount of spermatozoa in the lumen of seminiferous lobules (**Fig. 3.1.6c**).

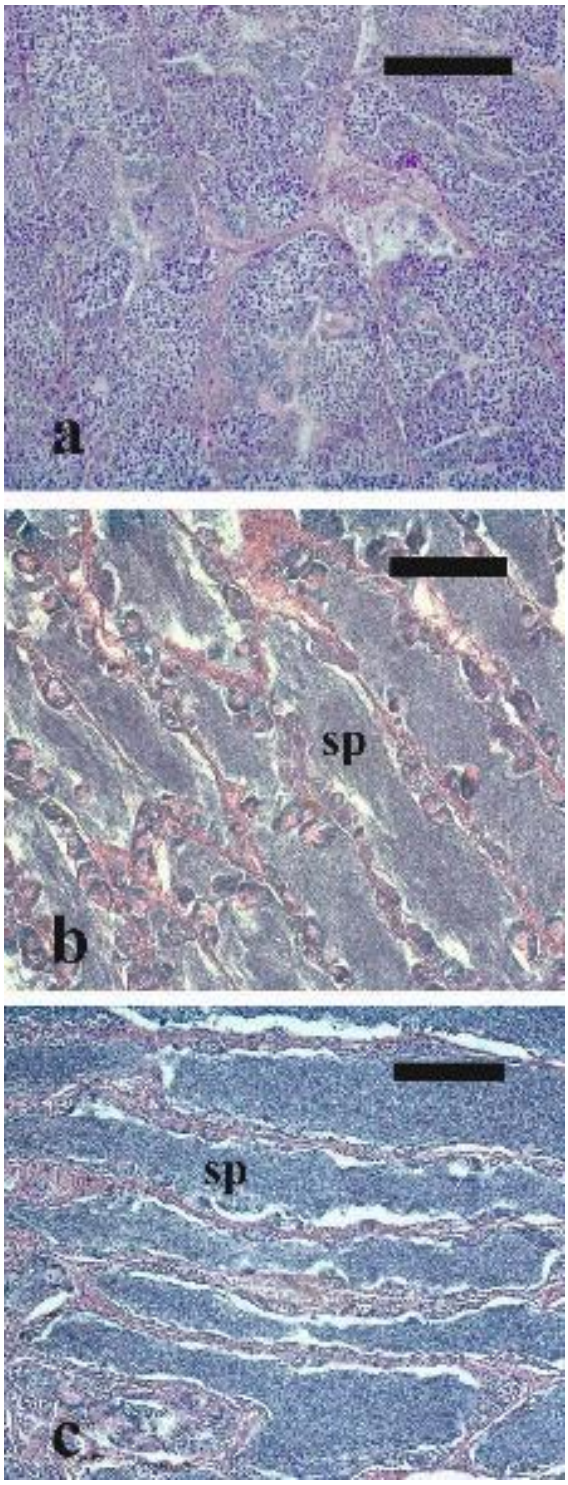


Figure 3.1.6. Micrographs of testis sections of captive-reared greater amberjack sampled in ARGO in three periods of the reproductive season. a) Testis section from an individual sampled in early gametogenesis period showing the presence of all stages of spermatogenesis. (b) Testis section from a fish sampled in the advanced gametogenesis period showing an arrested spermatogenesis state, with residual sperm cysts in the germinal epithelium and abundant spermatozoa in the lumen of seminiferous lobules. (c) Testis sections from a specimen caught during the spawning period showing a moderate amount of spermatozoa in the lumen of seminiferous lobules. Haematoxylin-eosin staining. Magnification bars = 100 µm in (a), 200 µm in (b) and (c). sp: spermatozoa in the lumina of seminiferous lobules.

3.1.4 Sex-steroid plasma level measurement

For the quantification of testosterone (T), 11-Ketotestosterone (11-KT) and 17,20β-dihydroxypren-4-en-3-one (17,20β-P) in the plasma, already established and well-described enzyme-linked immunoassays (ELISA) were used with some modifications. For the quantification of 17β-estradiol (E2), an ELISA kit was used. For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing for 3 min. After vortexing, samples were frozen for 10 min at -80°C and the supernatant organic phase was collected in new tubes and evaporated under a stream of nitrogen Samples were reconstituted in reaction buffer for running in the ELISA.

In wild females, plasma levels of T, E2 and 17,20β-P increased significantly from EARLY to ADVANCED, while in the case of T they significantly decreased during SPAWNING (**Fig. 3.1.7**). In captive-reared females, both T and E2 increased significantly from EARLY to ADVANCED and then decreased during SPAWNING (**Fig. 3.1.7a,b**), while plasma 17,20β-P did not decrease significantly at the SPAWNING stage (**Fig. 3.1.7c**). Significantly higher T and E2 plasma levels were found in wild compared to captive-reared animals at the ADVANCED and SPAWNING stage (**Fig. 3.1.7a,b**). Plasma 17,20β-P levels were significantly higher in wild compared to captive-reared fish during the in EARLY and ADVANCED (**Fig. 3.1.7c**).

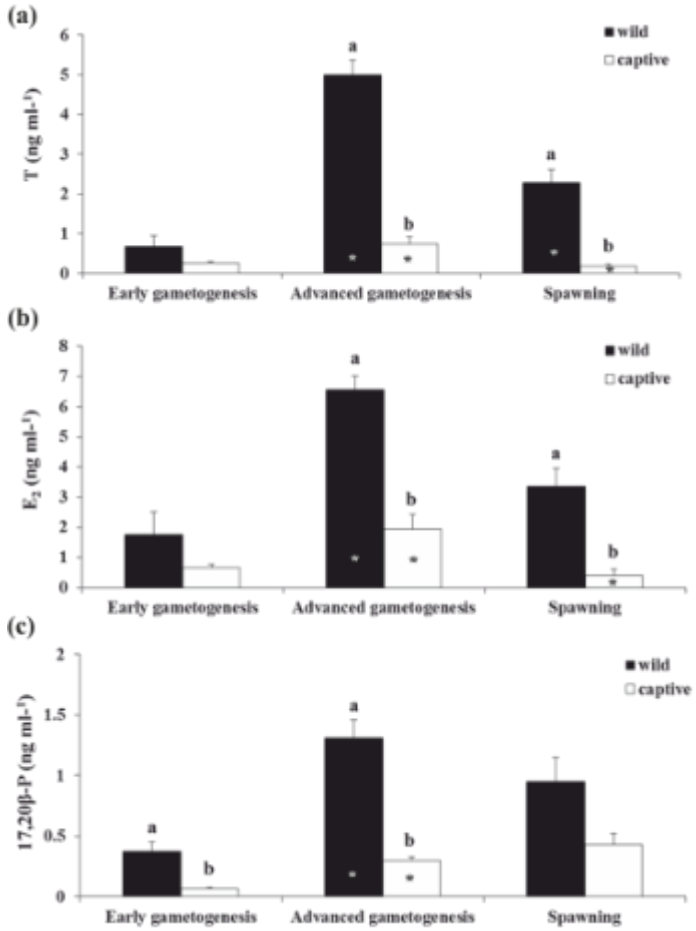


Figure 3.1.7. Mean (±SE) plasma (a) Testosterone (T), (b) 17-β Estradiol (E2) and (c) 17,20β-P plasma in wild and captive-reared greater amberjack females at three phases of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period within the same group. Different letters indicate significant differences between wild and captive-reared individuals in the same period of the reproductive cycle (ANOVA, $P < 0.05$).

In wild males, T and 11-KT plasma levels increased significantly from EARLY to ADVANCED and decreased thereafter in case of only 11-KT (**Fig. 3.1.8**a,**b**). Plasma levels of 17,20β-P showed a significant increase from EARLY to ADVANCED, and the same trend was observed in SP, even though a significant difference was not found (**Fig. 3.1.8c**). In captive-reared fish, both T and 11-KT plasma levels showed a decreasing trend from EARLY to SPAWNING (**Fig. 3.1.8a,b**), whereas plasma 17,20β-P levels showed a significant increase from ADVANCED to SPAWNING (**Fig. 3.1.8c**). In general, plasma levels of all the three analysed steroids were higher in wild than in captive-reared fish.

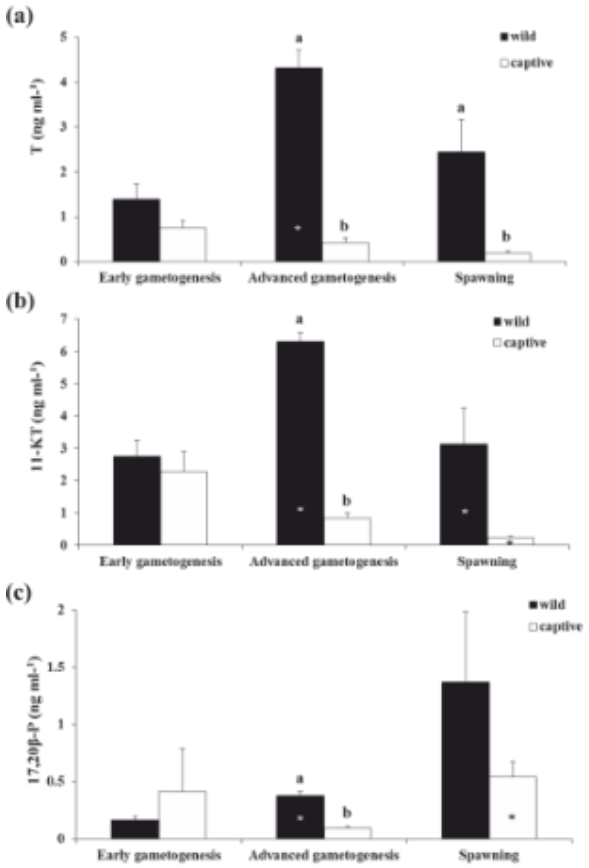


Figure 3.1.8. Mean (±SE) plasma (a) Testosterone (T), (b) 11-Ketotestosterone (11-KT) and (c) 17,20β-P in wild and captive-reared greater amberjack males at three periods of the reproductive season. Asterisks indicate statistically significant differences versus the preceding period within the same group. Different letters indicate significant differences between wild and captive-reared individuals in the same period of the reproductive cycle (ANOVA, $P < 0.05$).

3.1.5 Seminiferous lobule diameter and male germ cell proliferation and apoptosis

At least 50 seminiferous lobules were selected randomly from five-µm thick testis sections used for germ cell proliferation and apoptosis analyses (see below), and measured by using an image analysis software used for oocyte yolk accumulation. The diameter of seminiferous lobules of both wild and captive-reared greater amberjack showed significant changes, with an increase from EARLY to ADVANCED, followed by a decrease during the SPAWNING period. The diameter of seminiferous lobules was significantly larger (ANOVA, $P \leq 0.05$) in wild than in captive-reared greater amberjack during both the EARLY (May-Jun) and SPAWNING (Jun-Jul) periods (**Fig. 3.1.9**).

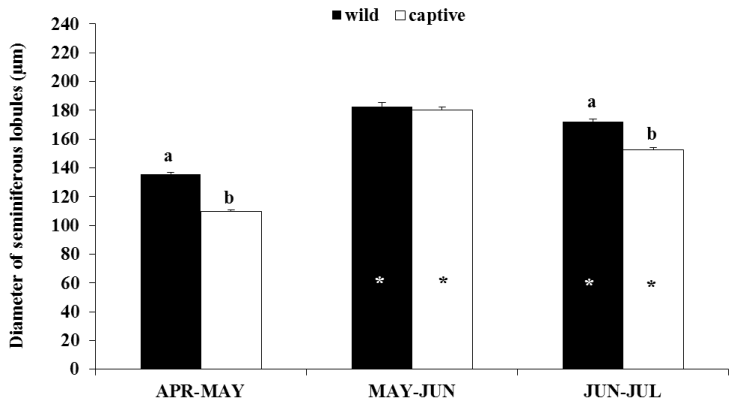


Figure 3.1.9. Mean (±SE) seminiferous lobule diameters of wild and captive-reared greater amberjack sampled in three periods of the reproductive season. Asterisks indicate statistically different diameter versus the preceding period within the same group. Different letters indicate a statistically significant difference between wild and captive individuals within the same sampling period (ANOVA, $P < 0.05$).

The identification of proliferating germ cells was performed on five-µm thick testis sections from all the greater amberjack wild and captive-reared males sampled by using the immunohistochemical localization of PCNA (Proliferating Cell Nuclear Antigen). Quantification of germ cell proliferation was performed by measuring: (a) the density of anti-PCNA positive spermatogonia (number of cells mm^{-2} testis tissue), and (b) the density of anti-PCNA positive spermatocysts (i.e. number of cysts containing spermatogonia or primary spermatocytes mm^{-2} testis tissue), on 5 randomly selected fields of each testis section. Measurements were performed by using the same equipment used for oocyte yolk accumulation. The density of anti-PCNA positive spermatogonia and anti-PCNA positive spermatocysts were compared between wild and captive greater amberjack individuals by using one-way ANOVA ($P \leq 0.05$).

Anti-PCNA immunopositivity was detected in single spermatogonia, as well as in cysts containing spermatogonia and primary spermatocytes (**Fig. 3.1.10**). A weak staining of the nuclei of secondary spermatocytes was also observed; however, these cells were not taken into consideration in the comparative analysis of germ cell proliferation. In captive-reared greater amberjack, anti-PCNA positive spermatogonia were stable from EARLY to ADVANCED and decreased significantly during the SPAWNING period (**Fig. 3.1.11a**). A constant, statistically significant decrease of anti-PCNA positive spermatocysts density was observed in captive-reared specimens throughout the examined periods of the reproductive cycle (**Fig. 3.1.11b**). The density of anti-PCNA positive spermatocysts was higher in wild than in captive-reared specimens both during the EARLY and the SPAWNING periods (**Fig. 3.1.11b**).

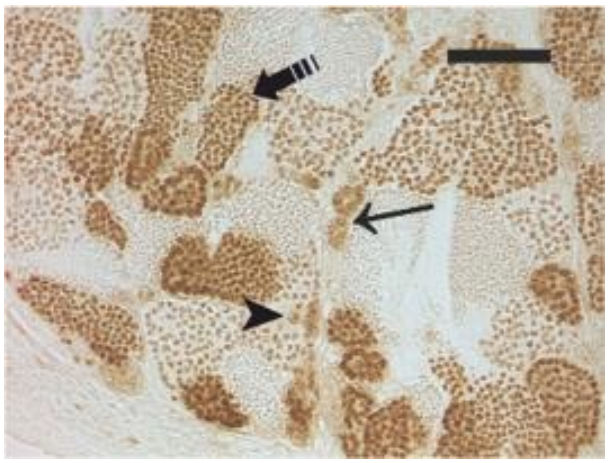


Figure 3.1.10. Micrograph from greater amberjack testis sections labelled with antibodies against PCNA. Nuclei of proliferating cells are stained in brown. Wild greater amberjack sampled on 1 May 2015. Magnification bars = 40 µm. Arrowhead: single spermatogonium; dashed arrow: spermatocyte cysts; single arrow: spermatogonial cysts.

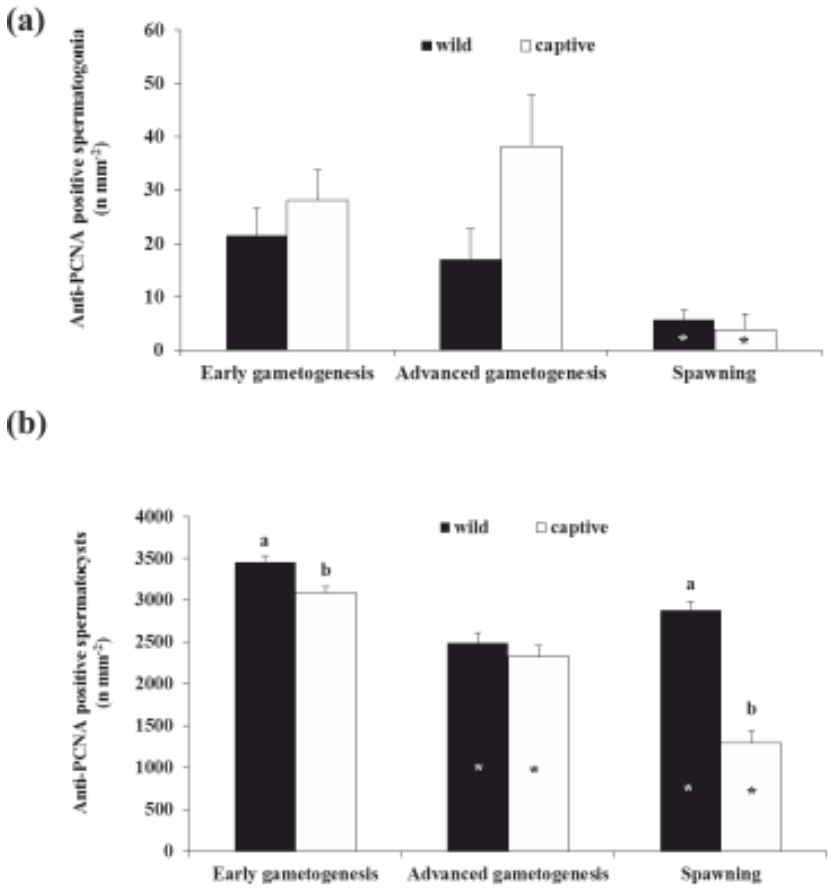


Figure 3.1.11. Changes in mean (±SE) anti-PCNA positive germ cell density during three periods of wild and captive-reared greater amberjack reproductive cycle. (a) Anti-PCNA positive single spermatogonia. (b) Anti-PCNA positive spermatocysts. Asterisks indicate statistically different mean density versus the preceding period within the same group. Different letters represent significant differences between wild and captive individuals within the same sampling period (ANOVA, $P < 0.05$).

Detection of apoptotic germ cells was carried out by using the terminal deoxynucleotidyl transferase-mediated d'UTP nick end labeling (TUNEL) method. Apoptotic cell labelling was obtained with an in situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany) used in accordance with the manufacturer's instructions. Quantification of apoptosis was performed by measuring the surface occupied by TUNEL positive apoptotic cells (μm^2 mm^{-2} testis tissue), measured on 5 randomly selected fields of each testis section. All these measurements were performed by using the same equipment used for oocyte yolk accumulation. Surface occupied by apoptotic germ cells, was compared between wild and captive greater amberjack individuals by using one-way ANOVA ($P \leq 0.05$).

TUNEL-positive germ cells were observed in the germinal epithelium of the majority of the specimens analyzed. Apparently, the TUNEL reaction involved mainly spermatogonia and primary spermatocytes (**Fig. 3.1.12**). In wild greater amberjack, the surface occupied by apoptotic germ cells increased significantly from EARLY to ADVANCED and remained stable thereafter (**Fig. 3.1.13**). In captive-reared individuals, the surface occupied by apoptotic cells was stable during the three sampling periods and comparable to the highest levels of the wild specimens (**Fig. 3.1.13**).

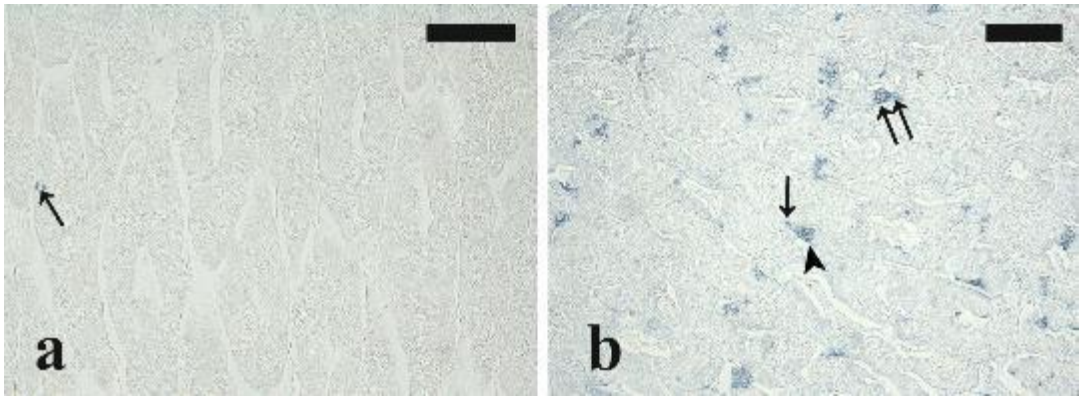


Figure 3.1.12. TUNEL-stained testis sections from wild (a) and captive-reared (b) greater amberjack sampled during the early gametogenesis phase. Apoptotic cells appear as dark blue dots. Magnification bar = 150 µm. Arrowhead: spermatogonial cyst; single arrow: single spermatogonia; double arrows: spermatocyte cysts

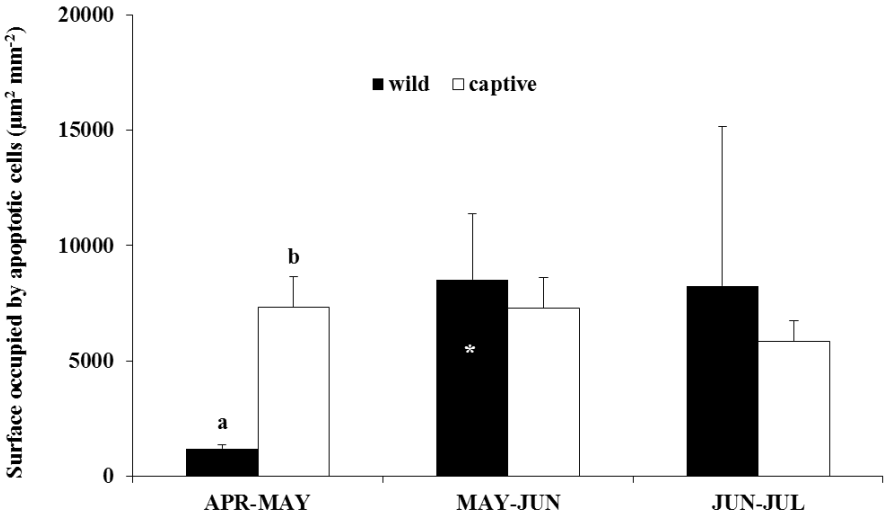


Figure 3.1.13. Changes in mean (±SE) surface occupied by apoptotic germ cells in wild and captive-reared male greater amberjack sampled in three periods (EARLY, ADVANCED and SPAWNING) of the reproductive cycle. Asterisk indicates statistically significant difference versus the previous period within the same group. Different letters indicate significant differences between wild and captive individuals sampled in the same period (ANOVA, $P < 0.05$).

3.1.6 Comparative analysis of gonadotropins, liver vitellogenins, vitellogenin receptor and leptin in wild and captive-reared greater amberjack

Comparative analyses of FSHβ and LHβ, vitellogenin (VgA, VgB and VgC), vitellogenin receptor (VgR) and leptin (Lep) gene expression between wild and captive-reared greater amberjack were performed by qRT-PCR established in the previous year (see Deliverables D3.1 and D3.3). Pituitary and plasma LH levels were measured by IOLR using an ELISA developed for striped bass LH and modified for greater amberjack. Ninety-six well polystyrene plates were coated with recombinant LH (r-LH; 2.4 ng per well) and incubated overnight at 4°C. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl per

well) for 0.5 h at 37°C. The primary antibody (anti-striped bass LH) was diluted 1:80,000 in PBST containing 2% normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4°C. Then the content in each tube was dispensed into the antigen-coated wells (100 µl per well in duplicate). Following an incubation (overnight at 4°C), AffiniPure Goat anti-Rabbit IgG (H+L) in 1% NGS-PBS T was added (100µl per well) for 0.5 h at 37°C. The wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) was added (100 µl per well). The reaction was stopped after 20 to 40 min at RT by the addition of 100µl of 1N phosphoric acid and the absorbance was read at 450 nm.

The FSHβ expression profiles in captive-reared greater amberjack specimens declined steadily with the progression of the reproductive period, yet revealed no significant variations during the equivalent period in the wild fish (**Fig. 3.1.14a**). Conversely, the LHβ expression levels in captive-reared amberjack did not vary significantly throughout the reproductive period, yet were elevated upon spawning in the wild fish (**Fig. 3.1.14b**).

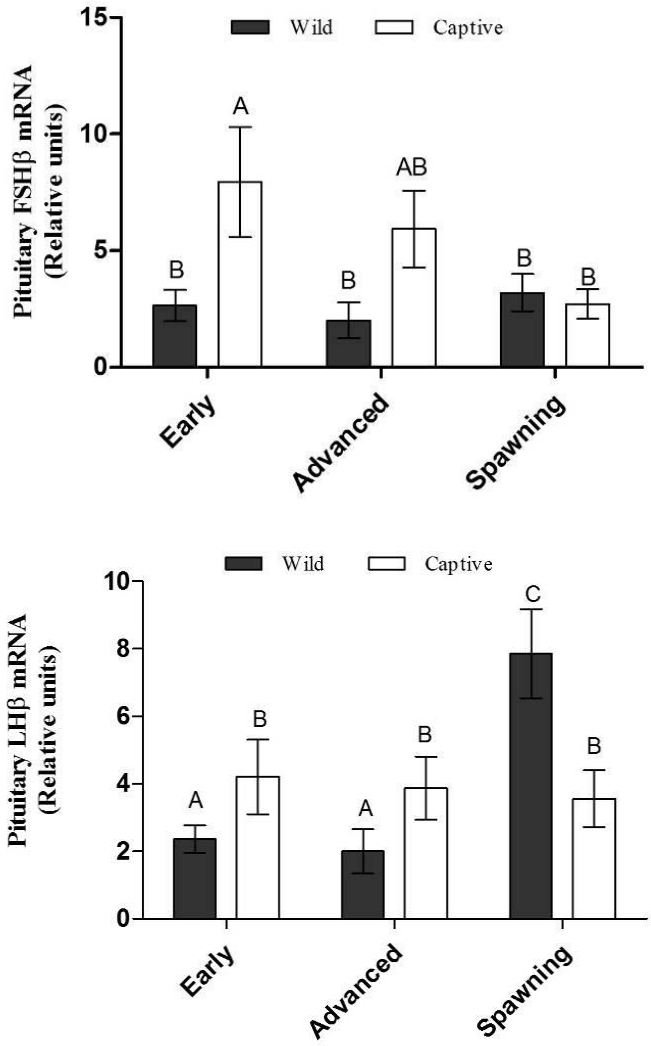


Figure 3.1.14. Relative transcript levels of pituitary FSH (a) and LH (b) β-subunits in wild and captive greater amberjack fish undergoing reproductive cycle. Levels (mean ± SE) are expressed as relative units, normalized to the amount of 18S rRNA. Different letters above bars indicate significant differences between means ($P < 0.05$).

Transcript levels of Vgs in captive-reared females were higher than those of wild fish in early gametogenesis (eg; late April-early May) and lower in advanced gametogenesis (AG; late May-early June) and spawning (SP; late June-July). In particular, VgA, VgB and VgC levels dramatically dropped in captive-reared individuals during SPAWNING (**Fig. 3.1.15**). The low Vgs levels in these specimens were congruent with the cessation of the reproductive state, which was shown by the histological analysis of the ovaries. VgR mRNA relative levels in captive-reared greater amberjack ovaries were higher in EARLY and lower in ADVANCED, compared to the wild counterpart (**Fig. 3.1.16**).

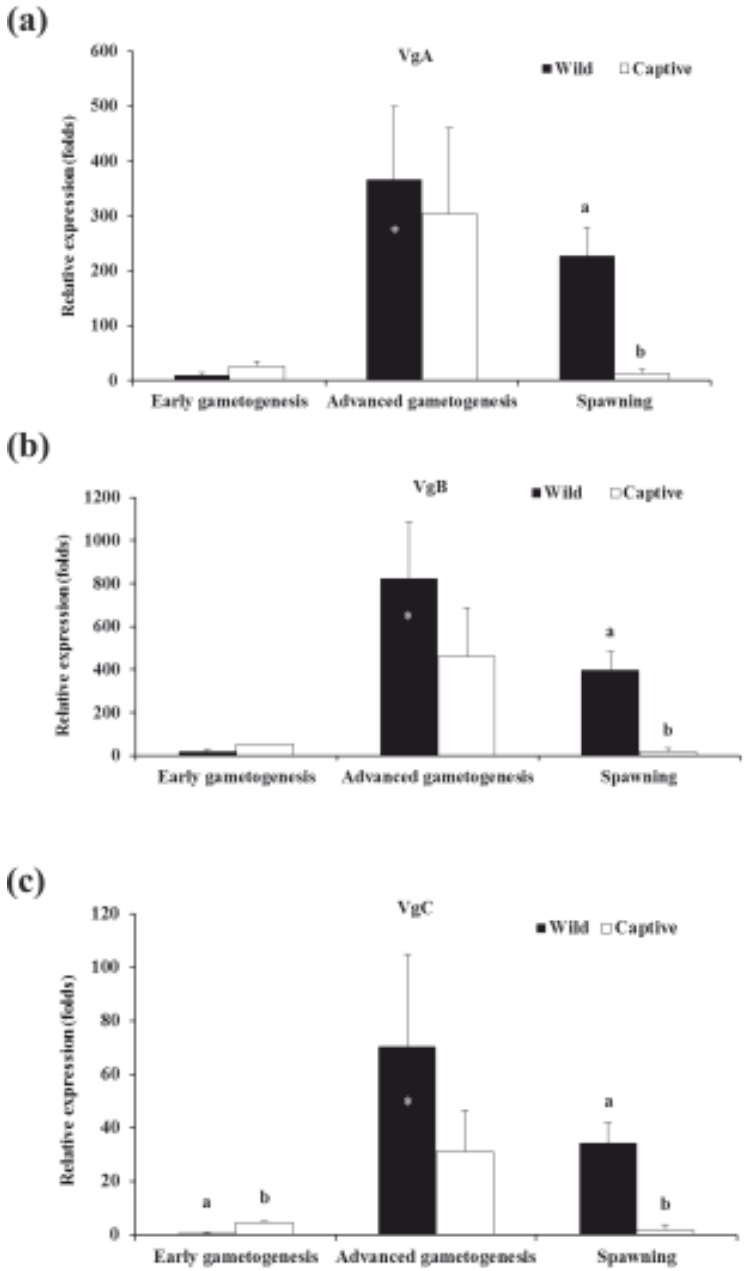


Figure 3.1.15. Mean (±SE) transcription levels of (a) VgA, (b) VgB and (c) VgC during three periods of the reproductive cycle of wild and captive-reared greater amberjack. Asterisks indicate statistically different transcription levels compared with the previous sampling period within the same group. Different letters above bars indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period (ANOVA, $P < 0.05$).

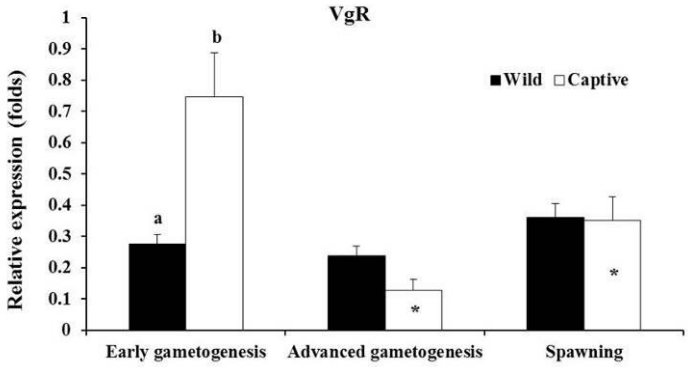


Figure 3.1.16. Mean (±SE) transcription levels of vitellogenin receptor (VgR) during three periods of the reproductive cycle of wild and captive-reared greater amberjack. Asterisks indicate statistically different transcription levels compared with the previous sampling period within the same group. Different letters above bars indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period (ANOVA, $P < 0.05$).

Quantitative real-time PCR (qRT-PCR) analysis of liver leptin mRNA demonstrated that transcript levels in both wild and captive-reared fish were minimal during ADVANCED and maximal at SPAWNING (**Fig. 3.1.17**). Interestingly, during EARLY, liver leptin mRNA levels were significantly higher in captive females than in cognate wild females (**Fig. 3.1.17a**). Nonetheless, the equivalent gene expression levels in captive and wild males did not significantly differ from each other (**Fig. 3.1.17b**).

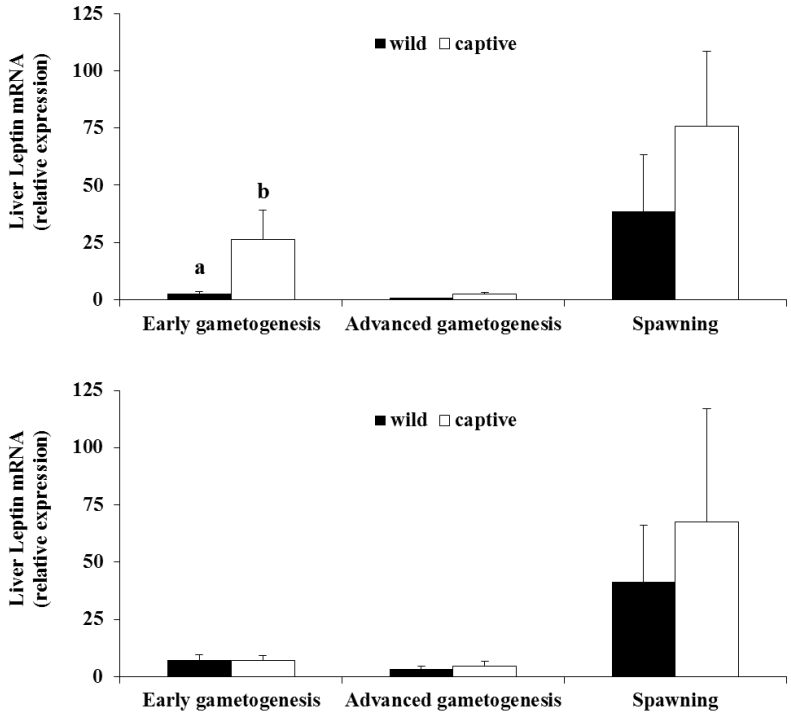


Figure 3.1.17. Mean (±SE) transcription levels of liver leptin during three periods of the reproductive cycle of wild and captive-reared greater amberjack females (upper) and males (lower). Asterisks indicate statistically different transcript levels between wild and captive-reared specimens within the same sampling period. (ANOVA, $P < 0.05$).

In order to test the possibility of using the heterologous striped bass *Morone saxatilis* (stb)LH ELISA for LH measurement in the greater amberjack, displacement curves obtained with serial dilutions of pituitary and plasma extracts from greater amberjack were compared with the r-LH standard curve (**Fig. 3.1.18**). On log–logit transformed data, linear responses were found in the serial dilutions of greater amberjack pituitary extract (**Fig. 3.1.18a**) and plasma (**Fig. 3.1.18b**) corresponding to a r-LH response (1.25–80 ng/ml). The ELISA was established with a sensitivity (IC50) and limit of detection of 19.6 ng/ml and 0.625 ng/ml, respectively.

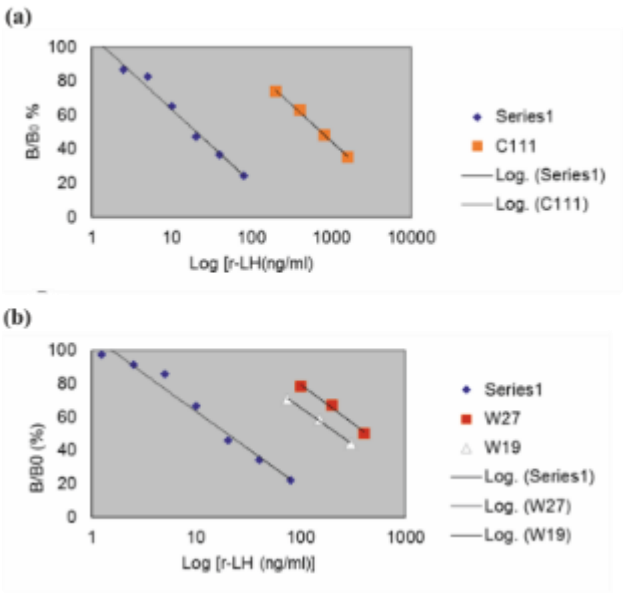
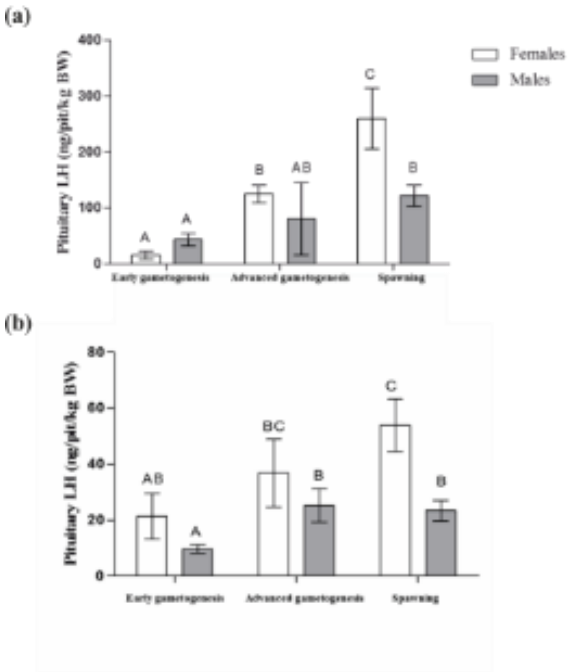


Fig. 3.1.18. Displacement curves for standard r-LH and serial dilution of pituitary extract (a) and plasma (b) samples from wild greater amberjack specimens (W19 and W27). The LOGIT function was utilized to transform standard curve to a linear plot. Each point is a mean of two determinations.



Pituitary LH content in wild (**Fig. 3.1.19a**) and captive-reared (**Fig. 3.1.19b**) greater amberjack broodstock steadily increased with the progression of the reproductive season reaching maximal levels during late June coinciding with the SPAWNING period. At that period of time, the pituitary LH levels, in both wild and captive broodstocks, exhibited sex dimorphic pattern and were 2-fold higher in females (wild: 260.31 ± 54.86 and captive: 53.91 ± 9.35 ng pit^{-1} Kg^{-1} BW, respectively) as compared with cognate males (wild: 122.52 ± 18.69 and captive: 23.42 ± 3.69 ng pit^{-1} Kg^{-1} BW, respectively). Nonetheless, during spawning time, the pituitary LH levels in wild fish (regardless of their sex) were 5-fold higher than those measured in the captive ones.

Figure 3.1.19. Pituitary LH content in wild (a) and captive (a) mature greater amberjack males and females during reproductive cycle (from early May to late June). Levels (mean ± SE) are expressed as total amount (ng) per pituitary per kg body mass. Different letters above bars indicate significant ($P<0.05$) differences between means.

Plasma LH levels did not vary significantly between the sexes, and were therefore, combined to increase the statistical power (**Fig. 3.1.20**). Plasma LH levels, in both wild and captive-reared fish, were maximal (wild: 76.56 ± 26.83; captive: 26.38 ± 8.36 ng ml^{-1}) and minimal (wild: 24.41 ± 5.56; captive: 9.6 ± 2.25 ng ml^{-1}) during EARLY and ADVANCED, respectively. Yet, the detected levels were approximately 1.5-fold higher in the wild fish as compared with the captive ones.

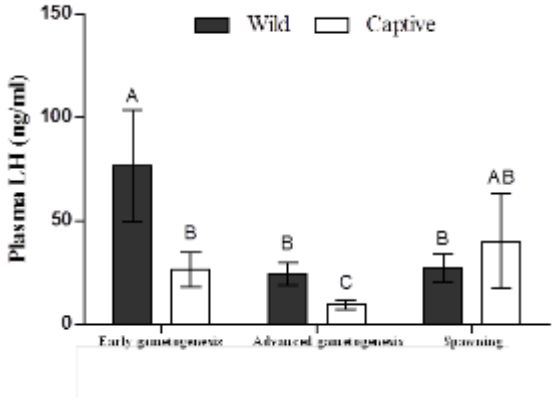


Figure 3.1.20. Plasma LH levels in wild and captive mature greater amberjack specimens during the reproductive cycle. Levels (mean ± SE) are expressed as total amount (ng) per ml plasma. Different letters above bars indicate significant (P<0.05) differences between means.

3.1.7 Nutritional state assessment

Gonads, muscle, and liver proximate and fatty acid composition; gonads carotenoid contents

To evaluate broodstock nutritional status, pieces of gonad, muscle and liver from captive and wild greater amberjack, were immediately frozen and kept at -80ºC, until analyses that were performed in ULL laboratories. Dry matter, and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC, 2012). Total lipid (TL) was extracted according to the method of Folch et al. (1957). Analysis of lipid classes (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and quantified by scanning densitometry using a dual-wavelength flying spot scanner Shimadzu CS-9001PC (Shimadzu, Duisburg, Germany) (Olsen & Henderson, 1989). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation and purified by TLC 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 carrier gas and temperature programming was 50–50°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). Carotenoids were obtained only from gonads at advanced gametogenesis and spawning periods, according to the method of Barua et al. (1993). Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

Tissues biochemical analyses are reported as means ± standard deviation (SD). All values presented as percentage were arcsine transformed. Normal distribution was checked for all data with the one-sample Kolmogorov–Smirnoff test and homogeneity of the variances with the Levene test. Differences between pairs of means were tested using Student's t-test. To compare more than two means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal-Wallis test was accomplished. The significant level for all the analysis was set at P<0.05. All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com).

The rearing conditions were not associated to any significant change in the general proximate composition of ovaries, with the exception of moisture content of captive fish, which was slightly higher than that of wild gonads during spawning period (SPAWNING; late June-July) (**Fig. 3.1.21a**). The evolution pattern of ovaries proximate composition during the reproductive cycle was quite similar between wild and captive counterparts, with a clear decrement of moisture and proteins, associated to a significant increment of lipids at advanced gametogenesis (ADVANCED; late May-early June) compared to early gametogenesis (EARLY; late April-early May) and and SPAWNING (**Fig. 3.1.21a,b,c**).

The rearing conditions did not seem to be associated to any change in the general proximate composition of testes, with the exception of moisture content of captive fish that was slightly lower than that of wild fish during ADVANCED and SPAWNING (**Fig. 3.1.22a**). The evolution pattern of testes proximate composition of wild and captive individuals during the reproductive cycle was quite similar, with no significant differences in terms of lipid contents and only a slightly significant increment of proteins observed in wild fish sampled during ADVANCED compared to EARLY (**Fig. 3.1.22b,c**). Proximate composition was more variable in ovaries than in testes, with lipid contents ranging from 6-8% to 20%.

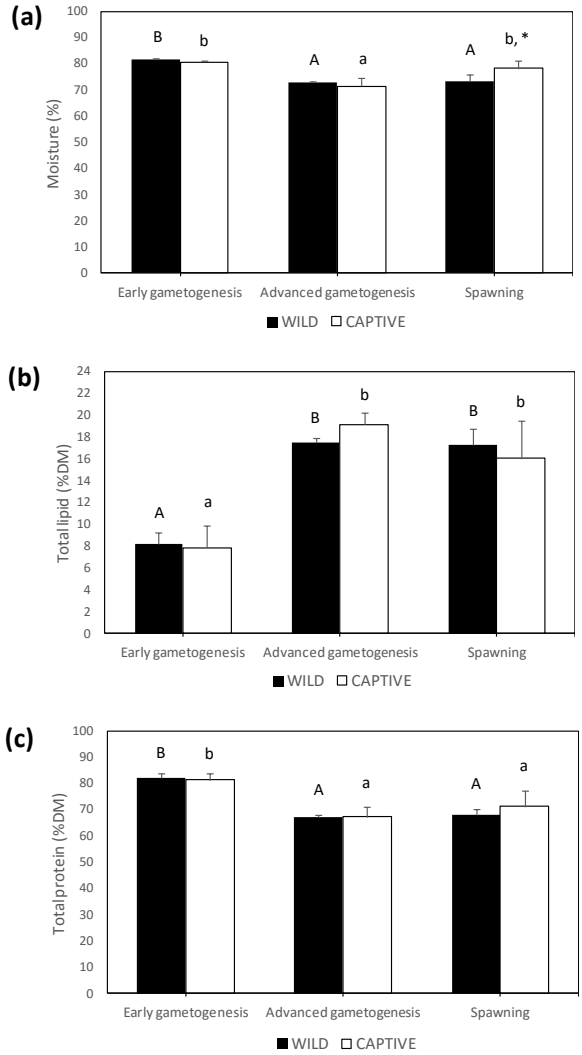


Figure 3.1.21. Mean (±SD) levels of ovaries (a) Moisture, (b) Total Lipids and (c) Total Proteins of wild and captive-reared greater amberjack broodstock at three periods of the reproductive cycle. Asterisks indicate statistical differences between wild and captive-reared specimens within a specific sampling period (Student`s t-Test, $P < 0.05$). Different uppercase and lowercase letters indicate statistical differences among the three periods for wild or captive fish, respectively (ANOVA, $P < 0.05$).

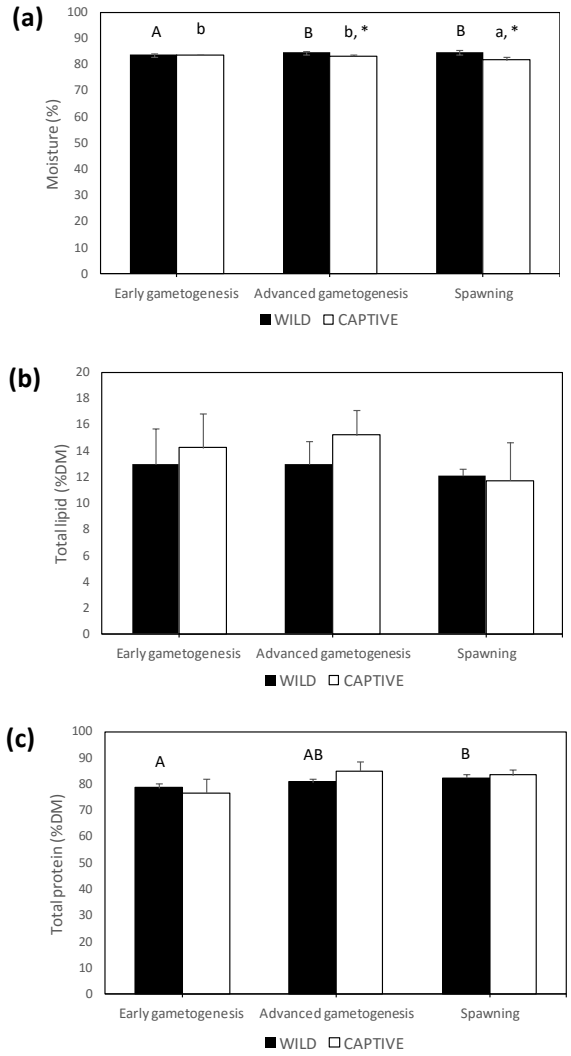
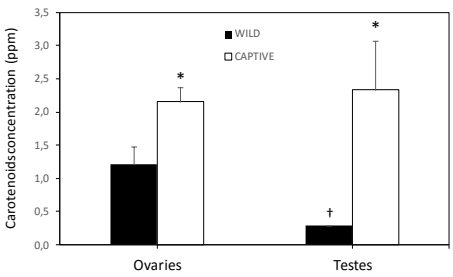


Figure 3.1.22. Mean (±SD) levels of testes (a) Moisture, (b) Total Lipids and (c) Total Proteins of wild and captive-reared greater amberjack broodstock at three periods of the reproductive cycle. Asterisks indicate statistical differences between wild and captive-reared specimens within a specific sampling period (Student`s t-Test, $P < 0.05$). Different uppercase and lowercase letters indicate statistical differences among the three periods for wild or captive fish, respectively (ANOVA, $P < 0.05$).

Carotenoid contents in ovaries and testes sampled at advanced gametogenesis period



The dietary regime of captive-reared fish, which consisted of a commercial extruded broodstock diet (Vitalis-Cal, Skretting SA, Norway) provided a significantly higher mean level of carotenoids than in wild specimens (**Fig. 3.1.23**). Surprisingly, carotenoid content in testes from captive-reared fish was the reverse of the wild fish where ovaries were significantly richer than testes in these antioxidant pigments.

Figure 3.1.23. Mean (±SD) levels of carotenoids in ovaries and testes of wild and captive-reared greater amberjack sampled during the spawning period. Asterisks indicate statistically different levels between wild and captive-reared specimens; (†) denotes differences between sexes (Student`s t-Test, $P < 0.05$).

Gonad lipid classes and fatty acid composition

The main lipid classes and fatty acid compositions varied amongst the gonads from wild and captive adult greater amberjack males and females and across the three different periods of the reproductive cycle (**Table 3.1.3 and 3.1.4**). The rearing conditions failed to provide the nutritional requirements to the testes of captive-reared fish to resemble wild testes content of total polar lipids and specific lipid class proportions. This was particularly evident for males sampled at EARLY as the testes displayed more than three times the amount of triacylglycerols (TG) present in the wild counterparts, with the consequent decrement in proportions of PE and PS. Total Polar Lipids and PE were especially high in fish testes and tended to be preserved at ADVANCED and SPAWNING in wild and captive fish. A marked decrease in PI was evident from EARLY to ADVANCED in wild testes and ovaries. On the contrary, PI levels exhibited a significant increase from EARLY to SPAWNING in captive-reared testes.

Table 3.1.3. Mean values of main lipid classes of gonads from wild and captive adult greater amberjack males and females sampled at three different periods of the reproductive cycle.

	Early gametogenesis				Advanced gametogenesis				Spawning period			
	Male		Female		Male		Female		Male		Female	
	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive
PC	27.6±2.9	20.5±1.8 *	21.6±1.5 b	18.4±2.2	25.8±1.1	22.9±3.0	18.2±1.0 a	17.7±1.8	27.8±0.8	23.1±0.9 *	17.2±1.1 a	17.3±0.6
PS	7.8±1.2 a	4.6±0.9 *,a	4.5±1.2 b	4.5±1.7 b	12.3±0.9 b	8.8±1.3 *,b	1.8±0.3 a	1.5±0.3 a	9.7±1.7 a	8.8±0.8 b	1.4±0.4 a	3.0±0.9 *,b
PI	5.9±0.4 b	5.0±1.1 a	4.9±0.8 b	3.8±0.7	1.7±0.6 a	6.8±0.7 *,b	2.4±0.4 a	2.9±0.4	5.6±3.1 b	7.1±0.3 b	2.4±0.3 a	3.4±0.7 *
PE	20.0±0.9 a	14.1±2.0 *,a	12.2±1.0 c	11.8±2.3 c	22.1±0.5 b	21.2±2.6 b	7.0±0.1 b	5.9±0.5 *,a	21.4±0.7 a	21.9±1.4 b	6.1±0.8 a	7.9±1.5 b
TPL	64.6±4.3 ab	47.4±5.3 *,a	47.8±2.8 b	43.8±7.1 c	63.5±0.5 a	63.8±7.4 b	32.3±1.2 a	30.8±0.6 *,a	67.6±3.4 b	65.7±1.6 b	28.6±3.1 a	35.6±3.8 *,b
Chol	18.7±1.0 a	16.5±2.3 a	16.2±1.3 b	15.1±2.1 ab	22.5±1.4 b	22.5±2.3 b	13.0±0.9 a	14.6±0.9 *,a	18.4±8.5 a	22.9±1.4 b	12.8±0.7 a	19.2±4.2 *,b
TG	8.7±1.9 c	28.9±8.6 *,b	21.0±1.5 a	26.3±8.6 b	3.0±0.9 b	2.8±0.6 a	24.7±0.5 b	26.4±1.8 b	1.8±0.7 a	4.4±1.6 *,a	22.2±1.8 a	21.3±2.6 b
TNL	35.4±4.3 ab	52.6±5.3 *,b	52.2±2.8 a	56.2±7.1 a	36.5±0.5 b	36.2±7.4 a	67.7±1.2 b	69.2±0.6 *,b	32.4±3.4 a	34.3±1.6 a	71.4±3.1 b	64.4±3.8 *,a

Asterisk indicates significant differences for a particular lipid class and period between wild and captive-reared broodstock (t-Student, $P < 0.05$). Letters within a row denote significant differences along the reproductive cycle for each sex ($P < 0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; Chol, Cholesterol; TG, triacylglicerides; TNL, total neutral lipids.

Table 3.1.4. Mean values of main fatty acids of gonads from wild and captive adult greater amberjack males and females sampled at three different periods of the reproductive cycle.

	Early gametogenesis				Advanced gametogenesis				Spawning period			
	Male		Female		Male		Female		Male		Female	
	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive	Wild	Captive
16:0	22.9±1.0	19.6±1.0 *,a	20.3±0.9 b	17.9±0.5 *,b	23.1±0.4	22.1±0.3 b	16.3±1.8 a	15.2±0.9 a	22.6±1.3	22.2±2.3 b	17.4±0.7 a	17.5±3.4 b
18:1[1]	19.6±2.9	23.7±3.1 b	17.9±2.1 a	22.1±3.0 a	16.4±2.3	18.3±2.3 a	24.8±1.6 c	26.7±1.3 b	17.2±0.6	18.8±1.9 a	22.1±1.1 b	23.4±4.2 ab
18:2n-6	1.1±0.2 b	7.1±0.9 *,b	1.0±0.2 a	6.5±0.6 *,a	0.8±0.1 a	5.0±1.2 *,a	1.0±0.3 a	10.1±0.5 *	1.0±0.1 b	5.8±2.0 *,a	1.8±0.2 b	8.1±2.1 *,a
20:4n-6	4.1±0.8	2.3±0.4 *,a	5.9±1.0 b	3.4±1.2 *,b	4.3±0.4	2.7±0.5 *,a	3.7±0.5 a	2.1±0.4 *,a	5.4±1.0	5.0±1.4 *,b	4.9±0.6 b	4.0±0.6 b
20:5n-3	3.7±0.4 b	4.6±0.8	3.9±0.4 ab	5.3±0.5 *	2.9±0.4 a	4.8±1.0 *	3.3±0.6 a	5.1±1.3 *	2.8±0.5 ab	3.8±0.8	4.3±0.5 b	4.3±0.6
22:6n-3	26.2±3.6 a	18.1±3.0 *,a	27.3±1.8 b	19.4±3.9 *	32.9±1.9 b	26.9±3.5 *,b	31.2±3.5 b	21.1±1.6 *	26.9±2.2 a	24.6±3.6 b	22.9±1.9 a	23.3±2.9
DHA/EPA	7.1±0.6 a	3.9±0.3 *,a	7.1±1.2 b	3.6±0.5 *,a	11.3±1.0 b	5.7±0.7 *,b	9.4±0.9 c	4.4±1.3 *,ab	10.0±1.9 b	6.7±0.7 *,b	5.4±0.8 a	5.8±0.6 b
ARA/EPA	1.1±0.2 a	0.5±0.0 *,a	1.6±0.4	0.6±0.2 *,a	1.5±0.3 ab	0.6±0.1 *,b	1.1±0.2	0.4±0.1 *,a	2.0±0.7 b	1.5±0.8 c	1.2±0.2	1.0±0.2 b

Asterisk indicates significant differences in fatty acids between wild and captive-reared broodstock (t-Student, $P<0.05$). Letters within a row denote significant differences along the reproductive cycle for each sex ($P<0.05$). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Differences in terms of fatty acid composition, from wild and captive gonads were particularly evident at EARLY and ADVANCED periods, with testes and ovaries of captive fish displaying around 30-40% less docosahexaenoic acid (DHA) and arachidonic acid (ARA), and clearly higher contents of linoleic acid 18:2n-6. As a consequence, DHA/eicosapentaeonic acid (EPA) and ARA/EPA ratios, also suffer marked decrements in the gonads of the captive fish. Among the three sampled periods, the most evident differences were a significant increase in DHA from EARLY to ADVANCED displayed by the testes of wild and captive-reared specimens. However, at SPAWNING the DHA content did not return to values observed at EARLY in the captive males, as it did in the wild fish. Similarly, the wild ovaries displayed an increase in DHA between EARLY and ADVANCED, returning at SPAWNING to EARLY levels. This DHA increment between EARLY and ADVANCED was not observed in the captive-reared females.

Liver and muscle proximate and fatty acid composition

Lipid content in liver and muscle of greater amberjack is shown in **Tables 3.1.5** and **3.1.6**, respectively. Lipid content was higher for the captive fish in both tissues, and as a consequence, moisture was generally lower. Whereas in the liver of the wild fish there was a progressive decrement of lipids from ADVANCED to SPAWNING, in the muscle the most evident change was a marked depletion of lipids at SPAWNING. None of these changes were evident for the captive fish.

Table 3.1.5. Lipid content in liver of wild and captive-reared greater amberjack sampled during the reproductive season (EARLY, EG; ADVANCED, AG; and SPAWNING, SP) in the Mediterranean Sea.

	EG		AG		SP	
	Female		Female		Female	
	Wild	Captive	Wild	Captive	Wild	Captive
Moisture (%)	69.5±4.8	62.2±2.9 *	73.6±0.6	65.9±4.3 *	74.1±0.8	66.2±3.8 *
TL (%DM)	25.4±6.2 b	35.2±3.0 *	20.7±0.2 ab	30.0±6.4 *	15.2±1.8 a	29.0±7.4 *

	EG		AG		SP	
	Male		Male		Male	
	Wild	Captive	Wild	Captive	Wild	Captive
Moisture (%)	63.6±5.3 a	56.1±9.9	73.0±1.0 b	58.6±4.5 *	73.6±1.5 b	66.2±6.9
TL (%DM)	34.4±9.9 b	45.8±12.3	19.2±6.0 a	37.1±8.7 *	18.9±2.6 a	25.9±13.0

Table 3.1.6. Lipid content in muscle of wild and captive-reared greater amberjack sampled during the reproductive season (EARLY, EG; ADVANCED, AG; and SPAWNING, SP) in the Mediterranean Sea.

	EG		AG		SP	
	Female		Female		Female	
	Wild	Captive	Wild	Captive	Wild	Captive
Moisture (%)	72.6±2.4	72.7±2.7	71.8±0.6	69.5±0.9	75.7±2.4	68.7±2.0
TL (%DM)	10.8±4.7 ab	11.4±5.0	18.8±9.5 b	18.4±5.2	5.0±2.4 a	18.9±5.1 *

	EG		AG		SP	
	Male		Male		Male	
	Wild	Captive	Wild	Captive	Wild	Captive
Moisture (%)	71.3±2.0 a	65.9±4.2	72.9±1.5 ab	70.6±2.0	76.0±1.4 b	71.2±2.9 *
TL (%DM)	13.2±2.9 b	28.2±3.6 b,*	15.4±4.5 b	17.6±6.1 a	4.7±0.8 a	30.4±4.4 b,*

Differences in terms of fatty acid composition, from liver and muscle of greater amberjack are shown in **Tables. 3.1.7** and **3.1.8**, respectively. As for gonads, compared to the wild counterparts, captive fish tissues are characterized by higher contents of 18:1n-9 and 18:2n-6, relatively lower contents of 16:0 and 20:5n-3 (EPA) and much lower contents of 20:4n-6 (ARA) and 22:6n-3 (DHA), which also affect the DHA/EPA and ARAEPA ratios. All these differences were more evident in the liver than in the muscle. As a consequence, the evolution of tissue fatty acid composition along the reproductive season clearly differs among the wild and captive fish. In this sense, the decrements of 18:1n-9 observed in liver and muscle of wild fish from EARLY to SPAWNING are not displayed by the captive fish. Similarly, the increments of ARA and DHA achieved by the wild fish also from EARLY to SPAWNING are much less evident in the captive counterparts.

Table 3.1.7. Fatty acid composition in liver of wild and captive-reared greater amberjack sampled during the reproductive season in the Mediterranean Sea.

	EG		AG		SP	
	Female		Female		Female	
	Wild	Captive	Wild	Captive	Wild	Captive
16:0	21.6±2.0	16.3±0.6 *	19.7±1.8	18.9±2.2	21.5±2.3	19.3±0.8
18:1[1]	23.3±3.4 a	38.7±2.5 *	23.9±5.0 a	34.4±3.1 *	13.1±1.9 b	34.5±4.5 *
18:2n-6	1.5±0.3	10.3±1.8 *	1.9±0.2	9.8±1.0 *	1.1±0.1	10.0±1.5 *
20:4n-6	4.6±1.4 a	0.8±0.1 *	3.8±1.2 a	1.4±0.5 *	7.0±1.3 b	1.6±0.3 *
20:5n-3	4.4±1.2	3.4±0.6	6.3±0.8	3.5±0.7 *	4.4±1.0	3.8±1.6
22:6n-3	21.6±3.2 a	5.4±0.4 a,*	19.3±0.8 a	9.2±3.0 b,*	26.4±2.7 b	9.0±2.5 b,*
DHA/EPA	5.2±1.3 ab	1.6±0.2 *	3.1±0.5 a	2.8±1.3 *	6.3±1.8 b	2.5±0.8 *
ARA/EPA	1.1±0.4 ab	0.2±0.0 *	0.6±0.3 a	0.4±0.2 *	1.7±0.5 b	0.4±0.1 *

	EG		AG		SP	
	Male		Male		Male	
	Wild	Captive	Wild	Captive	Wild	Captive
16:0	19.5±2.4	15.3±0.9 a,*	22.8±2.1	16.6±0.8 ab,*	20.4±1.5	18.3±1.4 b
18:1[1]	29.3±2.6 b	33.9±2.1	21.5±6.1 a	31.9±0.7 *	16.4±2.9 a	28.2±4.0 *
18:2n-6	2.2±0.4	12.4±0.4 *	1.9±0.7	11.9±1.9 *	1.8±0.4	10.4±1.3 *
20:4n-6	3.0±0.4 a	0.9±0.2 a,*	4.5±1.0 ab	1.1±0.1 ab,*	5.7±1.1 b	2.0±1.1 b,*
20:5n-3	5.9±0.6 b	4.5±0.6 *	3.9±0.5 a	4.4±0.9	4.9±1.1 ab	4.0±1.1
22:6n-3	16.4±2.4 a	6.8±0.5 a,*	23.1±6.5 ab	7.6±0.3 ab,*	23.4±3.9 b	12.5±5.8 b,*
DHA/EPA	2.8±0.5 a	1.5±0.1 a	5.9±1.3 b	1.8±0.3 ab,*	5.0±1.6 b	3.4±1.9 b,*
ARA/EPA	0.5±0.1 a	0.2±0.0 *	1.2±0.2 b	0.3±0.0 *	1.3±0.6 b	0.5±0.4 *

Asterisk indicates significant differences in fatty acids between wild and captive-reared broodstock. Letters within a row denote significant differences along the reproductive cycle for each sex ($P<0.05$). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Table 3.1.8. Fatty acid composition in muscle of wild and captive-reared greater amberjack sampled during the reproductive season in the Mediterranean Sea.

	EG		AG		SP	
	Female		Female		Female	
	Wild	Captive	Wild	Captive	Wild	Captive
16:0	21.7±1.0	17.2±1.0 *	21.4±1.2	17.3±0.7 *	21.6±1.2	16.8±0.7 *
18:1[1]	28.3±2.3 b	29.5±3.5	29.9±1.1 b	30.6±1.5	15.1±5.3 a	32.1±2.0 *
18:2n-6	1.3±0.5	9.3±1.9 *	1.6±0.2	9.5±0.6 *	1.6±0.4	9.6±1.1 *
20:4n-6	2.1±0.5 a	0.9±0.2 *	2.0±0.1 a	0.8±0.0 *	3.8±0.8 b	0.8±0.2 *
20:5n-3	4.0±0.3	3.8±0.5	3.6±0.4	3.5±0.4	3.6±0.2	3.3±0.5
22:6n-3	17.8±3.2 a	14.0±3.5	15.4±0.6 a	12.1±1.4	26.8±7.5 b	11.0±3.5 *
DHA/EPA	4.5±1.0 a	3.7±0.5	4.3±0.6 a	3.5±0.5	7.5±2.3 b	3.3±0.7 *
ARA/EPA	0.5±0.1 a	0.2±0.0 *	0.6±0.0 a	0.2±0.0 *	1.1±0.3 b	0.2±0.0 *

	EG		AG		SP	
	Male		Male		Male	
	Wild	Captive	Wild	Captive	Wild	Captive
16:0	21.9±0.3	16.5±0.2 *	22.0±2.1	17.5±0.5 *	20.5±0.5	16.5±0.4 *
18:1[1]	31.0±1.1 b	31.0±1.2	25.8±8.9 b	30.2±1.3	17.6±5.3 a	32.3±0.8 *
18:2n-6	1.5±0.1	10.1±0.8 *	1.6±0.4	9.3±0.9 *	2.1±0.7	10.1±0.7 *
20:4n-6	1.5±0.1 a	0.7±0.0 *	2.1±0.6 b	0.8±0.1 *	3.6±0.4 c	0.7±0.1 *
20:5n-3	4.1±0.2 b	3.9±0.2	3.3±0.5 a	3.5±0.2	2.8±0.2 a	3.4±0.7
22:6n-3	17.8±1.6	11.8±1.1 *	19.7±9.9	12.3±3.7	24.8±8.9	10.1±1.0 *
DHA/EPA	4.4±0.4 a	3.0±0.2 *	5.8±2.2 ab	3.5±1.0 *	7.6±3.1 b	3.0±0.6 *
ARA/EPA	0.4±0.0 a	0.2±0.0 *	0.6±0.1 b	0.2±0.0 *	1.1±0.2 c	0.2±0.1 *

Asterisk indicates significant differences in fatty acids between wild and captive-reared broodstock. Letters within a row denote significant differences along the reproductive cycle for each sex ($P<0.05$). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

3.1.8 Evaluation of captive-reared greater amberjack sperm quality

Sperm quality was assessed through physical and biological features. In order to make a relation between physiological status of the gonad and gamete quality, sperm was collected from the same captive fish as those sampled in ARGO for gametogenesis evaluation. Sampling for sperm assessment was performed by HCMR and IFREMER personnel who attended every sampling session (April 20-25th, June 3-8th and July2-4th) after a short training and intercalibration work held at the HCMR facilities in Crete, using the broodstock maintained there. After vain attempts to collect sperm by stripping the fish, samples of gametes were collected directly from the dissected gonads during the fish slaughtering. Samples were stored dry or after dilution in modified Leibovitz until they were processed for the different following purposes: concentration, motility, ATP content and viability assessments. The resulting dataset was complete and allowed studying

the variations of the different quality indexes between the 3 sampling times by ANOVA [after angular transformation in the case of percentage analysis (motility)]. The CASA processing and statistical analysis were co-performed by HCMR and IFREMER. Due to variation of recording quality on sampling site, the settings of CASA were adjusted to each sampling time, but only for image treatment. The settings associated to motility evaluation were common for all the analyses.

Sperm concentration

The concentration of sperm ranged from 2.3 to 4.6 10^{10} spz ml^{-1}, the usual range for sperm of marine fish. A significant difference between April (EARLY gametogenesis period) and July (SPAWNING period) was observed (**Tabla 3.1.9**).

Table 3.1.9. Crossed probabilities showing a significant difference in sperm concentration between April and July in captive-reared fish (n=4).

	Apr	**June**	**July**
Apr		0,193934	0,017906
June	0,193934		0,311706
July	0,017906	0,311706	

Sperm motility

The motion characteristics of sperm changed along the reproductive period. The analysis of the main motility features by CASA also showed that greater amberjack sperm behavior was comparable to all studied marine fish (**Fig. 3.1.26**). Within the first 20 seconds after activation by seawater, the maximal percentage of spermatozoa was reached whatever the rank in the season, but significant differences between sampling times were observed. The duration of motility, calculated by the time when any movement ceased, was between 1 and 11 minutes and also this parameter showed season-related significant differences (**Fig. 3.1.24**). Consequently, the swimming speed of spermatozoa showed significant variations (**Fig. 3.1.25**).

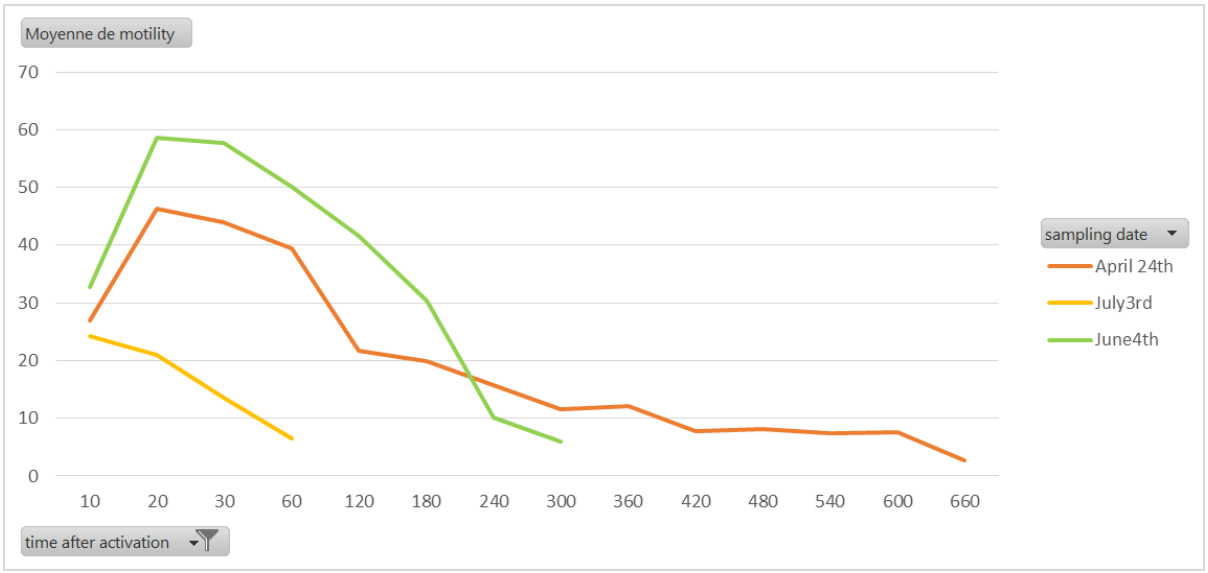


Figure 3.1.24. Motility of greater amberjack sperm at different periods of the reproductive season. A usual pattern of spermatozoa behavior was shown: general activation leading to maximum motility, followed by a decrease of the percentage of mobile sperm.

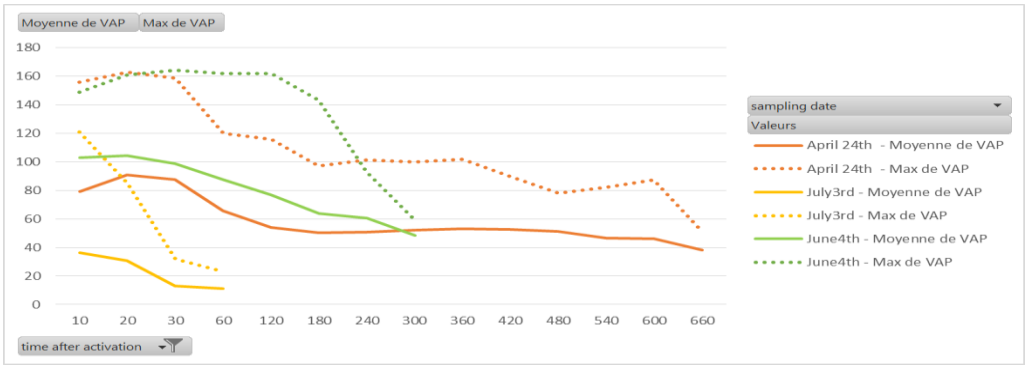


Figure 3.1.25. Mean (solid line) and maximum (dotted line) speed of spermatozoa (µm s^{-1}) at different sampling times during the reproductive period.

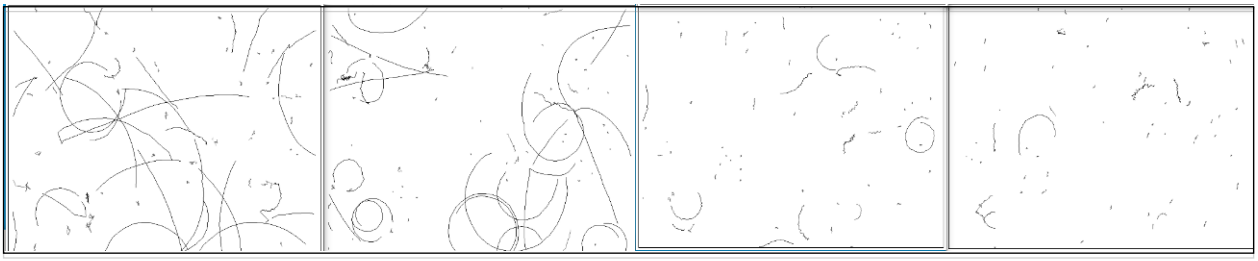


Figure 3.1.26. Illustration of greater amberjack spermatozoa tracks generated by CASA, showing the decrease of motility and speed with time after activation.

Sperm ATP content

The ATP content of spermatozoa was analyzed at the IFREMER laboratory in Brest. The ATP level in sperm at the different points of the season remained very low and very variable in the whole batch, and at the limit of detection, and therefore it was not possible to distinguish significant differences between samplings (**Fig. 3.1.27**). This result may be justified by the quite low performance of sperm regardless of the period during the reproductive season. However, a problem in sample conditioning or transportation cannot be excluded.

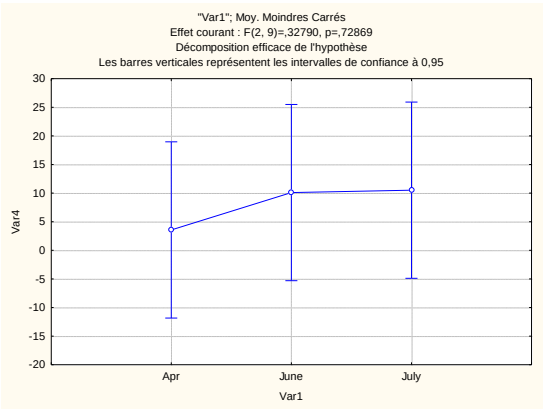


Figure 3.1.27. Mean ATP level (nmol 10^{-9}spz) with error bars showing 95% confidence interval (n=4) of sperm collected during three different phases of the greater amberjack reproductive season.

Sperm viability

The use of a dedicated molecular probe kit (live/dead sperm kit) showed that there was also a variation with time of the integrity of spermatozoa membranes. The use of fluorescent markers of DNA with differential penetration of the cell in relation to its live/dead status showed the presence of three different categories: live (green fluorescence), dying (green and orange) and dead (orange) (**Fig. 3.1.28**). A Nested Design ANOVA showed significant differences between randomly chosen males at each sampling time. Notwithstanding this individual variability, a significant increase of dead and live spermatozoa was observed from May to July, while dying spermatozoa did not show significant difference (P<0.05) (**Fig. 3.1.29**).

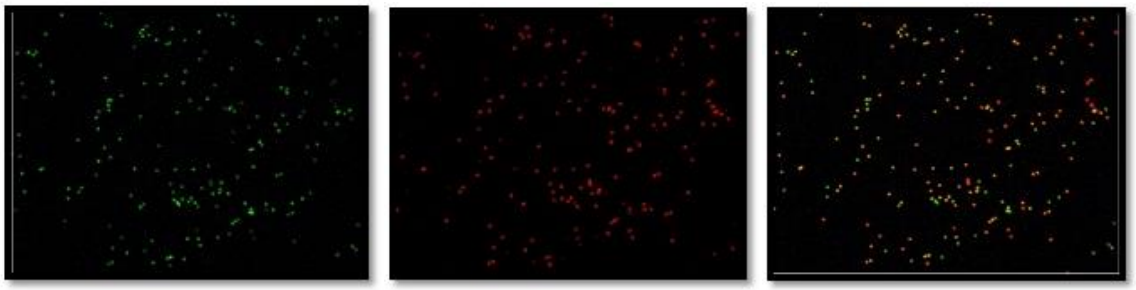


Figure 3.1.28. Estimation of greater amberjack sperm viability by differential fluorescence of Sybr 14 (green) and propidium iodide (red). The superposition of the 2 images allows distinguishing live (green), dead (red) and dying (both colors) spermatozoa.

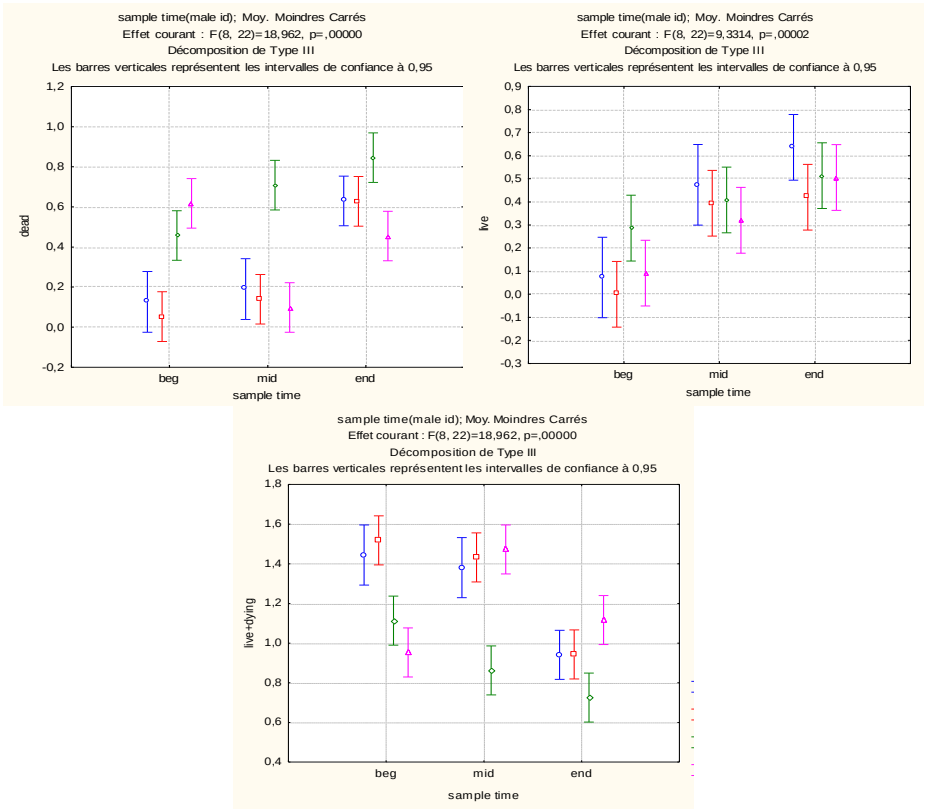


Figure 3.1.29. Mean ratios of live, dead and dying spermatozoa from each of the 4 males at each sampling period. The intra-male variability corresponds to the replicates of live/dead assessment.

This collaborative work was completed in terms of sampling and processing. The rough data and primary statistical analysis presented above have to be refined, explained and introduced in a larger perspective of fish gamete quality, which will be done in the appropriate deliverable.

3.1.9. Conclusions

To sum up, during Y2 important information was obtained on the effects of confinement on greater amberjack reproductive and nutritional state:

- Gonado-somatic index of captive-reared greater amberjack was significantly lower for both sexes in all the three considered periods compared to wild fish, thus indicating that the process of gonad development was somehow impaired in captivity.
- An extensive atresia affected late vitellogenic follicles of captive females, thus preventing any further oocyte development; all captive-reared males were spent during the spawning season of the wild population.
- A progressive decrease of germ cell proliferation occurred in captive-reared individuals together with a higher density of apoptotic germ cells during the EARLY compared to wild fish, indicating that spermatogenesis in captive-reared fish was already compromised at the beginning of the reproductive season.
- Captive-reared fish showed impaired pituitary expression of the gonadotropins (FSHβ and LHβ), and consequently lower concentration of LH in their pituitaries and plasma, compared to wild population.
- Captive-reared fish showed lower concentration of main sex steroids in their plasma, compared to wild population.
- Liver expression of Vgs were slightly lower in captive-reared compared to wild fish.
- A general decrease of crucial factors for reproductive success, such as specific lipid classes and fatty acids, were detected in gonads, liver and muscles of captive-reared greater amberjack.

All these data clearly indicate the occurrence of severe gametogenesis impairments in greater amberjack reared in captivity and these dysfunctions are possibly related to captivity-induced stress and husbandry manipulation, to the lack of natural conditions required for reproductive maturation and/or to nutritional deficiency.

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

According to the DOW, during Y2 of the project two different methods of spawning induction had to be examined, either multiple GnRHa injections given every 7 days or implants of sustained release of GnRHa. However, the HCMR broodstocks maintained in tanks did not achieve the appropriate maturation stage to start the experiment, as only a small number of fully vitellogenic females were seen at the different samplings (see details later). The same situation was observed for the FORKYS tank-reared broodstock, which did not complete gametogenesis and was thus not appropriate for spawning. The comparison between multiple injections and implants was planned to be conducted during Y3 (2016) either with the HCMR tank broodstock, if they develop properly this time after 3 years acclimation to our facilities, or with the ARGO cage broodstock, which will be moved to tanks for spawning after the GnRHa treatment. This activity was planned and implemented during Mo 31-32 (June-July 2016) and could not be analysed and reported in time for submission with this 2nd Periodic Report. Therefore, the spawning induction activities reported during the 2nd Reporting Period were limited to the use of GnRHa implants only, to induce spawning of fish maintained in sea cages at the different facilities and the acquisition and shipment of eggs for the larval rearing experiments (WP 15). Still, some of the breakthrough results obtained during Mo 31-32 will be mentioned here, but without the proper statistical evaluation and report.

3.2.1 Broodstock maintenance

A total of 124 breeders fish in 6 stocks were used for the spawning induction experiments (**Table 3.2.1**):
HCMR tanks: Breeders (n=27) were kept in two 35-m tanks under simulated natural temperature and photoperiod. Fish were fed on moist and then dry pellets (Vitalis Repro/Cal, Skretting, Spain), supplemented with raw fish (mackerel) three times a week.
HCMR cage: Breeders (n=13) were kept in a 40-m perimeter cage at the Souda Bay pilot cage farm, and were fed on moist pellets (Vitalis Repro/Cal, Skretting, Spain).
ARGO cage: Breeders (n=28) were kept in a 40-m perimeter cage at Salamina Island, Greece, and were fed on moist and then dry pellets (Vitalis Repro/Cal, Skretting, Spain), supplemented with raw fish.
FORKYS tank: Breeders (n=21) 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=28) were kept in a 40-m perimeter cage at Galaxidi, Greece and fed with live juvenile fish (seabass and seabream).
ITTICAL tank: 7 breeders were kept in a tank in the Panittica Pugliese S.A. facilities, Italy.

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

Stock	Location	Number of Individuals	Size at sampling (range in kg)	Feeding
HCMR	tanks	27	8.6-23.8	moist pellet, raw fish
HCMR	cages	13	9.9-18.4	moist pellet
ARGO	cages	28	10.7-19.5	moist pellet, raw fish
FORKYS	tanks	21	9.4-15.9	raw fish, squid
GMF	cages	28	9.0-18.0	live fish
ITTICAL	tanks	7	15.0-25.0	raw fish, squid

3.2.2 Evaluation of reproductive stage

Evaluation of reproductive stage begun in mid April 2015 in various broodstocks, based on the ambient temperature and observations on the maturation stage of the stock used for Task 3.1 at the ARGO facilities (the different stocks were maintained at different geographical locations as described in Section 3.2.1). 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.

21/4/2015 HCMR tank: Nine fish from tank S1 were sampled, 5 males and 4 females, with the males producing sperm, which was accessible only with a catheter; hence it was classified as intra-testicular sperm (IT sperm). The females had oocytes in vitellogenesis (Vg) with a diameter of 400-650 μm, but two of them had increased occurrence of atresia (AT).

8/6/2015 HCMR tank: Fourteen fish from tank S1 were sampled, 5 males and 9 females, with the males producing only IT sperm. Three of the females had Vg oocytes with a diameter of 300-650 μm (one in early Vg, one in mid Vg), but two of them had increased occurrence of AT. The remaining females were immature and had primary oocytes (po) (**Fig. 3.2.1.A**).

9/6/2015 ARGO cage: Male fish produced IT sperm, which was motile, having initial motility of 45-80%. Almost all females were in Vg with oocytes of 660-690 μm in diameter, with little occurrence of AT in one fish. Two female fish were found in Oocyte Maturation (OM) or just prior to Ovulation (Ov) stage with oocytes in 1000 μm in diameter (**Fig. 3.2.2.A,B**).

10/6/2015 GMF cage: All females were at Vg with oocyte diameters of 650-700 μm and occurrence of limited AT in 30% of the female fish (**Fig. 3.2.2.D**). Male fish produced IT sperm with initial motility of 50-85%.

18/6/2015 FORKYS tank: Only 1 female was in Vg with oocytes of 530 μm in diameter, while others were immature (containing only po) or in early Vg (eVg) having oocytes with a diameter of 300-400 μm, but in most of them AT was obvious (**Fig. 3.2.1.B,C**). Males produced IT sperm, but motility was not evaluated.

23/6/2015 HCMR Souda cage: The five sampled females were in Vg with a significant number of oocytes in early OM (eOM) with oocytes of 640-780 μm in diameter (**Fig. 3.2.2.C**). All sampled males produced IT sperm with initial motility of 75-100%, motility duration was 3.4 – 8.1 min and density was 1.56 – 3.04 x 10^{10} szoa ml^{-1}.

29/6/2014 HCMR tank: Thirteen fish from S2 tank were sampled. Females were immature containing only po. Males had IT sperm, but probably of small volume, so blood was also collected through the biopsy. Sperm was of bad quality with initial sperm motility 7.5-85% and density 1.10 – 3.96 x 10^{10} szoa ml^{-1}. Duration was not possible to be recorded due to spermatozoa death.

1/7/2015 GMF cage: Twenty fish were sampled. Females were again in Vg with oocytes of 550-780 μm, while some of them were in different stages of OM. One female found to be in post ovulation stage with increased occurrence of AT, and still Vg oocytes (**Fig. 3.2.3.B,C**). Males had IT sperm of 40-80% initial motility and motility duration of 4.07 - 8.97 min.

2/7/2015 ARGO cage: Male fish had IT sperm of 30-85% initial motility and motility duration of 0.85 - 4.05 min. Females were in Vg stage of 600-680 μm with some occurrence of AT. Some females were in post ovulation stage with po and occurrence of AT (**Fig. 3.2.3.A**).

14/7/2015 ITTICAL tank: All fish were sampled. Five females were immature having only po of 80-100 μm (**Fig. 3.2.1.D**). Male fish had IT sperm.

17/7/2015 HCMR Souda cage: Male fish had IT sperm. Initial motility of spermatozoa was 35-90% and motility duration 3.85 - 9.86 min, while density was 1.92 – 4.48 x 10^{10} szoa ml^{-1}. Females were immature having only po, while one fish had occurrence of AT (**Fig. 3.2.3.D**).

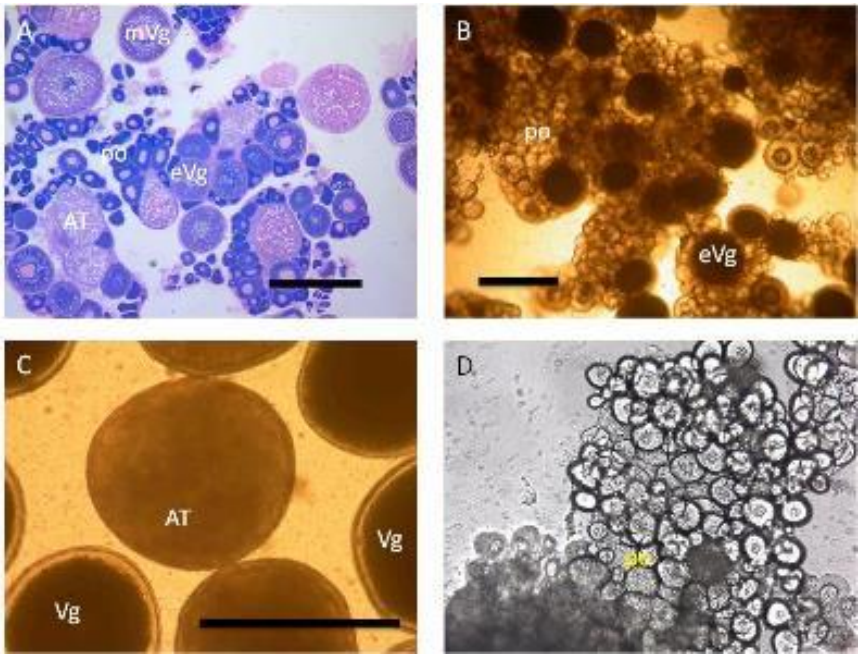


Figure 3.2.1. Female greater amberjack maintained in tanks. Microphotographs of histological section (A) from HCMR broodstock and wet mounts (B,C) from FORKYS and ITTICAL (D) broodstock. A: Female on 8/6/2015, in mid vitellogenesis (mVg) with a large number of primary oocytes (po) and high occurrence of atresia (AT). B, C: Females on 18/6/2015, in eVg and Vg with a large number of po and high occurrence of AT. D: Females on 14/7/2015 having only po of 80-100 μm diameter. Bar = 500 μm.

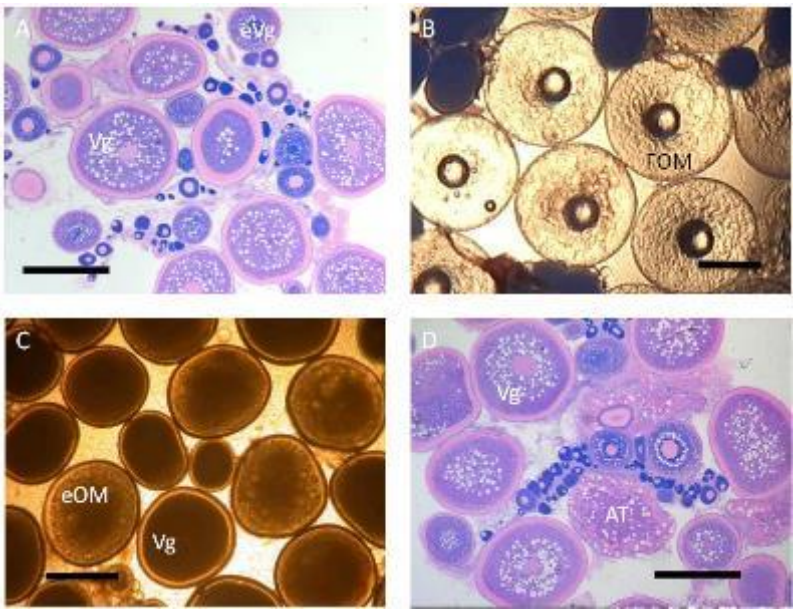


Figure 3.2.2. Female greater amberjack maintained in sea cages (1st sampling, June 2015). Histological sections (A,D) and wet mount photographs (B,C) of greater amberjack oocytes obtained from the ARGO (A,B), HCMR (C), and GMF broodstocks (D). A: Female on 9/6/2015 in vitellogenesis (Vg) having also early Vg oocytes. B: Female on 9/6/2015 in Final Oocyte Maturation (FOM). C: Females on 23/6/2015 in vitellogenesis (Vg) and some oocytes in early Oocyte Maturation (eOM). D: Females on 10/6/2015 in Vg and occurrence of AT. Bar = 500 μm.

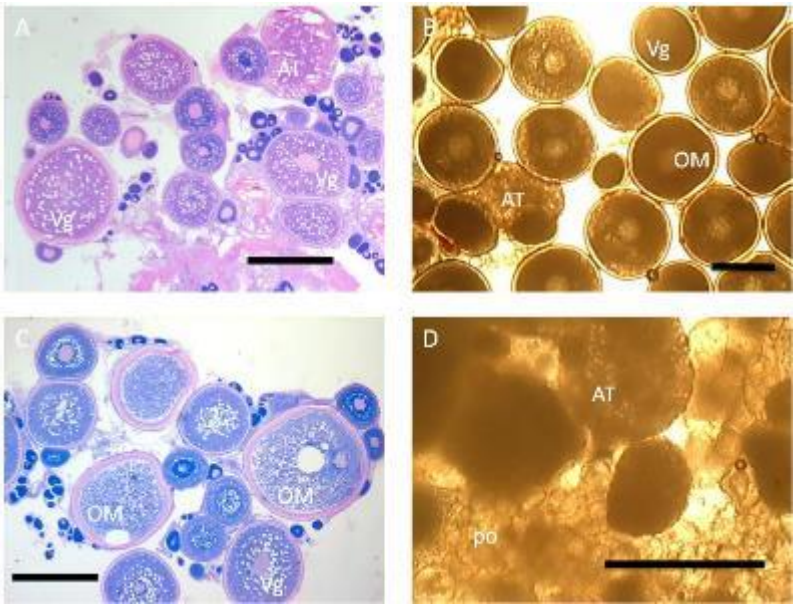


Figure 3.2.3. Female greater amberjack maintained in sea cages (2nd sampling, July 2015). Histological sections (A,C) and wet mount photographs (B, D) of greater amberjack oocytes obtained from the ARGO (A), GMF (B,C) and HCMR (D) at the second sampling of the broodstocks, after an initial induction of spawning with GnRHa implants. A: Female on 2/7/2015 in vitellogenesis (Vg) having also occurrence of atresia (AT). B,C: Female on 1/7/2015 in Oocyte Maturation (OM) having also Vg oocytes. D: Female on 17/7/2015 with primary oocytes (po) and occurrence of AT. Bar = 500 μm.

Overall from the monitoring of the above stocks during the period of the expected spawning season in Y2 and adding to the results of Y1 (1st Periodic Report), it was possible to draw some conclusions as to the reproductive capacity of greater amberjack in captivity and their response to hormonal therapy with GnRHa implants:

1. We found that reproductive maturation may occur in fish as small as ~6 kg in body weight. This particular individual (from GMF) had oocytes at an advanced stage of OM during the reproductive season.
2. The vast majority of sampled females maintained in sea cages were undergoing full vitellogenesis in May-June. Post vitellogenic oocyte diameters ranged between 650-700 μm (female body weights of 13-20 kg) with some occurrence of AT (apoptosis).
3. Temperature does not seem to be so crucial for spawning, since in ARGO and GMF fish were observed to be in eOM, OM and close to ovulation at temperatures lower than 20°C, and spawning induction was possible at this temperature.
4. All sampled males were producing sperm, but probably due to the muscular nature of the abdominal wall of this species or less produced volume of sperm, it was not possible to collect sperm with "stripping". The obtained IT sperm, however, showed good sperm motility characteristics in most cases.
5. Broodstock feeding did not seem to be a significant factor in the reproductive maturation of greater amberjack, and either live or raw fish produced comparable results with re-moistened (2014) or even dry (2015) commercial extruded feeds.
6. Fish maintained in cages had a significantly better degree of reproductive maturation, compared to fish maintained in tanks. In fact, only a small percentage of fish maintained in tanks in HCMR and FORKYS completed vitellogenesis, and upon spawning induction, fecundity was very low and fertilization success was nil. This may be related with water quality characteristics since in all sites well water is used, with a slightly lower pH and probably higher CO_2 content. Another possible explanation of this reproductive dysfunction could be the small volume of the tank (in relation with the size of the fish) or its architecture since in HCMR and ITTICAL 40 and 60 m^3 rectangular tanks are used respectively, and in FORKYS 25 m^3 circular tanks are used. Perhaps a raceway tank is more appropriate for a fast moving species of large size such as the greater amberjack, as tank reared, F1 fish developed and spawned successfully at the Canary Islands (see later Sections).
7. A small percentage of fish maintained both in tanks and in cages have the capacity of undergoing maturation and ovulation spontaneously, without the use of any hormones, as some post-ovulated oocytes were found in some females prior to the GnRHa spawning induction therapy.
8. Overall, it is concluded that wild-caught greater amberjack have the potential of undergoing gametogenesis and completing vitellogenesis to the stage that could be induced to spawn with hormonal therapies, depending on whether they are maintained in sea cages or in tanks (see below for spawning induction results). The difference in the success of gametogenesis observed between the sea cage stock used for Task 3.1 (see earlier) and the stocks used for Task 3.2 could be attributed to the lack of any handling or manipulation of the fish in Task 3.2, prior to the fish reaching post-vitellogenesis and inducing them to spawning with the exogenous hormones. So, it is possible for greater amberjack to complete vitellogenesis and spermatogenesis when maintained in sea cages in captivity, provided they are not handled or manipulated during the early gametogenesis period. Manipulation should be limited to the time of spawning induction, when the most advanced batch of oocytes has completed vitellogenesis and is ready to be induced to undergo OM and ovulation. This observation was confirmed again during Y3 (Mo 31-32) and will be reported fully in the 3rd Periodic Report and the appropriate Deliverable.

3.2.3 Spawning induction

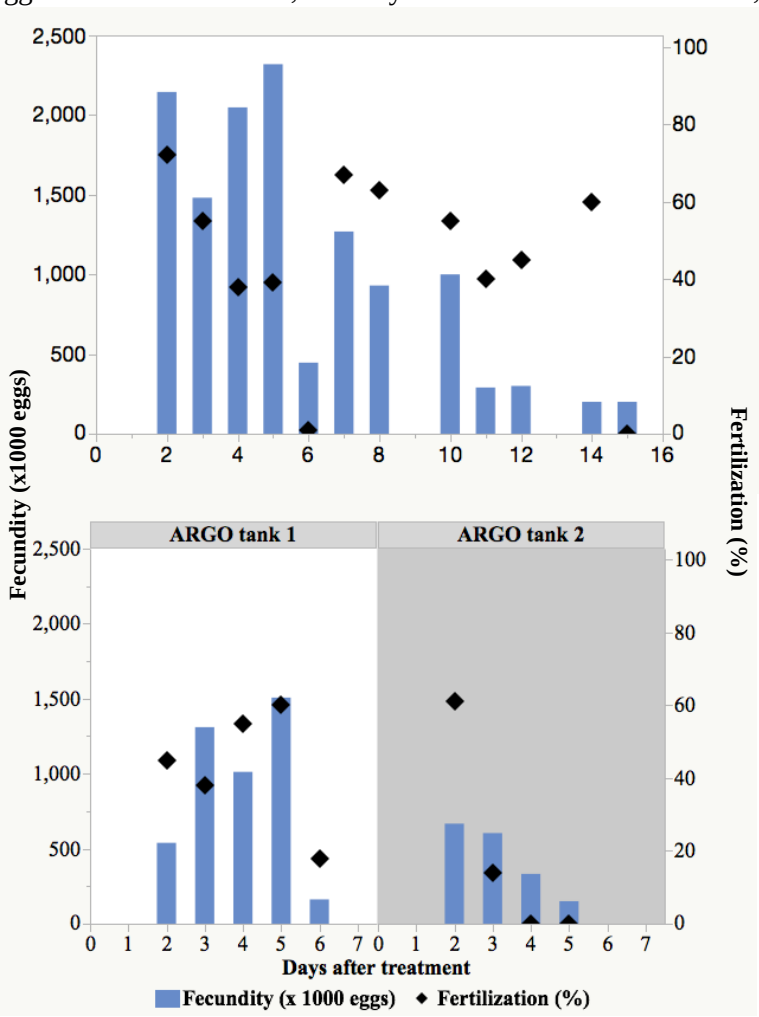
As explained above, a single dose of GnRHa controlled-release delivery systems (implants) was used, chosen based on previous experiments with greater amberjack, but also other marine fish (Mylonas et al., 2004, 2010). When female fish were in the appropriate stage of oocyte development, they were administered

with GnRHa implants, depending on their size, to obtain an effective dose of ~50 µg GnRHa kg^{-1} body weight. Similarly, males received GnRHa implants to obtain an effective dose of ~30 µg GnRHa kg^{-1} body weight. 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. When possible, a sample of eggs was transferred to microtiter plates for estimating the hatching and larval survival percentage as described in Panini et al., (2001).

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:

8/6/2015 and 29/6/2015 HCMR tank, and 18/6/2015 FORKYS tank: A hormonal treatment was given to a small number of females that were found to be the most developed of the population, even if their maturation stage was not what we would select for spawning induction if other individuals existed. In all cases, only a very small batch of eggs was produced 2-4 days later, and always of 0% fertilization success. So, practically no eggs were produced from hormonally treated females maintained in tanks throughout the year.

9/6/2015 ARGO cage: Fifteen female and twelve male fish were treated with GnRHa implants. Four females and three males were transferred in an indoor tank while the rest were left in the cage to spawn. Fish started spawning after 48 h, and they were spawning for 15 days after implantation. In the cage only 16,000 eggs were collected once, two days after treatment. In the tank, a total of 12,628,000 eggs were produced, with fertilization success between 0-72% (**Fig. 3.2.4**).



2/7/2015 ARGO tank: The fish that had been maintained in the tank after the previous spawning induction (9/6/2015) were evaluated for reproductive stage. Males had IT sperm, so they were treated with GnRHa implants and transferred to the tank 1 (see also "2/7/2015 ARGO cage" below). Females, after they had been producing a large amount of eggs (**Fig. 3.2.4**), had only poor AT oocytes and were removed from the population.

2/7/2015 ARGO cage: Twelve fish were treated with GnRHa implants for a second time and they were split in two indoor tanks with three males and three females in each tank. Since just a few eggs (16,000) were collected from the cage during the previous sampling, no fish were induced to spawn in the cage this time. Fish in tank 1 produced 4,530,000 eggs during a period of six days after implantation with fertilization success 18-60%, while fish in tank 2 produced a total of 1,753,000 eggs in 5 days after implantation with fertilization success 0-61% (**Fig. 3.2.4**).

Figure 3.2.4. Fecundity (x1000 eggs, blue bars) and fertilization (%, black diamonds) from greater amberjack stock maintained at the ARGO sea cage facility, induced with GnRHa implants at 9/6/2015 (top) and 2/7/2015 (bottom) and placed in tanks to spawn.

10/6/2015 GMF cage: Twenty-eight fish were treated with GnRHa implants. Six females and six males were transferred in two land-based tanks and were split equally, while the rest were left in the cage to spawn. Fish in the tanks started spawning after 48 h, and they were spawning for 5 days after implantation after which they were returned back to the cage. In tank 1, a total of 3,000,000 eggs were produced, with fertilization success between 67-100%, while in tank 2, 2,250,000 eggs were produced, with fertilization success between 80-100% (**Fig. 3.2.5**). In the sea cage no eggs were collected.

1/7/2015 GMF cage: Eighteen fish were treated with GnRHa implants for the second time. The cage contained also the fish from the previous spawning induction above that were placed in the tanks for spawning (moved after Day 5). Five females and five males were transferred in tank 3 and the rest were transferred in a plastic bag into the cage (which is used for anesthetizing fish), filled with seawater. In tank 3, a total of 5,630,000 eggs were produced with fertilization success 0-89%. In the anaesthetic bag, a total of 3,020,000 eggs were produced with fertilization success 0-75% (**Fig. 3.2.5**).

23/6/2015 HCMR Souda cage: Thirteen fish were treated with GnRHa implants. No eggs were collected.

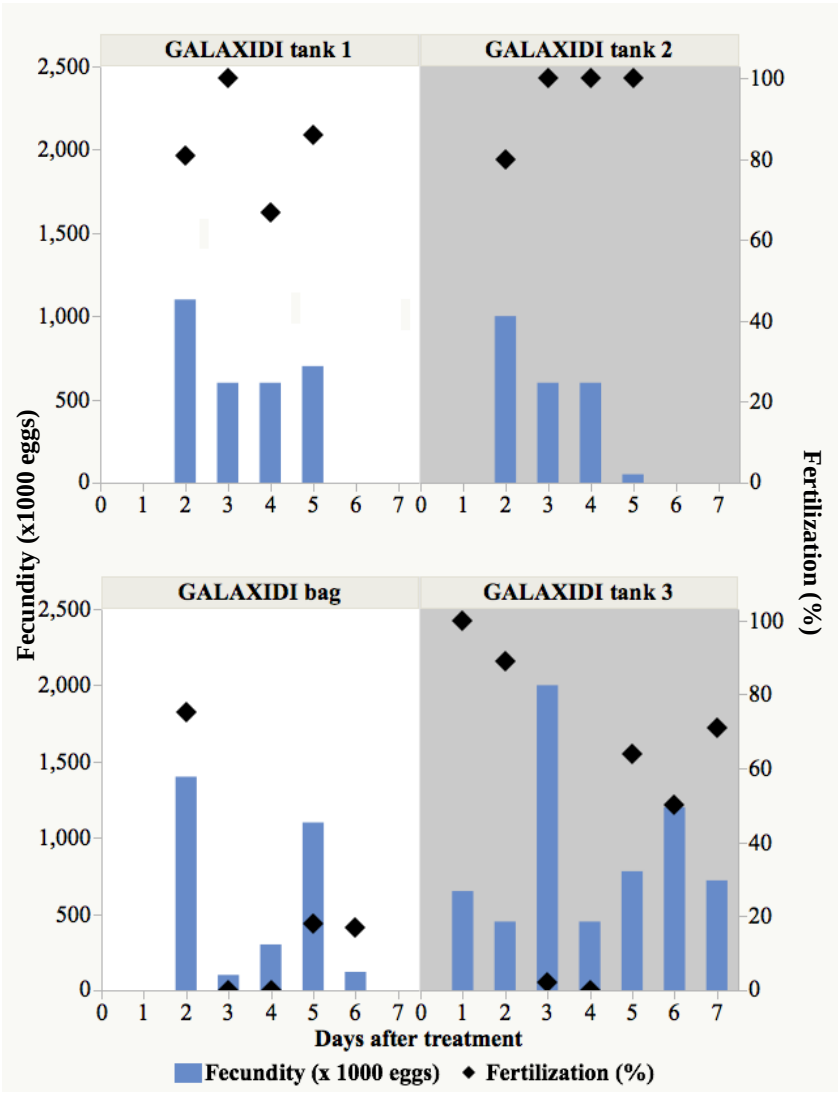


Figure 3.2.5. Fecundity (x1000 eggs, blue bars) and fertilization (%, black diamonds) from greater amberjack stock maintained at the GMF sea cage facility, induced with GnRHa implants at 10/6/2015 (top) and 1/7/2015 (bottom).

2016 ARGO: At Mo 31 (outside of this reporting period), the planned experiment of comparing two different methods of GnRHa treatments, was done at the P23. ARGO facilities. Fish were evaluated for reproductive maturation on 7th of June, and 28 fish (14 males and 14 females) were split in four tanks. The males were treated with GnRHa implants, while the females were treated either with liquid GnRHa solution (INJECTION 1 & 2) or with GnRHa implants (IMPLANT 1 & 2) (**Figure 3.2.6**). The injected females were treated on three consecutive weeks (day 0, 7, 14), while the implanted females received two treatments (day 0, 14). Even though the data are still under analysis, an obvious conclusion is that the implanted fish spawned more eggs in the experimental period as a total (12,423,000 eggs in IMPLANT 1 and 14,555,000 eggs in IMPLANT 2, while 4,192,000 eggs in INJECTION 1 and 6,208,000 eggs in INJECTION 2, respectively) and per day (the maximum daily fecundity in IMPLANT 1 was 3,010,000 eggs and in IMPLANT 2 was 4,242,000 eggs, while in INJECTION 1 was 2,062,000 eggs and in INJECTION 2 was 2,454,000 eggs, respectively).

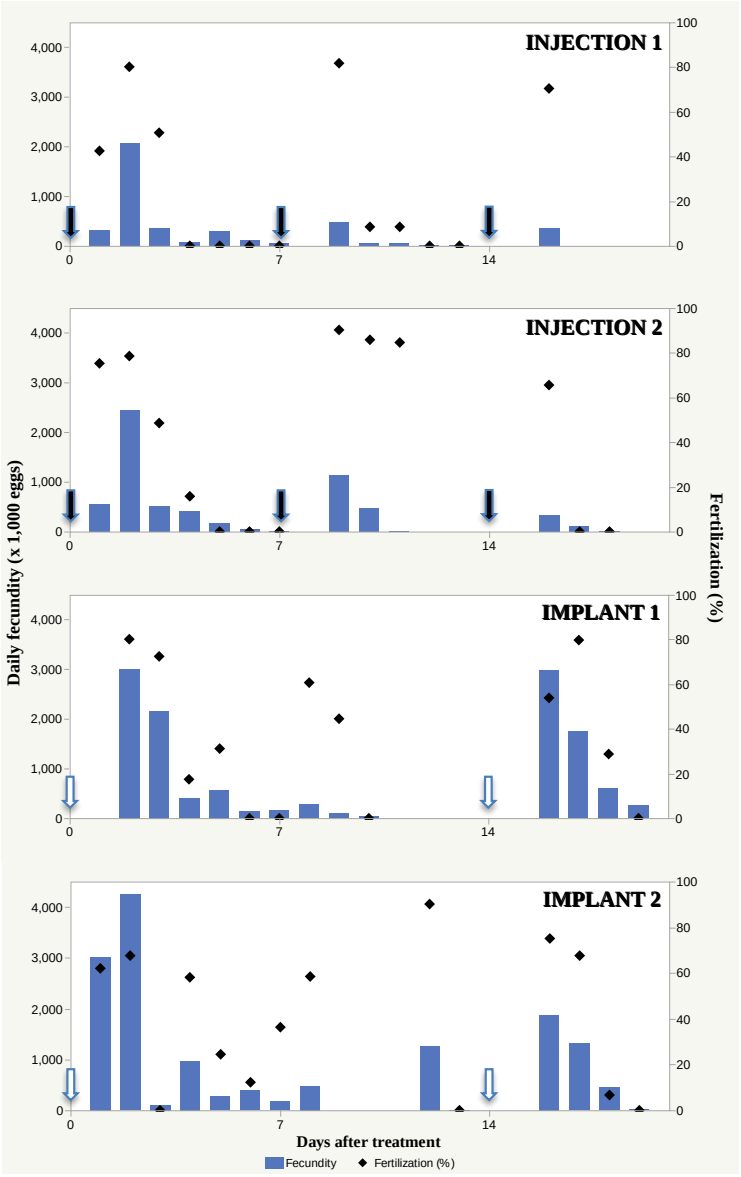


Figure 3.2.6. Fecundity (x1000 eggs, blue bars) and fertilization (%, black diamonds) from the greater amberjack stock maintained at the P23. ARGO sea cage facility, induced either with GnRHa in liquid form (INJECTION) or with solid GnRHa implants (IMPLANT). Black arrows indicate the time of injection treatments, while white arrows indicate the time of implant treatments.

To sum up, the spawning results so far (2014 and 2015, and partly from 2016) provide some promising insights into the development of methods for the control of egg production in greater amberjack:

1. Males produce sperm of good quality during the spawning period, albeit of limited stripping volume, and although it was not investigated if GnRHa treatment is absolutely necessary, males treated with GnRHa maintained good spermiation and quality parameters even after repeated spawning and production of fertilized eggs.
2. Females maintained in cages without any manipulation or handling during gametogenesis and moved to tanks after the hormonal therapy had a better response to the GnRHa treatment, producing eggs of higher fecundity and most importantly of better fertilization success, compared to females maintained in tanks throughout the year (gametogenesis and spawning induction). This was expected based on the achievement of better degree of reproductive development (post vitellogenesis) at the time of treatment (See previous section), compared to fish maintained in tanks.
3. Egg collection was possible from broodstocks maintained in cages during 2014, but the fecundity achieved was much less than from stocks spawning in tanks. On the contrary, just a few eggs were collected during 2015 in ARGO and no eggs were collected in HCMR and GMF cages. This is definitely due to significant loses of eggs from the cage, and more work needs to be done to optimize the egg collection process (See later in Task 3.5), before this method is proposed for commercial use.
4. An alternative broodstock management method resulting from the experience of the two years of the project could be the maintenance of the broodstock in sea cages during the year (gametogenesis) and their transfer to land-based tanks for spawning after GnRHa treatment. This ensures collection of all spawned eggs and thus higher effective fecundities. However, transfer to the tank affected the "long-term" reproductive condition of the females, thus shortening their reproductive season, since at the second re-evaluation all fish were undergoing atresia in their ovaries. Still, this method may be an effective approach for commercial production, producing large numbers of eggs within a short period of time.
5. Although not fully reported in the present report (as the experiments were undertaken during Mo 31-32 which are outside the scope of the 2nd Periodic Report), spawning induction in tanks, using fish that were maintained in sea cages during the year was again very successful in Y3, producing some 30 kg of eggs from ARGO and another 25 kg eggs from GMF, of very high quality. This enabled the production of a significant number of juveniles (Se WP 15), which will be used for the planned DOW experiments on Grow out husbandry. In addition, eggs were provided to a number of commercial hatcheries in Greece and Cyprus, in order to give them a chance to try their larval rearing expertise, and to pave the ground for the future commercial production of greater amberjack by the Mediterranean aquaculture industry.
6. The experiment on comparing the efficacy of GnRHa injections vs GnRHa implants, which could not be implemented during Y2, was undertaken in Y3 (Mo 31-32) at the facilities of ARGO, demonstrating that GnRHa implants are much more effective in producing large numbers of eggs or good quality, with less handling and manipulation. This is contrary to what has been observed in the Atlantic Ocean stock of greater amberjack, reported in the 1st Reporting Period by P2. FCPCT, and further examined here (see later). These results prompted the consortium to consider undertaking a study (at no extra charge to the project) of examining the genetic makeup of different broodstocks in the Mediterranean Sea and the Atlantic Ocean, in an effort to identify the existence of genetically different strains of greater amberjack, something that could explain the differences in reproductive biology between some of the stocks from Mediterranean Sea and the Atlantic Ocean.

In the following years, the experience acquired from the above experiments will be used to implement the planned work in order to optimize the spawning induction protocol in terms of time of application, method and effective dose of GnRHa.

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

Based on the results of the first reporting period, in which natural spawnings were more successful than induced spawnings and GnRHa injections were more effective than GnRHa implantations, the goal of the experiments carried out during the 2nd reporting period was to compare natural spawnings with GnRHa injection-induced spawnings.

In the present study 17 individuals of a group of 22 greater amberjack, captured in May 2011 in the southeast coast of Gran Canaria (Islas Canarias, España) were used. Fish (mean weight at capture 3.41±1.12 kg for females and 2.37±1.07 kg for males) were acclimated in 10 m^3 tanks (3 m x 3 m x 1,5 m in depth) located in the Planta Piloto de Producción de Alevines (AAPP) of Grupo de Investigación en Acuicultura (GIA), located in the facilities of P2. FCPCT. On January 2013, the fish (mean weight 8.27±1.11 kg for females and 8.12±1.82 kg for males) were transported to the new station of broodstock of the PCTM, where they were kept in round tanks of 40 m^3 (5 m x 2,35 m) and were used in the expreiments described in the 1st Periodic Report (2014).

Before the beginning of the experiments, at the end of March 2015, all fish were anesthetized with 50 ppm clove oil and body weight was determined. The same broodstock (3 males and 2 females) that spawned spontaneously in 2014 was used in Tank 1, while the other fish used in this trial were distributed in three circular tanks of 40 m^3 (Tanks 2, 3 and 4), at a ratio of 2 females and 2 males per each tank. All females used in this experiment spawned in the year 2014 and, to avoid stress, they were not cannulated, and no abdominal pressure to males was applied. The tanks of 40 m^3 have an open water system with a water exchange of 600% day^{-1} and salinity of 37 ‰. The photoperiod was natural, using the day period of the geographical position of 27º 59' 28" N; 15º 22'05" W. Temperature and oxygen was determined continuously, by means of a probe system controlled by a computer (Miranda, Innovaqua, Sevilla, España).

Fish in Tanks 2, 3 and 4, were injected intramuscular once a week with GnRHa (LHRHa, des-Gly10, [D-Ala6]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg kg^{-1}. The first natural spawn (Tank 1) occurred on 18 May 2015, at a temperature of 20.8ºC. Tanks 2, 3 and 4 contained two pairs, which were induced on different days. The first pairs in Tanks 2, 3 and 4 were induced to spawn on 02 June 2015, and the second pairs on 05 June 2015. The last natural spawn (Tank 1) occurred on the 09 October 2015, at a temperature of 24.6ºC. The last spawns from induced fish occurred on 08 October (tank 2 and 3) and on 28 October 2015 (tank 4), although three more inductions were done to each broodstock pair of each tank, and no spawns were obtained. The mean temperature during the spawning season ranged between 20.5±0.3ºC in May and 24.5±0.2 in October. The parameters indicative of spawning quality are reported in **Table 3.3.1.** Twenty-two natural spawns occurred in tank 1; 33 spawns were obtained in tanks 2 and 3 after 43 inductions per tank; 32 spawns were obtained in tank 4, after 46 inductions.

Table 3.3.1. Quality of spawn indexes (the values corresponding to the injected spawns, are the mean of the 3 induced tanks)

	% Fertilization	% Viable 24 h	% Hatching	% 4d Live	% 8d live
Spawn	P < 0.01	P > 0.05	P > 0.05	P < 0.01	P < 0.01
Natural	76.36±.19.89[a]	88.90±20.69	86.79±21.10	87.84±8.79[a]	7.80±4.58[a]
Injected	65.62±17.39[b]	90.70±7.78	88.40±9.19	71.15±15.67[b]	4.77±5.60[b]

*Different superscript letters in the same column indicate significant differences

Significant differences between the natural and induced spawns were observed in the fertilization rate and in larval survival after 4 and 8 days. No differences were found in the % of viable eggs at 24 h and at hatching. During all the spawning season, the total number of eggs was of 28.54 million in natural spawn and 12.21±7.66 million in the induced tanks, respectively. In **Figure 3.3.1** the number of eggs per spawn is reported, showing significant differences between the natural and the induced spawns (P<0.01). In **Figure 3.3.2**, the relative number of eggs per spawn and kg of female body weight is shown. Significant differences (P < 0.01) were observed between the natural and the induced spawns.

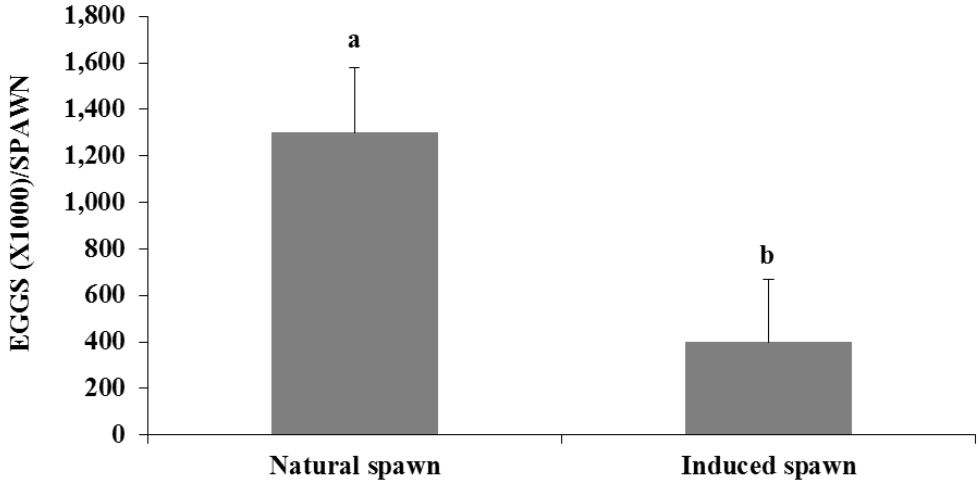


Figure 3.3.1. Number of eggs per natural and induced spawn. Different superscript letters indicate statistical significant differences (P < 0.01).

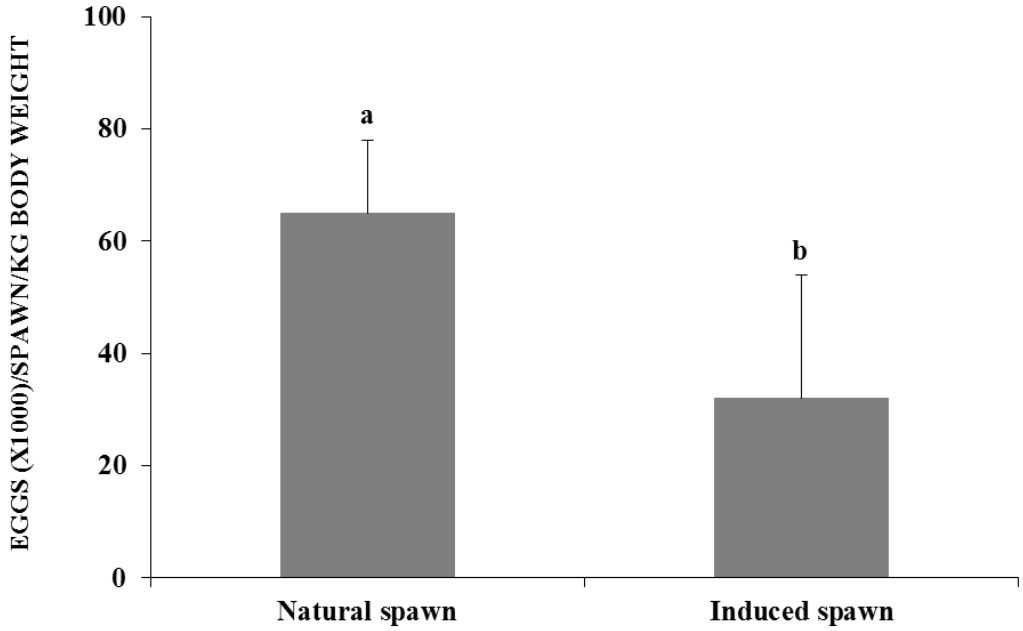


Figure 3.3.2. Number of eggs per spawn and female body weight. Different superscript letters indicate statistical significant differences (P < 0.01).

Task 3.4 Development of an optimized spawning induction protocol for F1 greater amberjack in the eastern Atlantic (led by IEO).

3.4.1 Experimental conditions

The experiments carried out in the present task involved IEO and HCMR staff. A group of 15 greater amberjack breeders born in captivity (average weight of 18.5±9.2 kg) were maintained in an outdoor covered raceway tank of 500 m^3 with continuous water supply (6 renewals day^{-1}) under natural photoperiod in the facilities of IEO in Tenerife, Canary Islands (Spain). Broodstock (7 males, 7 females and 1 undetermined sex) were tagged with passive integrated transponders (PIT tags).

All fish were sampled four times during the 2015 spawning season (May, June, July and September), and length and body weight were measured. 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 the 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 inserting 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 May, June and July. 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 ~50 µg GnRHa kg^{-1} body weight (**Table 3.4.1**). The dose of GnRHa implanted to males decreased from about 60 µg GnRHa kg^{-1} body weight in May to about 40 µg GnRHa kg^{-1} body weight in June and July (**Table 3.4.1**).

Table 3.4.1. Number of biopsied and treated fish (mean weight ± SEM) and dose of GnRHa kg^{-1} body weight implanted at each treatment sampling. All fish were treated with an GnRHa implant, and variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa.

Sex		Females					Males				
Sampling (Month)	Treatment	N		Dose (µg kg^{-1})			N		Dose (µg kg^{-1})		
		Biopsied	Treated				Biopsied	Treated			
May	First	7	4 (29.1±5.1)	53.9	±	10.9	7	7 (14.9±1.9)	67.9	±	20.3
June	Second*	7	7 (23.3±4.1)	54.4	±	8.5	7	5 (16.9±1.9)	38.5	±	4.1
July	Third	7	6 (23.9±3.6)	52.7	±	4.6	7	6 (13.9±1.9)	39.9	±	14.5
Septemb.		6					5				

Sperm quality parameters that were evaluated 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 2015), 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 and their lipid droplet were 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 the number of hatched larvae / the number of live embryos, and 2-5-d larval survival was calculated as the number of live larvae 2-5 d after egg collection / the number of hatched larvae.

For the quantification of 17 alpha, 20 beta-dihydroxyprogesterone (17,20 DHP), testosterone (T) and 17β-estradiol (E_2) in females, as well as of 17,20 DHP, T, and 11-Ketotestosterone (11-KT) in the plasma of males, already established and well described enzyme-linked immunoassays (ELISA) were used, with some modifications. 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 (Alanine transaminase (ALT)/Glutamic Pyruvic Transaminase (GPT), Aspartate transaminase (AST)/Glutamic Oxaloacetic Transaminase (GOT), Alcaline phosphatase, Cholinesterase 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). Plasma cortisol level was analyzed by radioimmunoassay using ELISA kits (Arbor Assay, Michigan, USA).

Mean sperm motility percentage was higher than 50% and remained unchanged throughout successive samplings while the duration of sperm motility was significantly higher in May (4.35 min) than in June (2.44 min) ($P<0.05$). The sperm density increased from 30.8 x 10^9 spermatozoa ml^{-1} in May to 78.0 x 10^9 spermatozoa ml^{-1} in September, although with elevated individual variability in September. Mean sperm motility was 58±21% during the reproductive period and no differences were observed between the samplings. On the contrary, motility duration was significantly higher at the 1st sampling (4.4±1.1min) comparing to the following 3 samplings (**Figure 3.4.1**).

The number of spawnings obtained in the successive post treatment periods decreased, 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 1st treatment period of 31 days of duration, between the first and last spawning, the eggs were collected almost daily (29 spawning events). However, after the 2nd treatment, a total of 15 spawnings were recorded during the first 16 days and no eggs were collected in the remaining 13 days. The eggs released after the GnRHa treatment were collected from 8 spawning events registered during the 9 days immediately after treatment.

Mean fertilization and hatching exhibited similar trends during the three spawning periods, reaching their highest values in the second period (June to July) (**Fig. 3.4.2**). However, no significant differences in embryo survival (1 day) were observed between periods after successive GnRHa treatment.

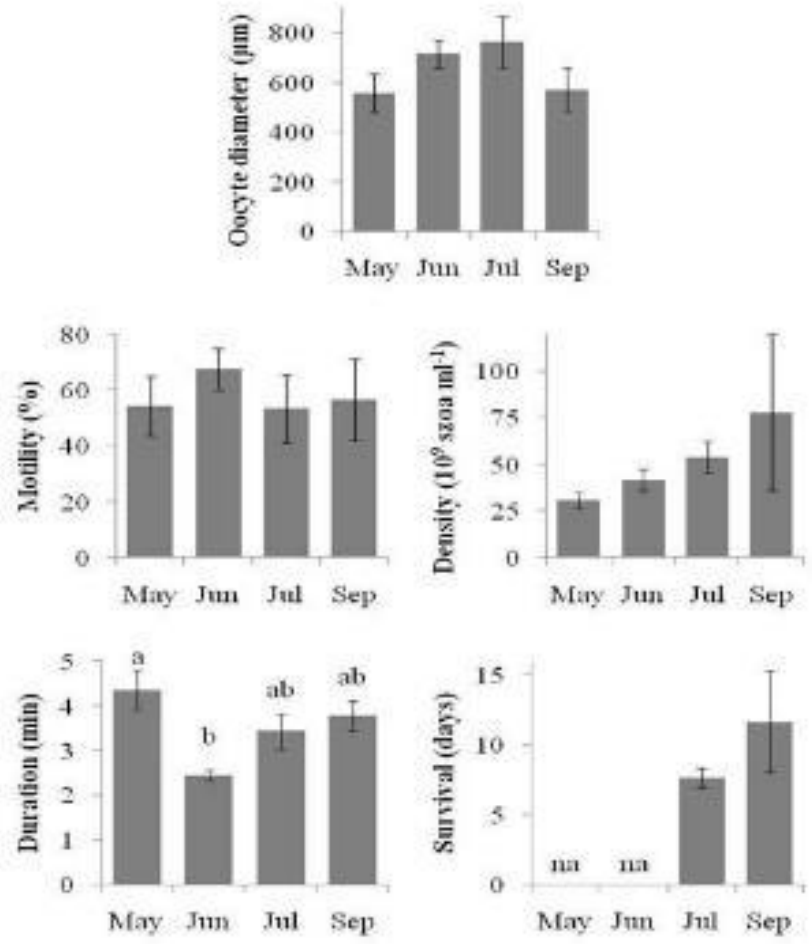


Figure 3.4.1. Mean (± SE) oocyte diameter and sperm quality parameters of greater amberjack at each sampling-treatment. Statistically significant differences among months are indicated by different lower case letters ($P < 0.05$).

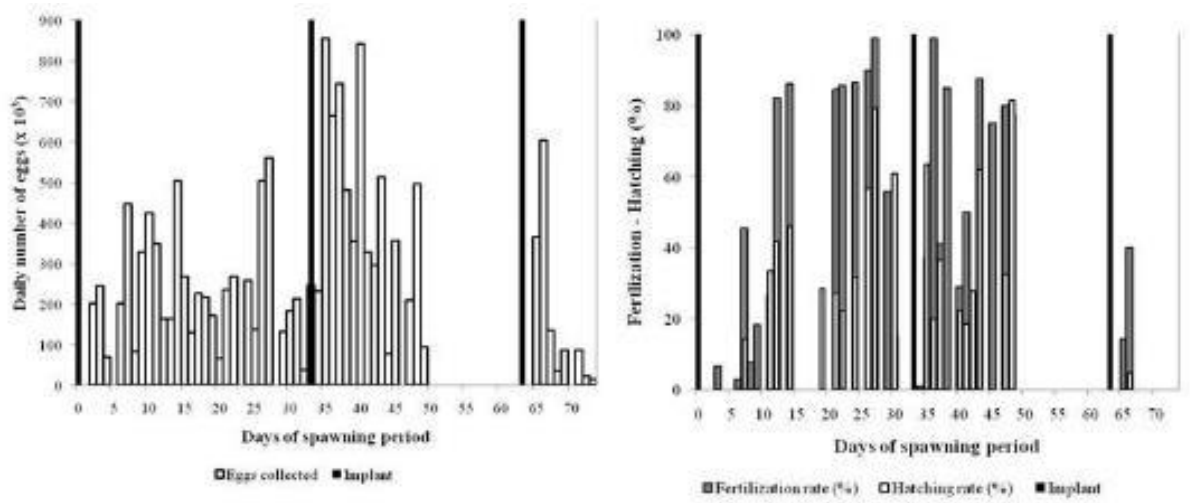


Figure 3.4.2. Daily number of eggs collected and fertilization and hatching rate registered during the experimental spawning period. The black bars indicate the day of the implant treatment - sampling.

Female plasma E_2 levels were high at the beginning of the spawning period (May), although with elevated individual variability as denoted by the high values of SE (**Fig. 3.4.3**). Thereafter, E_2 declined progressively until baseline values in July. The level of T in the female plasma was low along the spawning season, increasing significantly ($P<0.05$) in the sampling performed in September. The 17,20 DHP level remained at low values during all the experimental spawning period. In males, both plasma11-KT and T levels decreased progressively until reaching the lowest values in July; a significant increase of these hormones was observed in September ($P<0.05$); 17,20 DHP showed very similar pattern along the spawning season, with the highest level detected in September.

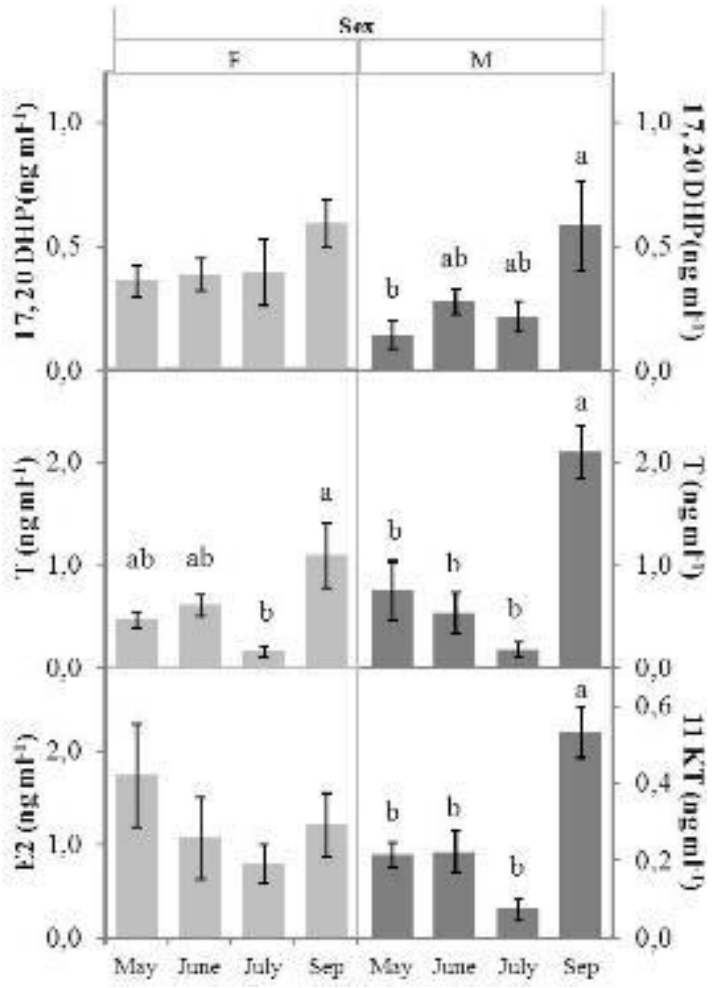


Figure 3.4.3. Plasma levels (means ± SE) of E_2, T and 17, 20 DHP in females (F) and plasma levels of 11 KT, T and 17,20 DHP in males (M) amberjack broodstock. Different letters indicate significant differences ($P<0.05$).

All blood parameters studied remained constant along the study and only erythrocytes, protein, cholesterol and hematocrit were lower at the end of the spawning season (**Table 3.4.4**). During chronic stress in fish culture, there are often characteristically high circulating levels of cortisol. In the present study, no significant differences ($P < 0.05$) in cortisol levels were observed along spawning season, although a trend to diminish was observed at the end of the spawning season (September). Furthermore, no differences were found in glucose and lactate, however, sodium showed lower values at the end of the spawning season.

Table 3.4.4. Erythrocytes (x10^4), leucocytes (x10^3), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), glucose (mg/dl), ALT/GPT (U/L), AST/GOT (U/L), alkaline phosphatase (U/L), cholinesterase (U/L), amylase (U/L), cortisol (ng/ml), lactate (mg/dl), sodium (mg/dl), potassium (mg/dl) along the spawning season.

	May		June		July		September	
Erythrocytes	347.78 ± 118.50	a	275.62 ± 72.75	a	149.31 ± 79.41	b	128.07 ± 52.50	b
Leucocytes	866.54 ± 475.47		653.21 ± 341.54		573.00 ± 260.08		694.54 ± 247.66	
Hematocrit	45 ± 10		52 ± 13		35 ± 15		37 ± 11	
Triglycerides	226.18 ± 58.52		172.14 ± 129.94		206.25 ± 104.28		221.44 ± 147.89	
Cholesterol	226.18 ± 58.52	ab	336.33 ± 170.63	a	275.65 ± 93.44	ab	177.86 ± 90.10	b
Protein	39.85 ± 10.41	ab	44.20 ± 12.60	a	49.93 ± 14.27	a	28.51 ± 8.68	b
Glucose	94.57 ± 26.27		74.94 ± 31.33		100.39 ± 34.85		107.84 ± 53.51	
ALT/GPT	12.92 ± 3.08		14.72 ± 7.55		13.53 ± 6.35		21.60 ± 11.25	
AST/GOT	23.96 ± 16.48		32.71 ± 27.36		31.03 ± 24.76		14.11 ± 4.33	
Alcaline phosphatase	63.11 ± 12.60	c	89.74 ± 20.90	bc	105.22 ± 19.49	b	142.50 ± 31.62	a
Cholinesterase	288.81 ± 235.19		186.44 ± 42.00		235.80 ± 125.87		243.72 ± 40.21	
Amylase	10.97 ± 2.69		15.09 ± 4.24		13.26 ± 17.99		1.98 ± 1.44	
Cortisol	10.82 ± 2.66		11.89 ± 4.30		32.79 ± 8.76		7.69 ± 3.37	
Lactate	39.20 ± 9.71		38.82 ± 7.34		40.09 ± 13.15		37.37 ± 11.84	
Sodium	435.57 ± 18.12	a	415.45 ± 11.67	a	516.06 ± 123.03	a	381.40 ± 10.11	b
Potassium	22.92 ± 6.61		15.98 ± 1.81		20.53 ± 7.39		14.41 ± 2.68	

Values are means ± SD. Different letters indicate significant differences (ANOVA, $P<0.05$)

Conclusions

Hatchery produced greater amberjack (F1 generation) were able to finalize vitellogenesis and spermiation, and they underwent repeated spawning for 3 months with a total production of almost 15 million eggs after treatment with GnRHa implants. These results, the first with successful reproduction of F1 greater amberjack broodstock, are a step towards the industrial aquaculture production of this valuable species.

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

As in 2014, egg collection in cages was tested in three different sites of ARGO, GMF and HCMR. The egg collectors were placed in each site after the initial sampling for reproductive evaluation (as described in Task 3.2), *i.e.* on 9/6/2015 in ARGO, 10/6/2015 in GMF and 23/6/2015 in HCMR. This time, the egg collectors were placed to an increased depth of 5 m (**Fig. 3.5.1**) compared to 3.5 m of the 2014 spawning season (1st Periodic Report). Also, the cage depth was reduced to 6-8 m to avoid loss of eggs from the bottom of the cage. The evaluation of the reproductive stage of development, spawning induction experiments and egg collection data was described in detail above (Task 3.2).

Figure 3.5.1. Egg collector in ARGO up to the depth of 5 m.

Despite the different device setup, the egg collectors did not work properly, as only a very small amount of eggs was collected in 2015, and only in the ARGO site (16,000 eggs), compared to the millions of eggs produced in the tanks by a smaller number of females. The failure to collect eggs in sea cages is perhaps due to low buoyancy of eggs immediately upon spawning (and until full hydration is completed) or strong currents in the cage facilities. In order to confirm that the fish left in the sea cages did actually spawn in response to the GnRHa therapy, a test was held with the anaesthetic plastic bag in GMF. After treating the fish with the GnRHa implants, the fish were put inside the anaesthetic bag for the few days after the spawning induction (**Fig. 3.5.2**). The amount of eggs collected using this method was comparable to the produced amount of the tank (**Fig. 3.2.5**), confirming that the lack of egg collection from the cages was not because of failure of the fish to spawn, but of failure to collect the spawned eggs, as they were carried away by the current.

Figure 3.5.2. The anaesthetic bag used for sampling the fish at GMF. At the conclusion of the sampling and GnRHa treatment, a small number of fish were placed in this bag and were left there for 4 days to verify that they would spawn.

The following 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 in 2015. 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. 3.5.3**). 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 3.5.3. 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.

The experiments were undertaken during Mo 31-32 (outside the period covered by this report) and will be reported fully in the 3rd Reporting Period. However, we can mention that again we did not succeed in obtaining the expected number of eggs, given the number of females induced to spawn, and further modifications are required to make this procedure applicable for commercial use.

References

AOAC, 2012. Official Methods of Analysis of the Association of Analytical Chemistry. (AOAC) International, Gaithersburg, MD, USA. 1766 pp.

Barua, A.B., Kostic, D., Olson, J.A., 1993. New simplified procedures for the extraction and simultaneous high-performance liquid chromatographic analysis of retinol, tocopherols and carotenoids in human serum. J. Chromatogr. 617B, 257–264.

Christie, W.W., 1982. Lipid analysis (2nd edn). Pergamon Press, Oxford.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of reproduction. Gen. Comp. Endocrinol. 165, 516-534.

Mylonas, C.C., Papandroulakis, N., Smboukis, A., Papadaki, M., Divanach, P., 2004. Induction of spawning of cultured greater amberjack (*Seriola dumerili*) using GnRHa implants. Aquaculture. 237, 141-154.

Olsen, R.E,. Henderson, R.J., 1989. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J. Exp. Mar. Biol. Ecol. 129, 189-197.

Panini, E., Mylonas, C.C., Zanuy, S., Carrillo, M., Ramos, J., Bruce, M., 2001. Incubation of embryos and larvae of marine fish using microtiter plates. Aquacult. Int. 9, 189-196.

Deviations from Annex I and their impact:

1. 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 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 HCMR tank facilities, as well as at the 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 ARGO, which last year matured well and responded adequately to the hormonal therapy. So, this experiment was indeed implemented during Mo 31-32 (outside the scope of this report) and is reported only briefly in this 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.

2. No new broodstock was acquired by ITTICAL and no effort was made by the company to examine the maturation status of their fish and induce spawning in Y3 (Mo 30 or 31). So, it is the opinion of the WP leader and the PC that this partner is underperforming in the consortium and a motion will be made to the Steering Committee to discontinue their participation in the project and transfer their activities to another partner (either ARGO which is already a partner in the consortium or GMF which is not a member of the consortium, but which has dedicated their broodstock and facilities to the experiments of WP 3 for no charge to the Consortium). This will be discussed with the EU Scientific Officer in the coming months. No negative impact on the success of the project is foreseen from this deviation, and in fact we believe that there will be an improvement of the work implemented, due to the closer proximity of any of the above proposed substitutes (ARGO or GMF) to HCMR, which is leading this Task, as well as the greater interest and commitment that that these SMEs have demonstrated so far.

4. Our observations made in the first 3 years of the project, lead us to believe that there are some significant differences in the reproductive biology of greater amberjack from the Mediterranean Sea (Italy, Greece) vs the Atlantic Ocean (Canary Islands). Specifically, Atlantic Ocean broodstocks (1) adapt much more readily to tanks (even of small diameter) and undergo successful gametogenesis, (2) have a greater propensity to undergo spontaneous spawning without the use of any exogenous hormones, (3) respond better to GnRHa injections (*i.e.* pulsatile stimulation) than GnRHa implants (*i.e.* sustained stimulation) and (4) reproduce for a much longer period of time, from May to September. So, we hypothesize that the greater amberjack populations in the Mediterranean Sea and the Atlantic Ocean, may not represent the same strain or stock, and

there may be significant genetic differences between them, which have not been examined so far. So, we decided to undertake such a population genetic study for greater amberjack, taking advantage the existence of a large number of broodstocks from different geographic locations (both captive and sampled from the wild as part of Task 3.1), as well as the access of the consortium to other broodstocks through professional contacts. This work will be undertaken at no extra charge to the project, and we already collected fin-clips from broodstocks of the various partners involved, such as FCPCT, IEO, UNIBA, HCMR, ARGO, FORKYS and other commercial hatcheries such as SAGRO Aquaculture (Cyprus) and KILIC FISHERIES (Turkey). This deviation will have a positive impact on the further contribution of the project to the knowledge of greater amberjack population structure and reproductive biology.

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), Dimitris Tsaparis (P1)			

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 progress towards objectives and details for each task (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.

Task 4.1 Evaluation of the genetic variation in available domesticated broodstocks of pikeperch (led by UL, Pascal Fontaine) This Task has been completed and reported during the 1st Reporting Period.

Briefly, DNA extractions were done for all domesticated samples following standard protocols (salt precipitation, Miller et al., 1988). The PCR amplifications have been focused in the Aquaculture/domesticated samples in order to first fulfill D4.1 (Month 12, i.e. end of November). In total, DNA was extracted and analyzed from 439 fish samples (**Table 4.1.1**); a wild population of 53 fish from Hungary was used as a reference for all population genetics parameters.

Table 4.1.1. List of domesticated pikeperch samples and number of fish per sample that were genotyped and analyzed; the first population marked in red is of wild origin.

A/a	Population	Sample size
1	Gyori Elore, HTSZ, Hungary	53
2	Szabolsi, Halaszati Kft, Hungary	50
3	VanMecklen, Holland, Aquapri A/S, Danemark	54
4	Czech Rep., Aquapri A/S, Danemark	38
5	Excellence fish, Hollande, Aquapri A/S, Danemark	14
6	Hungary, Aquapri A/S, Danemark	74
7	Mosso, Aquapri A/S, Danemark	19
8	IfB, Potsdam, Germany	48
9	FGFRI Kainuu fisheries research station, Finland	31
10	FGFRI Laukaa Fish Farm, Finland	20
11	ASIALOR, France	31
12	INAGRO, Belgium (German origin)	30
13	INAGRO, Belgium (Dutch Origin)	30

The microsatellite loci were chosen from other studies in pikeperch. Multiplex optimizations were performed for 22 loci (grouped into two multiplexes) that were reported in: Leclerc et al. (2000) for the yellow perch (*Perca flavescens* – Code: Pfla), Borer et al. (1999) and Wirth et al. (1999) in walleye *Stizostedion vitreum* – (Code: Zvi), and Dubut et al (2010) in the Rhone streber (*Zingel asper* – Code: Za). Those reported in pikeperch by Kohlmann & Kersten (2008) have shown low number of alleles (2-6 alleles in a population of 25 fish) and low expected heterozygosity (0.334 – 0.777) and have not been used previously in a multiplex to genotype populations. Microsatellite loci were first ordered by increasing size in base pairs (bp) and the size range (reported in the species described), and in each range one of the primers for each microsatellite locus the reverse (code: R) was fluorescently labelled with the dyes that conformed to the P1. HCMR's sequencing technology (ABI 3730).

The number of alleles per locus ranged from 6-7 (PflaL3 and PflaL9, respectively) to 20 (Za138). Therefore, microsatellite loci showed relatively high levels of polymorphism even though some samples were monomorphic (exhibited only one allele) for some loci like for Za199 and PflaL9 in the Excellence fish of Aquapri A/S (population 5), locus Za237 in Kainuu fisheries research station (population 9), and Za144 in Laukaa Fish Farm (population 10). For the thirteen populations analyzed, the least number of alleles was encountered in Aquapri's VanMecklen (2.64), Aquapri's Excellence fish and Laukaa Fish Farm (2.73) and

the greatest in Hungarian Aquapri's (7.91) and Halaszati Kft (7.55) stocks, which were greater than that in wild Hungarian stock (6.00). Likewise, expected heterozygosity (H_E) ranged from 0.3198 (in Aquapri's Excellence fish) to 0.7163 (in Aquapri's Hungarian fish).

A wide range of F_{IS} values were observed in the 13 populations analyzed. In principle, **positive** F_{IS} values indicate that individuals in a population are **more related** than you would expect under a model of random mating, whereas **negative** F_{IS} values indicate that individuals in a population are **less related** than you would expect under a model of random mating. The F_{IS} values are high and significant for Halaszati Kft (0.068), Aquapri's Mosso (0.0455) and ASIALOR (0.0658) samples. Such deviations from Hardy Weinberg equilibrium (HWE) may be due to i) the Wahlund effect, i.e. the reduction in the overall heterozygosity of a population as a result of subpopulation structures (that means if two or more subpopulations have independent allele frequencies then the overall heterozygosity is reduced, irrespective of whether those subpopulations are in Hardy-Weinberg equilibrium), ii) non-panmixia (inbreeding, groupings of relatives, selection against heterozygotes) or iii) to genotyping errors (null alleles and other scoring errors).

Inbreeding seems an explanation in domesticated and non-random mating is also likely in our case, as deficits were homogeneous among loci (all significant and all non-significant F_{IS} values). Selection against heterozygotes cannot be demonstrated from our results; although microsatellite loci are typically recognized as neutral genetic markers, it is possible that one or more loci are linked to genes or gene groups under selection. The Walhund effect could also explain the deficit of heterozygotes due to the mixing of genetically variable populations to form a new domesticated stock, which might be the case in some aquaculture companies' practices. Moreover, one of the above microsatellite loci (PflaL3) showed significant probability ($P > 0.05$) of "large allele dropout" or "stuttering". However, when this locus is excluded from the analysis the F_{IS} values are slightly changed but remain significant in any case.

Finally, F_{ST} values are frequently used as a summary of genetic differentiation among groups. It depends on the allele frequencies at a locus, showing specific properties linked to genetic diversity: higher values for biallelic single-nucleotide polymorphisms (SNPs) than for multi-allelic microsatellites, low values among high-diversity populations viewed as substantially distinct, and low values for populations that differ primarily in rare alleles. Due to these reasons, several authors argued that F_{ST} measures may be poor measures of genetic differentiation when the level of diversity is high. Estimated population differentiation across samples using the F_{ST} estimate by Weir & Cockerham's (1984) showed that the smallest values were between Hungarian samples (wild-1 and domesticated-2) and also showed (as expected) a close relationship (F_{ST} <0.11) between the above two populations with the Aquapri's Hungarian one (population 6). There was also a close relationship of Aquapri's VanMecklen (population 6) with the Czech population from the same company and that from IfB Potsdam (F_{ST} <0.15). Lastly, a close relationship was found between ASIALOR and INAGRO's Belgian samples (F_{ST} =0.14) and Aquapri's Mosso sample with that from INAGRO's Dutch samples (F_{ST} =0.16).

The full description of the work and results with domesticated broodstocks has been provided in *Deliverable* ***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).

The primary objective of this task was to use the microsatellite multiplex tools developed previously for the species in task 4.1 and evaluate the genetic variability of some wild pikeperch populations. Population genetics parameters from wild stocks are compared to those of captive broodstock in commercial RAS farms around Europe with the objective to define how a future genetic breeding program should be established for sustainable optimal performance through domestication of pikeperch. In addition to the thirteen cultured populations analyzed in D4.1, eight more populations were genotyped of which one was domesticated (Sweden). Therefore, current results refer to a total of 21 populations and more than 950 fish (**Table 4.2.1**). The Qiagen multiplex PCR kit was used for PCR with the two multiplexes (7-plex and 4-plex).

Table 4.2.1 List of the 21 domesticated and wild pikeperch populations, and number of fish per sample that were genetically analyzed; populations marked in blue were of wild origin and in red were cultured origin.

	Population	Sample Size
1	**Gyori Elore, HTSZ, Hungary**	53
2	Szabolsi, Halaszati Kft, Hungary	49
3	Aquapri A/S Denmark, VanMecklen, Netherlands	54
4	Aquapri A/S Denmark, Czech Rep.	38
5	Aquapri A/S Denmark, Excellence fish, Netherlands	14
6	Aquapri A/S Denmark, Hungary	73
7	Aquapri A/S Denmark, Mosso, Denmark	19
8	IfB Potsdam, Germany	46
9	FGFRI Kainuu fisheries research station, Finland	31
10	FGFRI Laukaa Fish Farm, Finland	20
11	ASIALOR, France	63
12	INAGRO Belgium, German origin	100
13	INAGRO Belgium, Dutch origin	100
14	**Tunisia**	59
15	Svensk Fiskodling AB, Hjalmaren Lake, Sweden	30
16	**Dom de Lindre, France**	51
17	**URAFPA-DAC, Czech Rep.**	70
18	**Sarag L., Poland**	14
19	**Wymoj L., Poland**	11
20	**Oulujarvi L., Finland**	32
21	**Hiidenvesi L., Finland**	31

Basic population genetics parameters & Cross-species microsatellite transferability

Considering a long term breeding program, it is fundamental to ensure sufficient genetic variation within populations, as this determines the potential for selection of desired traits or of adaptation to hostile changes in environmental /rearing conditions. In domesticated stocks, caution is required because the loss of genetic variability within the first generations of breeding practices limits the potential for future genetic improvement from selection practices.

Basic population genetics parameters (allelic richness, heterozygosity indices, inbreeding coefficients) were calculated for both wild and domesticated stocks. The total number of alleles per locus ranged from 8-9 (PflaL3 and PflaL9, respectively) to 23 (Svi4) (**Table 4.2.2**). Therefore, microsatellite loci showed relatively high levels of polymorphism even though some samples were monomorphic (exhibited only one allele) for some loci such as for Za199 and PflaL9 in the "Excellence fish" of Aquapri A/S (population 5), locus Za237 in Kainuu Fisheries Research Station (population 9), Za144 in Laukaa Fish Farm (population 10), Za024 in the Tunisia (population 14) and Pfla3 in Sweden (population 15). The Tunisian population (population 14), was the only wild population that appeared to have one allele at a locus (Za024). Moreover, the microsatellite loci used were developed in other phylogenetically close species and the polymorphism appeared to be related to the species from which the microsatellite originated. The most polymorphic were the loci from *Stizostedion vitreum* (Zvi, 2 loci average 21 alleles), followed by those from *Zingel asper* (Za, 7 loci average 14.85 alleles) and last from *Perca flavescens* (Pfla, 2 loci average 8.5 alleles). This was consistent with recent trends in taxonomy that proposed *Stizostedion* to be grouped into *Sander* genus and that *Zingel* species are the closest to this *Stizostedion-Sander* group, before the perches of the genera *Perca* (*P. fluviatilis, P. schrenkii* etc) and *Gymnocephalus* (GenBank taxonomy sequence sources). [*Sander lucioperca* was first described as *Stizostedion lucioperca* by Linnaeus, 1758]

For the 21 populations analyzed, the least number of alleles was encountered in Aquapri's VanMecklen (2.6), Aquapri's "Excellence fish" and Laukaa Fish Farm (2.8), Aquapri's "Mosso fish" (3.1), and the greatest in Hungarian Aquapri's (8.2), Halaszati Kft (7.8) and Inagro's German (7.2) stocks, which were greater than that in all wild stocks (3.7 to 6.2). Likewise, expected heterozygosity (H_E) ranged from 0.3408 in Aquapri's Excellence fish to 0.7194 in Aquapri's Hungarian fish; the later population showed higher values than those in the wild populations (**Table 4.2.3**).

Table 4.2.2 Number of alleles per locus; populations numbers follow those in **Table 4.2.1**.

Population	Locus										
	PflaL3	Svi18	Za199	Za138	PflaL9	Svi4	Za024	Za038	Za144	Za207	Za237
1	4	6	5	9	3	7	7	6	8	4	7
2	5	12	5	14	4	6	6	8	8	6	9
3	3	3	2	3	2	4	2	3	3	2	2
4	3	4	3	3	3	5	3	4	3	3	2
5	2	4	1	2	1	5	2	4	4	3	2
6	5	13	6	13	3	8	7	9	8	6	9
7	2	4	3	2	4	5	2	3	3	3	2
8	2	4	4	7	5	6	6	4	9	7	5
9	3	4	4	4	3	5	4	4	4	4	1
10	2	3	3	2	5	4	3	3	1	2	2
11	5	7	7	6	4	5	4	4	8	6	3
12	4	9	10	9	6	7	5	5	9	6	6
13	5	5	7	7	5	5	3	3	4	4	4
14	3	5	6	4	2	3	1	3	6	4	3
15	1	3	7	4	4	7	4	6	3	3	3
16	5	6	5	5	4	6	3	4	6	4	3
17	3	5	4	6	3	5	3	4	3	3	2
18	5	6	7	4	4	4	4	5	5	4	3
19	3	4	7	4	4	3	4	4	4	5	3
20	4	5	8	5	4	6	5	4	3	4	4
21	5	5	5	6	5	7	5	4	4	3	3
Total No. Alleles	**9**	**19**	**16**	**20**	**8**	**23**	**14**	**13**	**17**	**11**	**13**

Table 4.2.3 Basic population genetics parameters for all populations analyzed: mean number of alleles per locus, observed (H_O) and expected heterozygosity (H_E), and F_{IS} calculated in GENETIX v. 4.05 (Belkhir et al., 2004). Asterisks indicate significance at p=0.05**.** Populations marked in blue were of wild origin and in red were cultured origin.

	Population	Sample Size	Mean Nb of alleles	H_E	H_O	F_{IS}
1	**Gyori Elore, HTSZ, Hungary**	53	6.2	0.6826	0.7472	-0.08424
2	Szabolsi, Halaszati Kft, Hungary	49	**7.8**	0.7182	0.6759	0.06962*
3	Aquapri A/S Denmark, VanMecklen, Netherlands	54	2.6	0.4675	0.6796	-0.44607
4	Aquapri A/S Denmark, Czech Rep.	38	3.3	0.4616	0.4882	-0.04401*
5	Aquapri A/S Denmark, Excellence fish, Netherlands	14	2.8	0.3408	0.4100	-0.16229
6	Aquapri A/S Denmark, Hungary	73	**8.2**	0.7194	0.7165	0.01110*
7	Aquapri A/S Denmark, Mosso, Denmark	19	3.1	0.4169	0.3985	0.07185*
8	IfB Potsdam, Germany	46	5.7	0.5567	0.5502	0.02343*
9	FGFRI Kainuu fisheries research station, Finland	31	3.7	0.5257	0.5819	-0.09055
10	FGFRI Laukaa Fish Farm, Finland	20	2.8	0.4743	0.6032	-0.24757
11	ASIALOR, France	63	5.4	0.5940	0.5913	0.01261
12	INAGRO Belgium, German origin	100	**7.2**	0.7224	0.8099	-0.11621*
13	INAGRO Belgium, Dutch origin	100	4.7	0.6156	0.6465	-0.04510
14	**Tunisia**	59	3.7	0.4013	0.3585	0.11512*
15	Svensk Fiskodling AB, Hjalmaren Lake, Sweden	30	4.4	0.5250	0.5817	-0.08989
16	**Dom de Lindre, France**	51	4.6	0.5923	0.6706	-0.12237
17	**URAFPA-DAC, Czech Rep.**	70	3.8	0.4692	0.4382	0.07357*
18	**Sarag L., Poland**	14	4.6	0.5763	0.5643	0.05780*
19	**Wymoj L., Poland**	11	4.2	0.6149	0.6764	-0.05217*
20	**Oulujarvi L., Finland**	32	4.8	0.5946	0.5995	0.00787*
21	**Hiidenvesi L., Finland**	31	4.7	0.6034	0.5340	0.13148*

On average, domesticated populations exhibited a slightly higher number of alleles (2.634 versus 2.58, not significantly different with an F-test) (**Fig. 4.2.1**) and amongst the domesticated samples there were populations that were more polymorphic than any wild population [population 2 (from Szabolsi, Halaszati Kft, Hungary), population 6 (Aquapri's Hungarian) and population 12 (INAGRO's from Germany)]. Likewise, unbiased Expected Heterozygosity Estimates were slightly higher in wild population (0.573 versus 0.553, but again not significantly different with an F-test) (**Fig. 4.2.2**); values for wild populations were lower than 0.69 (in the first Hungarian one), whereas the three above mentioned domesticated stocks (populations 2, 6 and 12) showed values above 0.70.

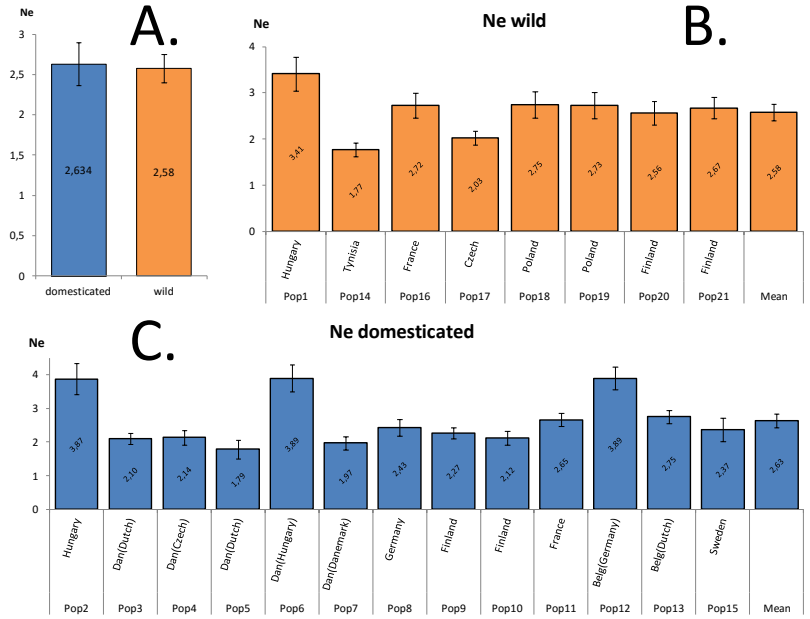


Figure 4.2.1 Mean number of alleles for domesticated and wild populations (A) and for each one separately (B & C).

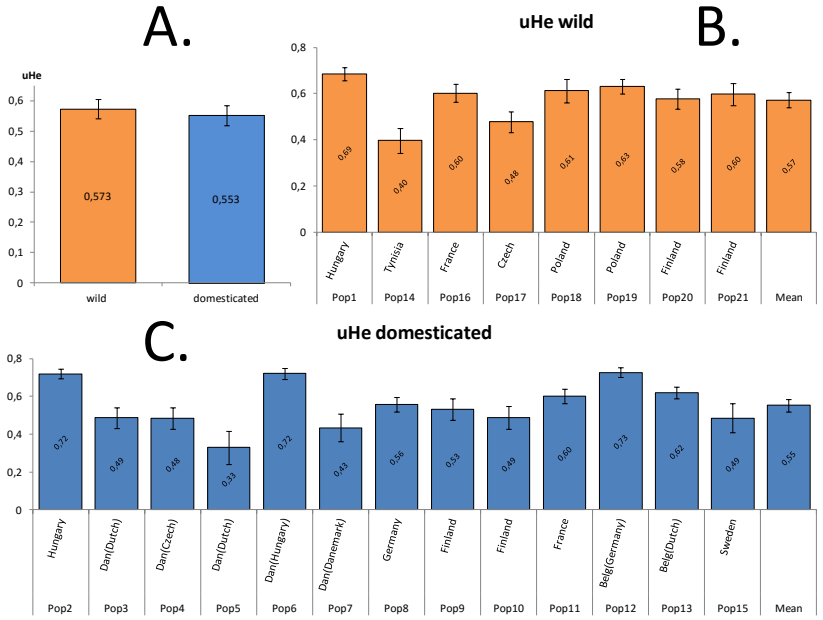


Figure 4.2.2 Estimates of Unbiased Expected Heterozygosity (uHe) for domesticated and wild populations (A) and for each one separately (B & C).

A wide range of F_{IS} values were observed in the 21 populations analyzed (**Tables 4.2.3** and **4.2.4**). In principle, **positive** F_{IS} values indicate that individuals in a population were **more related** than expected under a model of random mating, whereas **negative** F_{IS} values indicate that individuals in a population are **less related** than expected under a model of random mating. The F_{IS} values were high and significant for Hiidenvesi L. in Finland (0.131), in Tunisia (0.115), Halaszati Kft (0.069), Aquapri's Mosso (0.071) and almost all wild samples. Such deviations from Hardy Weinberg equilibrium (HWE) may be due to i) the Wahlund effect, *i.e.*, the reduction in the overall heterozygosity of a population as a result of subpopulation structures (that means if two or more subpopulations have independent allele frequencies, then the overall heterozygosity is reduced, irrespective of whether those subpopulations are in Hardy-Weinberg equilibrium), ii) non-panmixia (inbreeding, groupings of relatives, selection against heterozygotes) or iii) to genotyping errors (null alleles and other scoring errors).

Table 4.2.4 F_{IS} values per locus for the 21 pikeperch populations genotyped for 10 loci (locus PflaL3 is excluded due to null alleles); populations numbers follow those in **Table 4.2.1**.

Population	Svi18	Za199	Za138	PflaL9	Svi4	Za024	Za038	Za144	Za207	Za237	All
1	-0.409	0.064	-0.032	-0.242	0.003	0.022	0.024	-0.116	-0.161	0.010	-0.084
2	0.199	-0.202	0.330	0.038	-0.061	0.038	-0.057	0.069	0.017	0.230	0.070
3	-0.105	-0.377	-0.615	-0.293	-0.341	0.057	-0.603	-0.640	-1.000	0.000	-0.446
4	0.078	0.641	-0.091	0.395	-0.110	0.193	-0.341	0.037	-0.289	-0.158	-0.044
5	-0.305	NA	0.000	NA	-0.368	1.000	-0.051	-0.300	-0.189	-0.048	-0.162
6	-0.045	-0.013	-0.015	0.021	0.133	0.049	-0.075	0.007	0.088	-0.021	0.011
7	-0.003	-0.094	0.000	0.344	-0.485	0.000	0.402	0.465	-0.138	-0.029	0.072
8	-0.302	-0.001	0.271	0.182	0.150	0.100	-0.005	-0.114	-0.056	-0.161	0.023
9	-0.091	-0.192	-0.211	0.150	-0.122	0.117	-0.291	0.094	-0.230	NA	-0.091
10	-0.137	-0.238	-0.440	-0.080	-0.526	-0.109	-0.302	NA	-0.188	-0.166	-0.248
11	-0.389	0.013	0.303	0.206	0.644	-0.309	0.138	-0.103	0.022	-0.007	0.013
12	0.140	-0.199	-0.060	-0.175	-0.192	0.006	0.109	-0.043	-0.336	-0.471	-0.116
13	-0.217	0.024	-0.177	0.125	-0.004	0.121	-0.109	-0.008	-0.019	-0.186	-0.045
14	-0.169	0.121	0.022	0.269	0.064	NA	-0.056	0.273	0.316	0.172	0.115
15	-0.357	-0.108	-0.102	0.354	-0.306	-0.093	-0.111	-0.211	0.254	0.156	-0.090
16	-0.093	-0.220	-0.042	0.072	-0.130	-0.234	-0.172	-0.196	-0.085	-0.091	-0.122
17	-0.139	0.211	0.186	0.673	0.126	0.096	0.024	0.078	-0.095	0.056	0.074
18	0.227	0.167	-0.111	0.343	-0.324	0.150	-0.167	-0.087	0.122	0.328	0.058
19	-0.119	-0.087	0.209	0.259	-0.500	0.104	-0.240	-0.224	0.080	-0.022	-0.052
20	-0.060	-0.130	0.056	0.243	0.028	-0.133	0.055	0.222	-0.019	-0.221	0.008
21	0.077	0.251	0.005	0.235	0.057	0.221	0.105	0.024	0.128	0.223	0.131

Inbreeding seems an explanation in domesticated and non-random mating is also likely in our case, as deficits were homogeneous among loci (all significant and all non-significant F_{IS} values). Selection against heterozygotes cannot be demonstrated from our results; although microsatellite loci are typically recognized as neutral genetic markers, it is possible that one or more loci are linked to genes or gene groups under selection. The Walhund effect could also explain the deficit of heterozygotes due to the mixing of genetically variable populations to form a new domesticated stock, which might be the case in some aquaculture companies' practices.

Finally, F_{ST} values are frequently used as a summary of genetic differentiation among groups. It depends on the allele frequencies at a locus, showing specific properties linked to genetic diversity. Population differentiation was estimated across samples using the F_{ST} estimate by Weir & Cockerham's (1984) (**Table 4.2.5**). The smallest F_{ST} estimate values were between the two wild Finnish samples (0.021) and the Finno-scandinavian (wild and domesticated) samples in general (F_{ST} <0.18). Next, a close relationship was

observed (F_{ST} <0.11) between the two Hungarian populations with the Aquapri's Hungarian one (population 6) (**Table 4.2.5**). Also a close relationship was observed between the two Czech populations (F_{ST} =0.03 between Aquapri's population and the wild one) and that the two German ones (F_{ST} =0.16 between IfB Potsdam and Inagro's). Lastly, a close relationship was observed between the wild sample of Domaine de Lindre and INAGRO's German samples (F_{ST} =0.11) and Aquapri's Mosso sample with the wild from Wymoj L. in Poland (F_{ST} =0.087).

All results mentioned above based on F_{ST} values can also be visualized based on a Factorial Correspondence Analysis graph using the GENETIX v. 4.05 (Belkhir et al., 2004) software (**Fig. 4.2.3**).

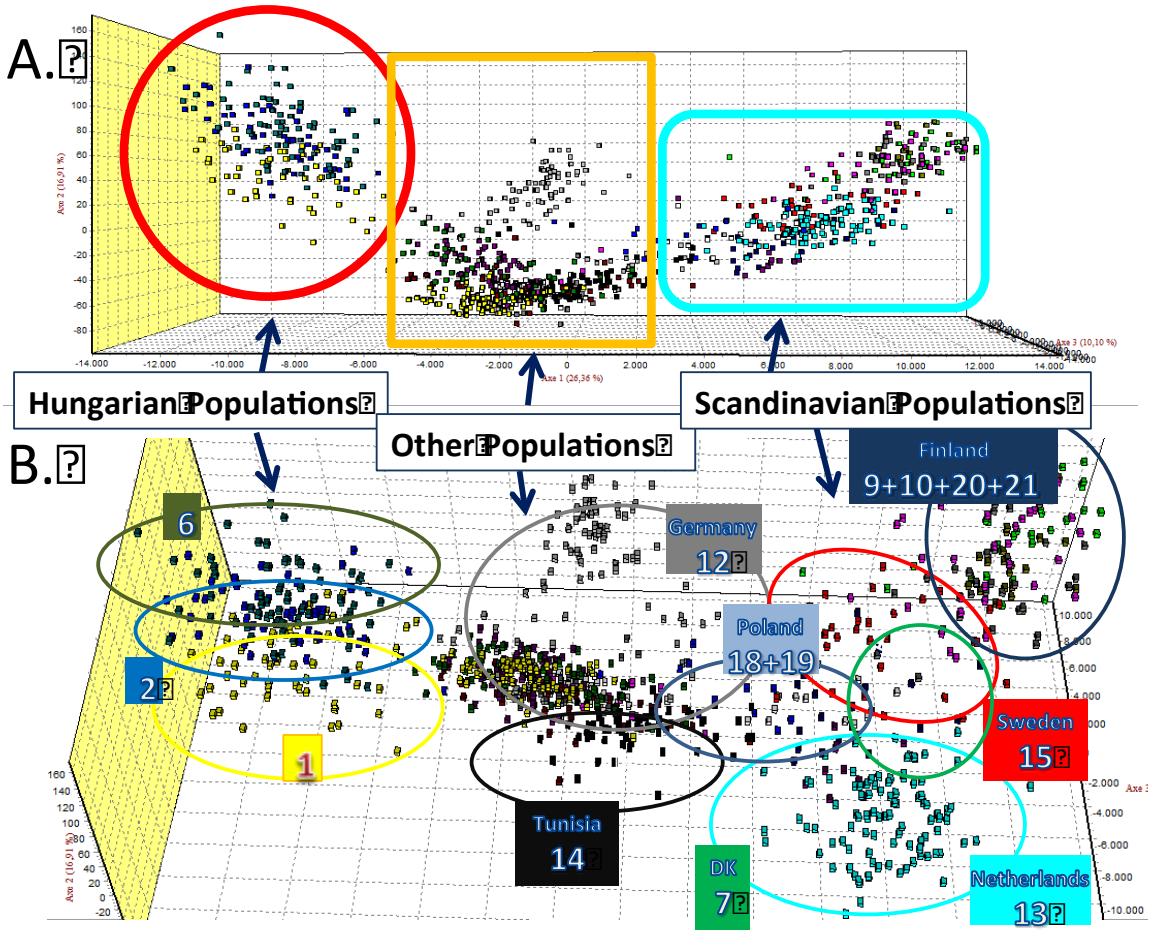


Figure 4.2.3 Factorial Correspondence Analysis (FCA) for all twenty-one populations and ten loci using the GENETIX v. 4.05 (Belkhir et al., 2004) software; populations numbers follow those in Tables 4.2.1.

Structure Analysis

Admixture analysis with STRUCTURE suggested a k value of two as the most likely number of existing clusters based on the Δ*K* criterion (**Fig. 4.2.4**). The first cluster comprises all the populations from Netherlands/Denmark/Poland and northwards to Sweden/Finland (light blue in **Fig. 4.2.5** for K=2) and the second one all the remaining populations (red in 4.2.5 for K=2). If we progressively take into account three and four clusters groupings in **Fig. 4.2.5**, the two above mentioned clusters are further divided to a Scandinavian Sweden/Finland cluster (dark blue for K=4) and a Hungarian one (red for K=4), respectively.

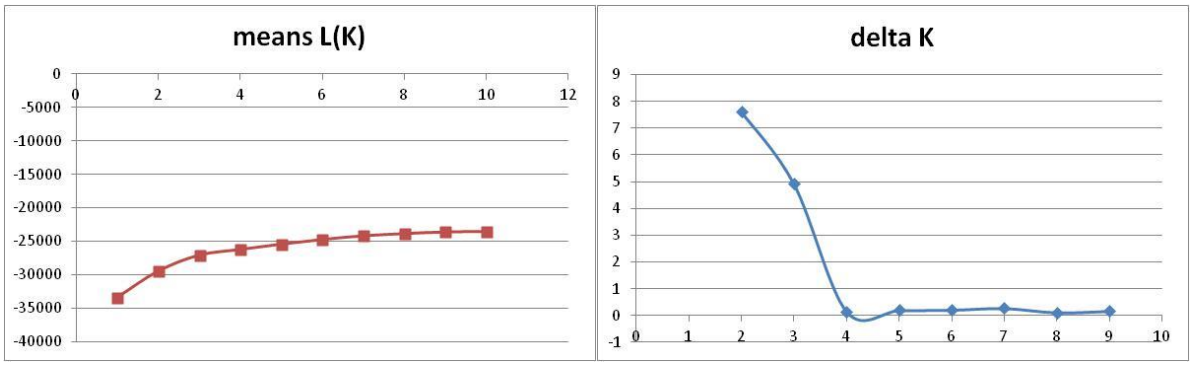


Figure 4.2.4 Admixture analysis revealed two genetic clusters as the most likely number, as indicated by a decrease in likelihood (A) and an increase in variance of calculated probabilities Δ(K) (B) Determination of the number of clusters (*K*) including all 10 repetitions for each *K* without geographical area as a prior. The highest peak denotes the most likely number of clusters according to the Pritchard Bayes formula. PD: probability of data.

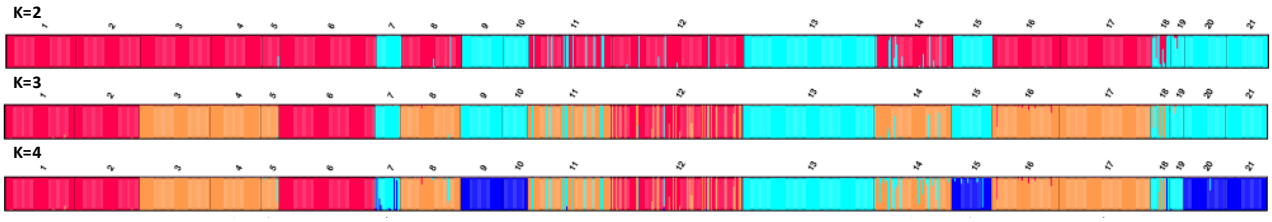


Figure 4.2.5 Bayesian individual assignment implemented in STRUCTURE for *K* = 2, 3 and 4 clusters without using geographical area as a prior. The *y*-axis represents the probability of assignment of an individual to each cluster and each colour. Population numbers follow those in **Tables 4.2.1**.

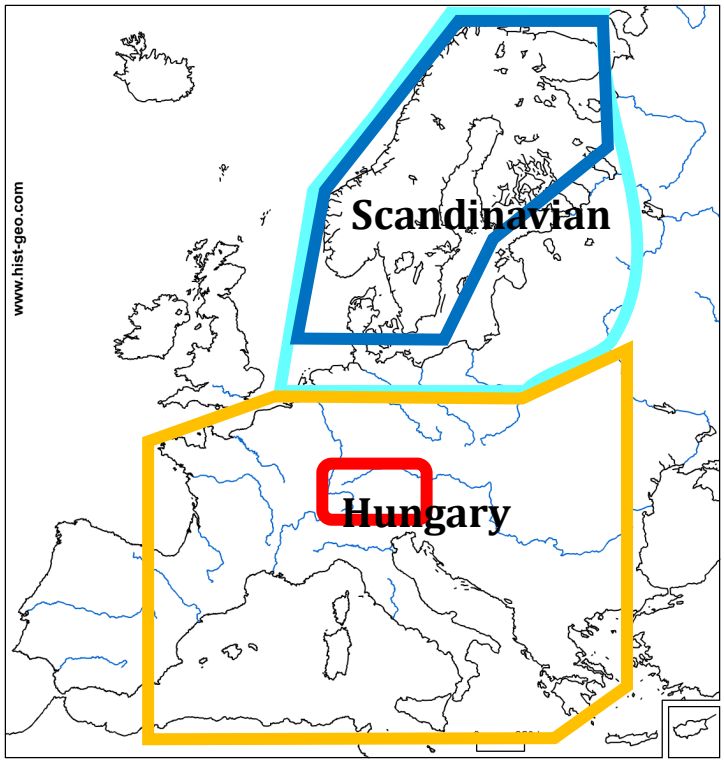


Figure 4.2.6 Map of Europe showing the major pikeperch genetic groups; colours follow those in **Fig. 4.2.5**.

The full description of the work and results with identified broodstocks is provided in the *Deliverable* ***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 second 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)			

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 progress towards objectives and details for each task (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 actually few differences between fecundity, fertilisation, hatching, egg size and hormone content between eggs from wild-caught and farmed females. However, although there were no 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.

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

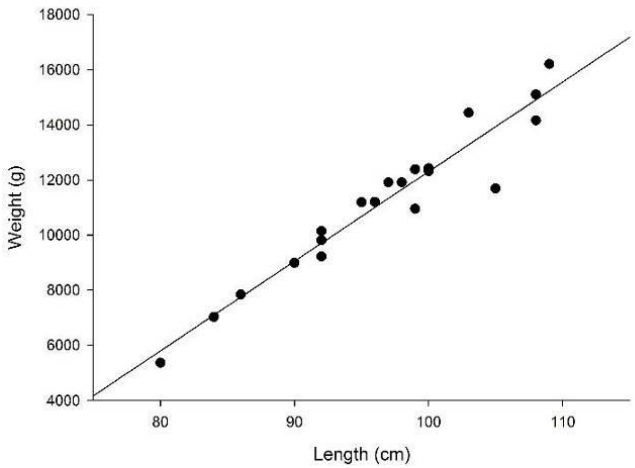
Introduction

Even though empirical data suggest a significant difference in spawning performance between wild-captured and farmed Atlantic halibut females, there currently is a lack of systematic documentation. 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-captured 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. There is, however, a lack of thoroughly documented evidence to support the hypothesis of such a reproductive dysfunction in farmed females. Consequently, reproductive performance of domesticated, wild-caught halibut and farmed (F1) females was compared. In addition, performance was documented and compared in four groups of F1 broodstock that were photo-manipulated to spawn at four different times of year.

Materials and methods

2015

Based on the information gathered in the first year of the project, one group of domesticated, wild-captured breeders, held in captivity for at least four years (n=4), and one group of farmed females (F1; n=5) were closely monitored during the spawning season of 2015 (February-April). All individual females were measured in January, before the start of the breeding season. Their weights were estimated based on our previous measurements of length and weight in farmed female Atlantic halibut (**Fig. 5.1.1**) and the weight-length chart provided for Pacific halibut by the International Pacific Halibut Commission (http://www.iphc.int/publications/bulletins/lenwtmet.pdf).



Eggs were stripped and fertilized according to previously described procedures (*e.g.* Norberg et al., 1991). The following parameters were recorded: egg batch volume, fertilization rate, batch interval (hours between ovulations), number of batches, total and relative fecundity. Fertilization rate was calculated on a subsample of ~200 eggs, using a dissecting Microscope (Leica Wild M10). Egg yolk content of cortisol and testosterone was analysed by Enzyme-linked immunoassay (ELISA) (Cuisset et al., 1994) in unfertilized eggs from all collected batches. Egg samples were frozen and kept at -80°C until analysis.

Figure 5.1.1. Weight-length relationship in female Atlantic halibut of the 2007 year-class at P7. IMR, Austevoll Research Station (r^2=0.925; P<0.0001)

2016

Based on the results obtained in 2015, egg characteristics (fertilization, diameter, cell division symmetry) and hatching success were compared in selected egg batches from the same three domesticated and five farmed females that were used the previous year.

Female Atlantic halibut, either wild-caught and domesticated for at least five years (n=3), or farmed, F1 generation individuals (n=5) were monitored for ovulation, strip-spawned and fertilized during the spawning season of 2016 (February-April), as described above. As the timing of stripping of the first two batches may not be optimal, the third or fourth egg batch was used in order to ascertain a high fertilization rate. Egg size and dry weight has been documented to decrease over time in batch spawners, so that eggs from the last batches are smaller than eggs from the first (Kjesbu et al., 1996). This made it necessary to use eggs from similar batch numbers, in order to make a valid size comparison.

Fertilized eggs were photographed at the 8-16 cell stage using a dissecting microscope and dark field (Leica MS5; 113.23 pixels/mm), for measurements of egg diameter and blastomere symmetry. Egg diameters were measured automatically in ImageJ (http://rsb.info.nih.gov/ij/) using custom made plug-in and macros http://simon.bio.uva.nl/ObjectJ/objectj.html). Eggs were classified according to the following scale:

1. Fertilized, OK
2. Dead
3. Asymmetric cell division
4. From previous batch
5. Unfertilized

Hatching rate was determined in the third or, in three cases, fourth egg batch collected from each female. Eggs were incubated at 6°C under standard hatchery procedures (Mangor-Jensen et al., 1998) for 72 day-degrees (11 days at 6°C). For calculation of hatching percentage, 300 eggs were collected and divided into three beakers containing 500 ml of sterile-filtered seawater (salinity 35‰, temperature 6°C) and incubated in darkness at 6°C for 72 hours. Hatched larvae and dead eggs were counted in a binocular microscope (Leica MS5). Larvae were also photographed in a dissecting microscope (Leica MS5, dark field), in order to document any possible aberrations from normal development.

Validation of ELISA for analysis of halibut egg yolk concentration of cortisol and testosterone

Samples of unfertilised eggs were collected from all batches, and were frozen and stored at -80°C until extraction. For steroid extraction, 500 µg of eggs were homogenized by ultrasonication. The homogenate was centrifuged at 14000x*g* for 2 min. Steroids were extracted from 200 µl of the supernatant as described by Pankhurst and Carragher (1992) and Kleppe et al. (2013). Extraction efficiency was determined by addition of a known amount of ^{3}H labelled steroid to the egg homogenate and was 67% for cortisol and 68% for testosterone. Final steroid concentrations were corrected in relation to extraction efficiency. Logit-log binding curves of serial dilutions of steroid standards and plasma samples were parallel showing that extracted egg samples were suited to the assay conditions. The ED80 and ED20 were 0.004 ng ml^{-1} and 0.08 ng ml^{-1} for testosterone (T), and 0.07 and 1.2 ng ml^{-1} for cortisol. Detection limits of the assays were 0.008 ng ml^{-1} for testosterone, and 0.07 ng ml^{-1} cortisol. Intra-assay variation was 6.3% for testosterone (n=6) and 5.4% for cortisol (n=7). Inter-assay variation was 3.4% for testosterone (n=2) and 6.4% for cortisol (n=3). Testosterone and cortisol 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-reactivity for testosterone and cortisol antisera are described by the manufacturer.

Results and Discussion

Monitoring of spawning cycles, fecundity and egg viability

The broodstock females consisted of 9 individuals -4 domesticated, wild-caught individuals and 5 farmed females of the F1 generation. The farmed females were second-time spawners, *i.e.* they matured and spawned for the first time in 2014. One domesticated female (named "Frida") developed a large skin lesion and was left undisturbed after the third stripping, due to animal welfare concerns. This individual was not included in the calculations of spawning performance. Biometric data and details on spawning performance are summarized in **Table 5.1.1**. Overall, the wild-domesticated females appeared to spawn fewer, larger egg batches with higher and more stable fertilization success. Relative fecundity, measured as amount of eggs in relation to body weight, did not differ between the two groups. Careful monitoring and timing of stripping, as close to ovulation of the whole batch as possible, was necessary in order to obtain high fertilization of eggs (by avoiding over-ripening). In cases where the whole egg batch could not be strip-spawned, domesticated females generally released the remaining eggs into the tank. Farmed females, in apparent contrast, tended to leave a small "residue" of eggs, typically 100-250 ml, which were held in the ovary. These eggs had to be stripped 6-12 hours after the main batch so that the overripe residue would not have a negative impact on the viability of the next, maturing cohort. Once this was established as an additional routine in strip-spawning eggs from farmed females, the fertilization success stabilized at levels above 75-80% in most individuals, with occasional batches having up to 90-94% fertilization.

Table 5.1.1. Biometric and spawning performance data of domesticated and farmed halibut breeders at IMR, Austevoll.

	Domesticated females	Farmed (F1) females
n	3 (4*)	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

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

Egg size and cell division symmetry

Egg diameters were in the range of 2670 to 3700 µm, with average diameters from 3013 to 3104 µm. There were no significant differences in egg diameter between any of the individual fish. When eggs were characterized according to development, most were either in category 1 (Fertilized, OK) or 2 (dead). Very few of the fertilized eggs showed signs of asymmetric cell division, and most eggs appeared to be cleared from the ovarian cavity when the batch was spawned (**Fig 5.1.2**).

Hatching percentage and development

Fertilization and hatching percentages are summarized in **Table 5.1.2**. Overall, eggs from wild-domesticated females appeared to have higher fertilization and hatching percentages, with less individual variation than farmed females. Due to high variation between egg groups from farmed females and a small number of

females used, this was not considered to be statistically significant. One farmed female, in particular, spawned eggs consistently with high (>90%) fertilization and hatching success (data not shown).

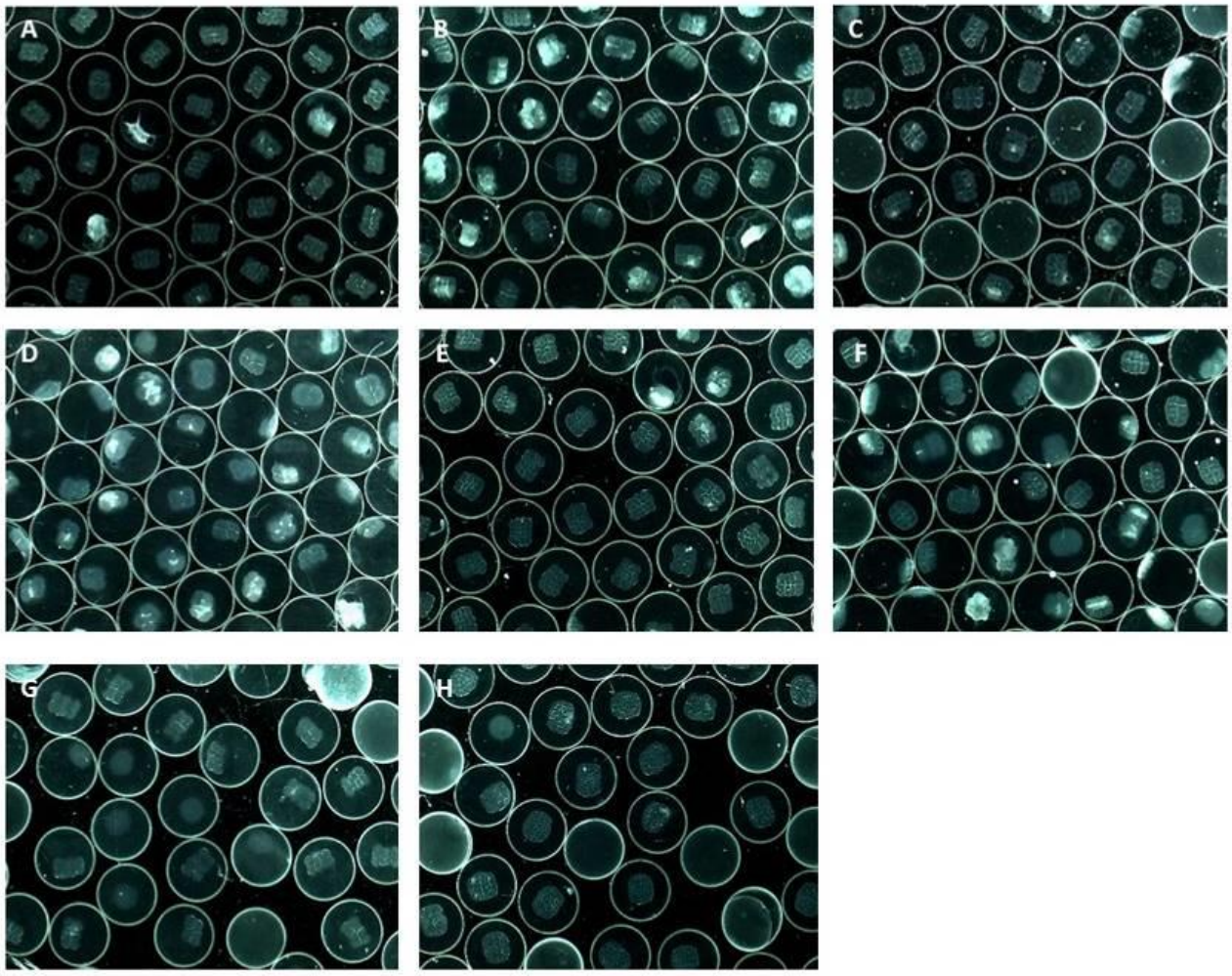


Figure 5.1.2 Fertilized eggs at the 8-16 cell stages from domesticated (A-C) and farmed (D-H) Atlantic halibut.

Table 5.1.2 Fertilization and hatching of eggs from domesticated and farmed Atlantic halibut females.

	Domesticated females	Farmed females
n	3	5
Average fertilization (%)	91.3 ± 5.7	78.4 ± 13.7
Average hatching (%)	97.3 ± 0.6	76.0 ± 10.9

Some differences were observed between eggs from the different females. eggs from farmed females generally appeared heavier, and would sink to the bottom of the incubator/beaker, while eggs from

domesticated females remained buoyant near the surface. In addition, dead or deformed larvae were observed more frequently when eggs from farmed females hatched (**Fig. 5.1.3)**. It is not clear what caused the deformities, but one possible cause may be mechanical damage of the heavy eggs, that sank and rested at the bottom of the beaker for two days. Further work is needed, however, in order to establish whether this is the cause or if there are genetic/epigenetic factors that may contribute to a higher rate of deformities in larvae from those females.

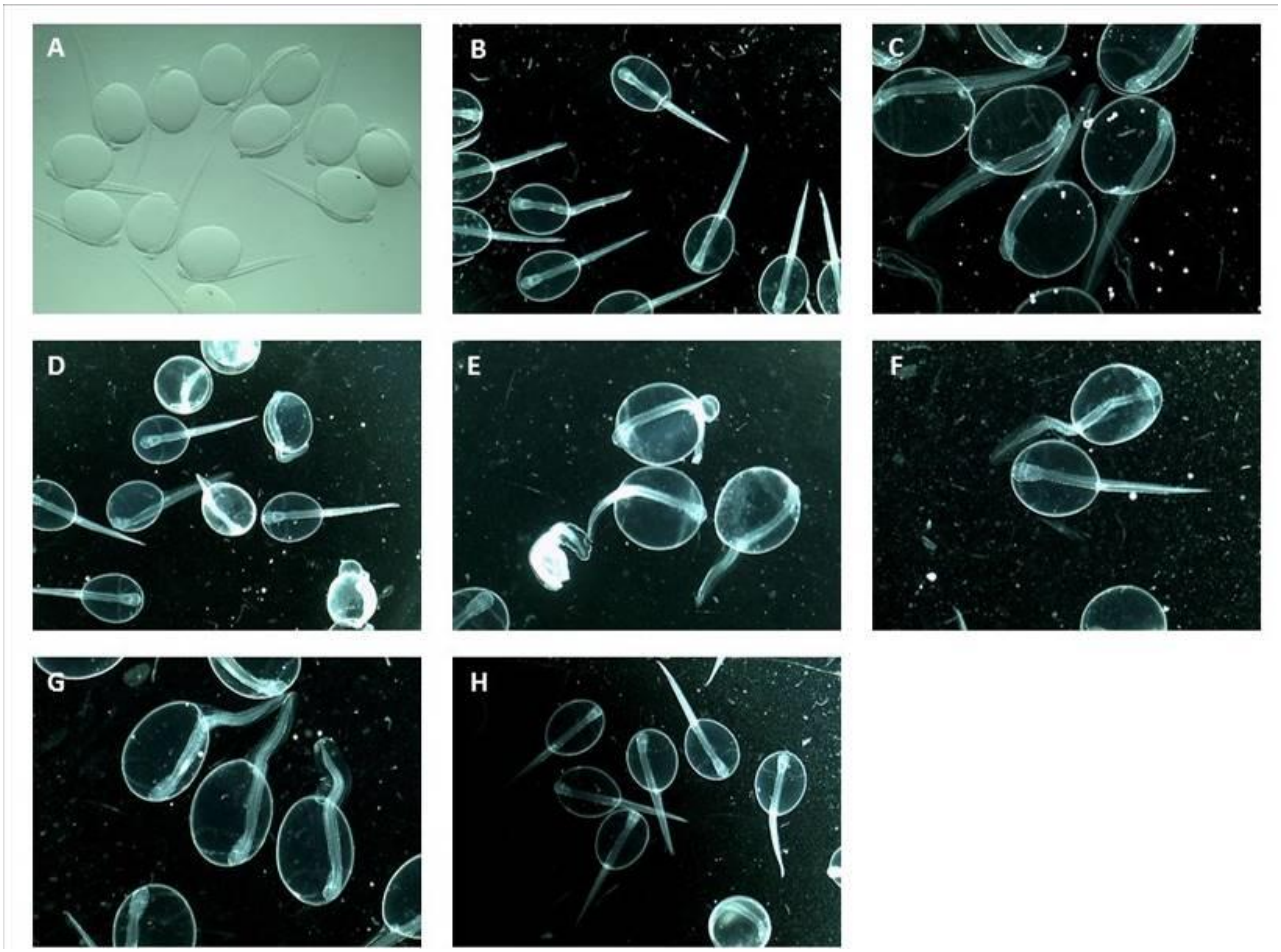


Figure 5.1.3 Newly hatched larvae from domesticated (A-C) and farmed Atlantic halibut females. Note spinal deformities in E, F and G.

Egg steroid content

No significant difference was found in average egg steroid content between domesticated and farmed female Atlantic halibut (**Fig. 5.1.4**.). There was a general trend towards decreasing egg content of steroids through spawning. Testosterone levels were low, in the range of 0-11 $pg \cdot egg^{-1}$, especially towards the end of spawning (**Fig. 5.1.5.A**). Egg cortisol content varied between females, but also between egg batches (**Fig. 5.1.5.B**). Overall, egg cortisol content was high, especially in farmed females, ranging from 110 to 450 $pg \cdot egg^{-1}$.

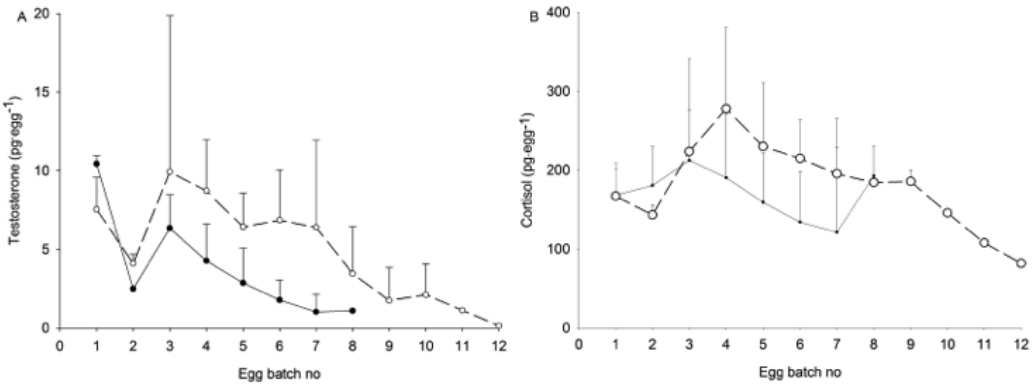


Figure 5.1.4. Average content of testosterone (A) and cortisol (B) in eggs from domesticated and farmed Atlantic halibut broodstock. Domesticated females: Black symbols, solid lines. Farmed females: Open symbols, dashed lines.

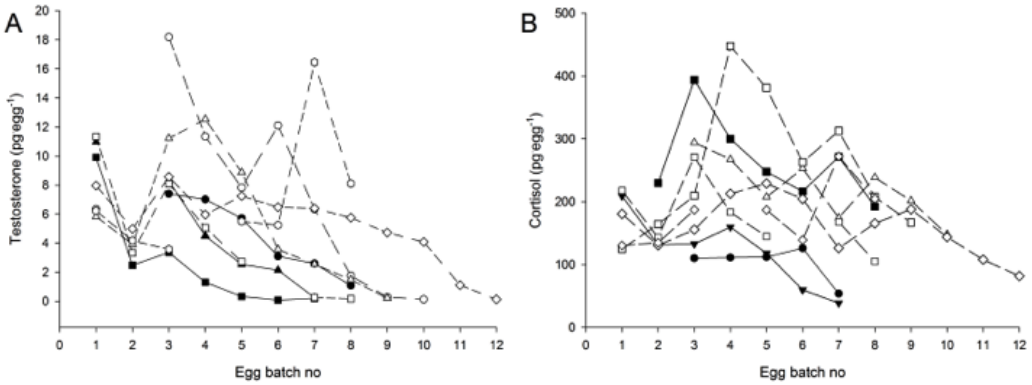


Fig 5.1.5 Individual profiles of testosterone (A) and cortisol (B) content in eggs from domesticated and farmed Atlantic halibut. Domestic females: Black symbols, solid lines. Farmed females: Open symbols, dashed lines.

The decrease in egg T content observed in most females through the spawning season is in accordance with previously reported changes in plasma concentration of the breeders (Methven et al. 1992, Björnsson et al., 1998). As spawning progresses, the capacity for sex steroid synthesis also becomes lower due to a reduced number of steroid-producing follicle cells. A decrease in egg T content over time would suggest that there is a passive influx of steroids during oocyte maturation, rather than an active sequestration.

Overall, cortisol content was high in Atlantic halibut eggs, but appeared to decrease towards the end of spawning. In a recently published study (Skaalsvik et al., 2015), a similar pattern was found and furthermore, high cortisol content was correlated with high occurrence of yolk sac edema. Cortisol implants in female Atlantic cod (*Gadus morhua*) resulted in increased cortisol concentrations in plasma, oocytes and eggs, but did not affect fertilization, cell division or hatching (Kleppe et al., 2013). However, genes linked to important developmental processes were differentially expressed in oocytes and blastula embryos in response to cortisol. Although no effects were detected in cod egg/embryo viability up until hatching, effects may appear later in development. Our study was limited to egg quality parameters and hatching rate, but

future work should address the possible impact of high egg concentrations of cortisol on Atlantic halibut larval development.

Commercial application at P22. SWH

Materials and methods

Sterling White Halibut (Partner 22. SWH) no longer uses wild-caught broodstock, but only farmed females. All females used were established spawners between 12 and 20 years of age. Data was collected from four spawning groups that were held at different photoperiods and spawned from December 2014 (C4) to August 2015 (C3) (**Fig. 5.1.6**). Individual spawnings were recorded, and females strip-spawned accordingly, although for practical purposes night-time stripping was not carried out. Total and relative fecundity, number and size of batches and fertilization rates were individually recorded in all females.

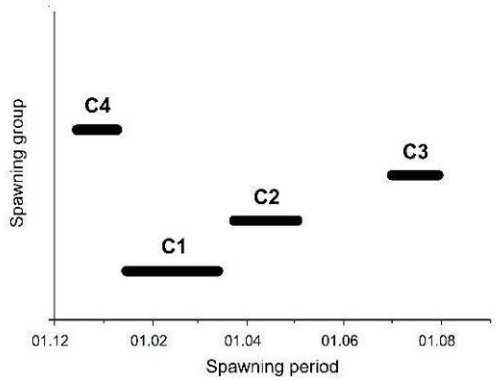


Fig 5.1.6. Spawning periods in the four broodstock groups (C1 – C4) followed in the DIVERSIFY study.

Results and Discussion

Biometric data and data on fertilization success are summarized in **Table 5.1.3**, and fecundity, average and median fertilization are presented in **Fig. 5.1.7**. Fish used in the experiments for Task 5.2 were chosen from group C1, based on the information obtained in the present task.

Overall, total and relative fecundity was similar in all groups except C4, where fewer egg batches were obtained from each female. Individual batch size was, however, similar in all groups. Relative fecundity of the SWH females in groups C1-C3, 355-385 $ml \cdot kg^{-1}$, was similar to that of females held at IMR, which was 347 and 349 $ml \cdot kg^{-1}$ in domesticated and farmed females, respectively (**Table 5.1.1**).

Fertilization success did not differ between the four groups, and was highly variable between batches, as well as between individual females. However, groups C2 and C3 had a larger proportion of high quality eggs, *i.e.* eggs with more than 80% fertilization. Overall, the lowest fertilization success, as well as lowest proportion of high quality eggs were found in group C1, which was chosen for treatment with GnRHa.

Table 5.1.3 Biometric data and fertilization success for broodstock held at SWH and used in Task 5.1.

	C1	C2	C3	C4
n	25	12	21	13
Age (year class)	1995/96 + 2003	1998 + 2002	2002	1995/96 + 2003
Average weight (kg)	27.3 ± 4.9	33.3 ± 6.3	30.6 ± 4.7	24.7 ± 4.4
Number of batches spawned	7 ± 3	6 ± 2	6 ± 1	3 ± 2
Average fertilization (%)	44.6 ± 28.0	59.5 ± 25.8	57.0 ± 27.0	48.0 ± 30.6
Eggs ≥80% fertilization (%)	13	25	24	18

In all, 412 egg batches, with a total volume of 714.6 l, were strip-spawned at SWH in 2015. Of those, ~139 l had >80% fertilization and were classified as high quality eggs. A few females in each group had consistently higher fertilization success than the others. The two groups that had the oldest fish also had a lower proportion of <80% fertilization. It was not possible to directly relate high age with low egg quality in the present study. However, concentrating on high-quality breeders may be useful in order to reduce the very considerable effort connected with spawning and egg collection of halibut.

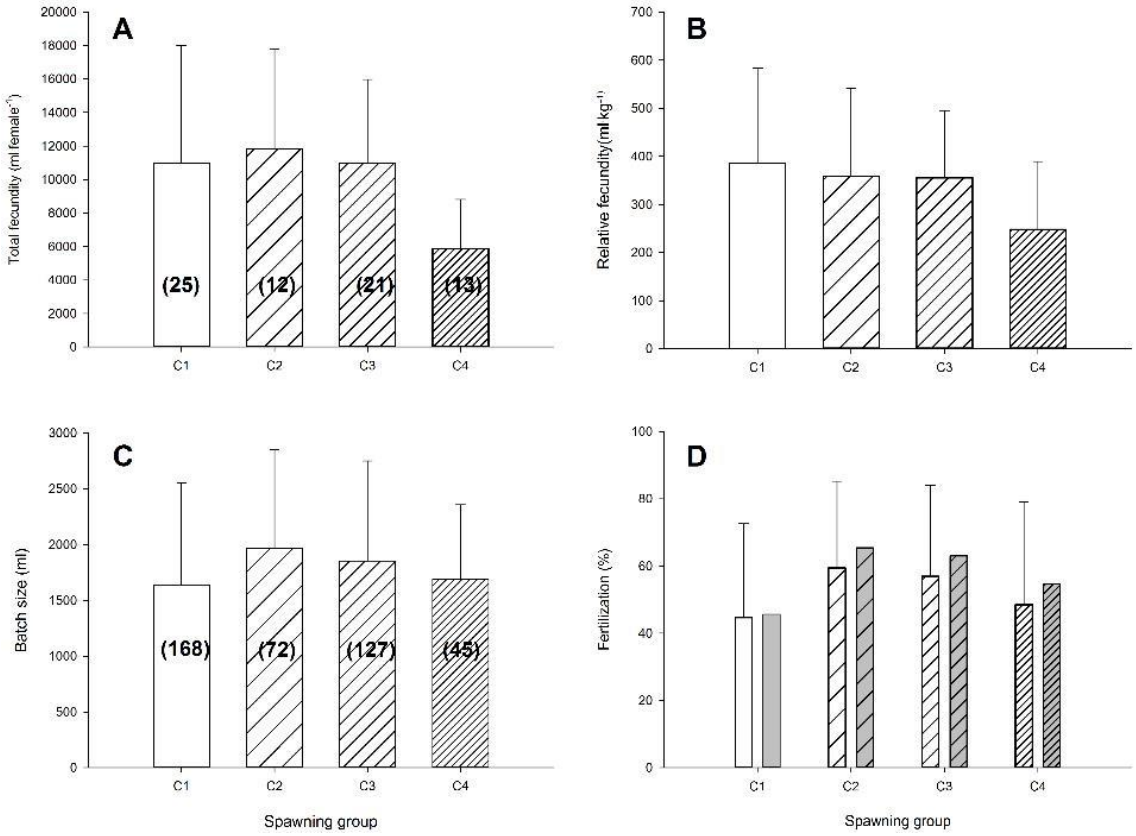


Fig 5.1.7 A. Total fecundity (eggs·female^{-1}, number in bars=n (females)), **B**. Relative fecundity (eggs·kg^{-1}), **C**. Average batch size (ml; number in bars=n (batches)) and **D**. Average (white bars ± SD) and median (grey bars) fertilization in four broodstock groups with different spawning periods at Partner SWH.

Conclusions

Wilddomesticated females were predictable spawners that consistently gave eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified and stripping carried out close to ovulation. However, for commercial, as well as breeding purposes, it is not practical to rely on wild-caught females. As at both IMR and SWH, relatively few farmed females produced eggs with fertilization rates >80-85% consistently, it may be necessary to include wild-caught broodstock also in future breeding groups in order to ensure a broad enough genetic material. 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 of Atlantic halibut.

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

Two commercial trials were run at the SWH hatchery (Reipholmen) during the reporting period. Both were performed on broodstock that were photo-manipulated and had a spawning period that was advanced by 1-2 months. Experimental fish for the second trial were chosen based on the data collected in Task 5.1, and were spawners that had performed below average in the previous season. The employed GnRHa implants were prepared as described in the 1st Periodic Report and in the submitted ***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.***

The release from the implants was evaluated using an *in vitro* release system, incubated at 6°C. The release kinetics were similar in both GnRHa doses, and as expected the implants begin with a high release as soon as they come into contact with the *in vitro* assay buffer (and hence the fish body fluids when administered *in vivo* (**Fig. 5.2.1**)). Thereafter, the release was reduced significantly and after 3-4 days it stabilized to about 3-4 % of the total loaded amount per day. In the *in vitro* assay, the implants continued to release GnRHa for a period of at least 32 days, which was the duration of the assay. The total amount of GnRHa released from the implants during this period was estimated at between 75 – 118% of the estimated loaded hormone (data not shown). The two implants (*i.e.* 500 and 750 µg GnRHa) were used in combination depending on the weight of the treated fish, in order to give an effective dose of ~50 or 100 µg GnRHa kg^{-1}.

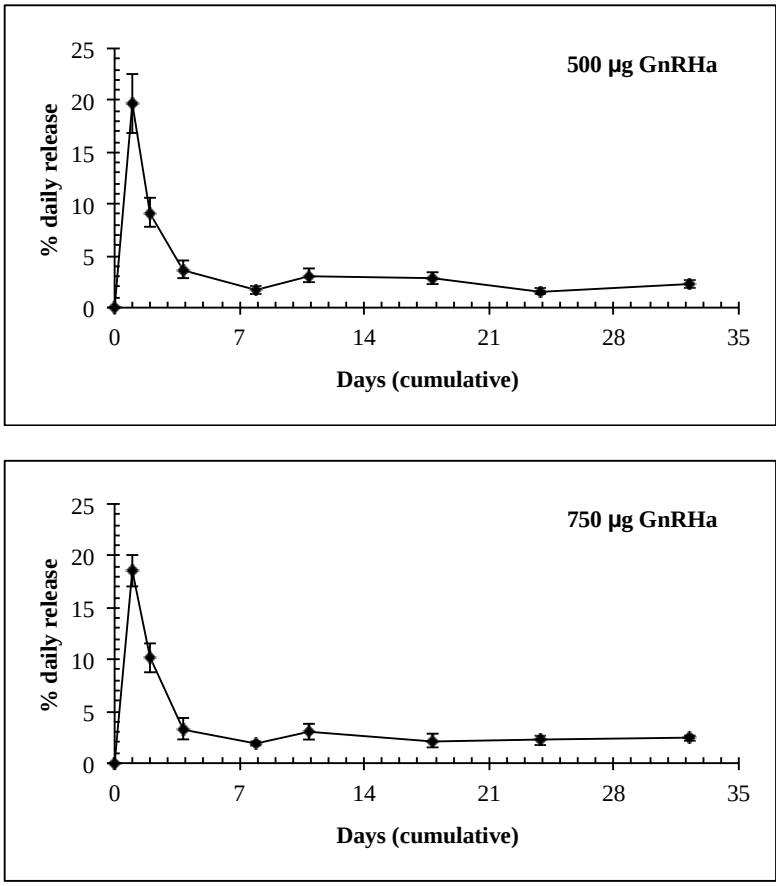


Figure 5.2.1. Mean (±SD) GnRHa released *in* vitro from the GnRHa implants (n = 4) loaded with 500 or 750 µg GnRHa and maintained at 6°C.

Commercial trial 1 (SWH, Reipholmen)

In order to test GnRHa implantation in a commercial system, two trials were run at the Reipholmen hatchery of P22. SWH. Eight females with an average weight of 26 ± 1.3 kg were chosen for implantation based on

outer signs of maturation: ovary visible on exterior of fish but not enlarged near the ovipore, degree of swelling and color of ovipore. In the first pilot trial, 4 females were implanted on Jan 16 2015, with 100 µg GnRHa kg^{-1} and 4 females were sham-injected as Controls. All fish were held in the same tank, with a diameter of 11 m and a volume of 76 m^3. The water supply was from 150 m depth and the temperature throughout the year fluctuated between 7.8 and 8.2°C. One month prior to expected start of spawning, the water temperature was lowered to 5.5-6°C and held constant until all females were spent.

All implanted females and two Control females ovulated within three weeks of treatment. The remaining Control females did not ovulate during the observation period, which lasted until March 1. There was no difference in start or end date of spawning between implanted females and Control females that spawned. Spawning periods lasted for 17±3.3 days in GnRHa-treated fish and for 17±1.4 days in Control females. Realized relative fecundity was 294 ± 163 ml·kg^{-1} in implanted fish (n=4) and 403 ± 218 ml·kg^{-1} (n=2) in Control females, respectively. Average fertilization rates were 36.8 ± 18.8% and 24.6 ± 30.8%, respectively. Of the two Control females that spawned, one consistently gave eggs with fertilization rates <4%, while the other had >55% fertilization in 5 out of 7 egg batches. Implanted females spawned between 5 and 8 batches, while the Control females each gave 7 egg batches.

Commercial trial 2 (P22. SWH, Reipholmen together with P7. IMR)

Ten females were chosen using the same criteria as in the pilot trial, and based on documented spawning performance (see Task 5.1). All females chosen had given average to low amounts of eggs in previous seasons. On 14 January 2016, five females, with an average size of 27 ± 1.4 kg, were implanted with 75 µg GnRHa kg^{-1}. Five females, with an average weight of 32 ± 6.1 kg, were sham-injected as Controls. The fish had not been treated previously, but were held in the same tank and under the same conditions as in the pilot experiment. One Control female died 8 days after treatment and is not included in calculations of fecundity and egg viability (fertilization success).

GnRHa-implanted females ovulated between January 25 and February 17, a period of 23 days and were stripped of eggs for fertilization. The ovulation period in treated females lasted for 16±3.5 days. Measured relative fecundity of stripped eggs was 187±72 ml eggs ·kg^{-1} and average fertilization success was 46.9±0.3%. However, actual realized fecundity may have been higher, as the presence of ovulated eggs in the water indicated at least one of the females spontaneously released several egg batches in the tank. Control females ovulated and were stripped between February 8 and March 16, or during a period of 37 days. The spawning period lasted for 25±9.7 days in Control females. Measured relative fecundity was 311±176 ml·kg^{-1} and average fertilization success was 53.0±0.3%.

Although GnRHa implantation did not advance spawning time significantly in Atlantic halibut females, there was an apparent synchronization in spawning time between individuals in experiment 2, as all treated females had completed spawning 1 month before all Control fish were spent. Spawning in Atlantic halibut normally occurs during a period of 2 to 3 months both in captive broodstock and in natural populations (Norberg et al, 1991; Haug 1990). This is most likely an adaptation that will ensure production of viable offspring independent of year-to-year fluctuations in temperature and feed availability for larvae. In a commercial production, however, synchronization between individuals can be an advantage as staff efforts can be concentrated to a relatively short period. Atlantic halibut females ovulate and release their eggs (*i.e.* spawn) in captivity, but fertilization of eggs released in the broodstock tank happens only occasionally. Therefore, Atlantic halibut breeders need to be monitored for ovulation and stripped on a regular basis, and eggs are fertilized *in vitro*. As a consequence, the use of GnRHa implantation offers a logistic advantage to the commercial broodstock management of the species, by reducing the duration of the spawning season.

On the other hand, spawning performance in terms of fecundity per female and fertilization success was not significantly affected by GnRHa treatment in Atlantic halibut females. At this stage the use of GnRHa therapy to increase fecundity and/or fertilization success is not confirmed. Apparently, spontaneously maturing and ovulating females may produce as many eggs as GnRHa treated individuals. However, GnRHa was demonstrated to be highly effective in ensuring that all females matured and ovulated, as all treated females ovulated at least 3 to 4 egg batches, whereas in all trials some of the Control fish did not ovulate and

appeared to resorb their ovaries. These results indicate that GnRHa implantation may be a useful tool to ensure that all females in a broodstock group reach maturation and ovulation, increasing parentage contribution to the next generation and increasing overall broodstock fecundity, without having deleterious effects on egg viability.

The full description of the work done in Task 5.2 has been described in ***Deliverable 5.2: GnRHa implant therapy as a means to improve spawning performance.***

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

No work done during the reporting period. See explanation under "Deviations".

References cited

Kjesbu O S, Kryvi H and Norberg B (1996) Oocyte size and structure in relation to blood plasma steroid hormones in individually monitored, spawning Atlantic cod (*Gadus morhua* L.) J. Fish Biol. **49**(6): 1197-1215.

Kleppe, L., Karlsen, Ø., Edvardsen**,** R. B., Norberg, B., Andersson, E., Furmanek, T., Taranger, G. L., and Wargelius, A. (2013). Cortisol treatment of prespawning female cod affects cytogenesis related factors in eggs and embryos. Gen. Comp. Endocrinol. **189**: 84-95.

Mangor-Jensen, A., Harboe, T., Hennø, J .S., and Troland, R. (1998). Design and operation of Atlantic halibut, *Hippoglossus hippoglossus* L., egg incubators. Aquaculture Res. **29**(12):887-892

Pankhurst, N. W. & Carragher, J. F. (1992). Oocyte maturation and changes in plasma steroid-levels in snapper *Pagrus* (*chrysophrys*) *auratus* (Sparidae) following treatment with human chorionic-gonadotropin. Aquaculture **101**, 337-347.

Skaalsvik, T. H., Bolla, S. L., Thornqvist, P-O. and Babiak, I. (2015). Quantitative characteristics of Atlantic halibut (*Hippoglossus hippoglossus*) egg quality throughout the spawning season. Theriogenology **83**, 38-47.

Deviations from Annex I and their impact:

Due to a prolonged sick leave for the WP leader Dr Birgitta Norberg from May to December 2015, some of the work in Task 5.1 was moved from 2015 to 2016. As a consequence, ***Deliverable 5.1*** will be delayed by a maximum of three months. All the data collection in Task 5.1 is done, as is reported here, but statistical analyses remain and will be completed shortly. Sterling White Halibut no longer keeps wild-caught broodstock and the collected data are from farmed females only. They were compared to the results obtained by IMR, and also used when Task 5.2 was planned.

Due to the delay in Task 5.1, Task 5.3 and ***Deliverable 5.3 Fecundity regulation***, have been postponed by one year (sampling starting in August 2016) and the Deliverable will be submitted in M48. We do not expect any impact on the overall work and achievements of the WP.

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			
Lead Scientist preparing the Report (WP leader):	Tito Peleteiro			
Other Scientists participating:	Constantinos Mylonas, Ioannis Fakriadis, Maria Papadaki and Irini Sigelaki (P1), Christian Fauvel (P14), Fatima Linares, José Luis Rodríguez (P19), Antonio Vilar (P32), Blanca Álvarez-Blázquez, Pedro Domingues, Rosa Cal, Montse Pérez, Evaristo Pérez, Nuria Lluch (P8), Neil Duncan (P3).			

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 progress towards objectives and details for each task (13-30 Mo):

During this 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.

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

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. Fish were transported by sea on a ship with flow-through water until O Grove Aquarium 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 P14. IEO facilities in Vigo in March 2016. These two juveniles (4.86 and 0.94 kg in body weight) will be maintained separated from the existent stock at the P14. IEO, until they become adults. Simultaneously, we are following the growth and development of the three juvenile specimens captured during 2014, two held at the P14. IEO **(Fig. 6.1.1.**), and the third at the Acuario de O Grove. Furthermore, we are monitoring the development of the Acuario O Grove wreckfish broodstock, which is constituted by 7 fish: 2 females, 3 males and 2 undetermined with an average weight of 11.57±1.86 Kg.

Fig. 6.1.1. *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 makes it difficult to obtain specimens of wild wreckfish to establish new wreckfish broodstocks.

Task 6.2 Describe reproductive cycle (led by IEO, Tito Peleteiro).

HCMR: The broodstock of P1. HCMR is constituted by three fish, two males of 7.2 kg and 11.0 kg, and a female of 13.1 kg. In continuation of the sampling that began and was reported in the 1st Periodic Report, these breeders were followed during Y2-3 of the project (between 25/2/2015 and up to the 9/5/2016) and blood, sperm and ovarian biopsies were collected as described in the 1st Periodic Report.

In February 2015, the female was undergoing vitellogenesis (Vg), having oocytes of 1,025 µm in diameter (**Fig. 6.2.1.A**). In March, most of the oocytes were in Vg, with the maximum oocyte diameters between 950 and 1075 µm. There were some more developed oocytes with clear lipid coalescence (lc), which were

between 1125 and 1250 μm in diameter. In addition, there were very few oocytes that looked much more clear, were larger (1,450 μm) and they looked at Oocyte Maturation (OM) (**Fig. 6.2.1.B**). The same situation was observed during April, but the number of Vg oocytes had increased. On 24/4/2015, the female started spawning spontaneously a small number of dead eggs (**Fig. 6.2.2**). On the morning of 1/5/2015, the female had spawned spontaneously 82,000 eggs with 56% fertilization success, while 35,000 eggs were stripped manually from the fish and were inseminated artificially. Eggs were transferred to incubators and photos were taken from floating eggs every day (**Fig. 6.2.3**). Eggs from the spontaneous spawn were floating for 4 days, while from stripped spawning were floating/viable for 2 days. At this time (1/5/2015), the female fish also contained many Vg and early OM oocytes (**Fig. 6.2.1.C, D**) and was induced to spawn using GnRHa implants (see below in Section 6.3 for details). In response to this therapy, the fish spawned up to 22/5/2015 (**Fig. 6.2.2**) and an artificial insemination was also performed on 4/5/2015 (see Section 6.3 for details). The biopsy taken on 25/5/2015 showed the presence of apoptotic oocytes and overripe eggs, while no Vg oocytes were found. In July 2015, the occurrence of atresia was high and some early Vg oocytes were found (**Fig. 6.2.1.E**). In October 2015, fish started the vitellogenic cycle for the next spawning season with Vg oocytes of 630 μm in diameter, having also leftover yolk in its ovaries (**Fig. 6.2.1.F**). The same situation was observed in December 2015 with Vg oocytes of 900 μm in diameter.

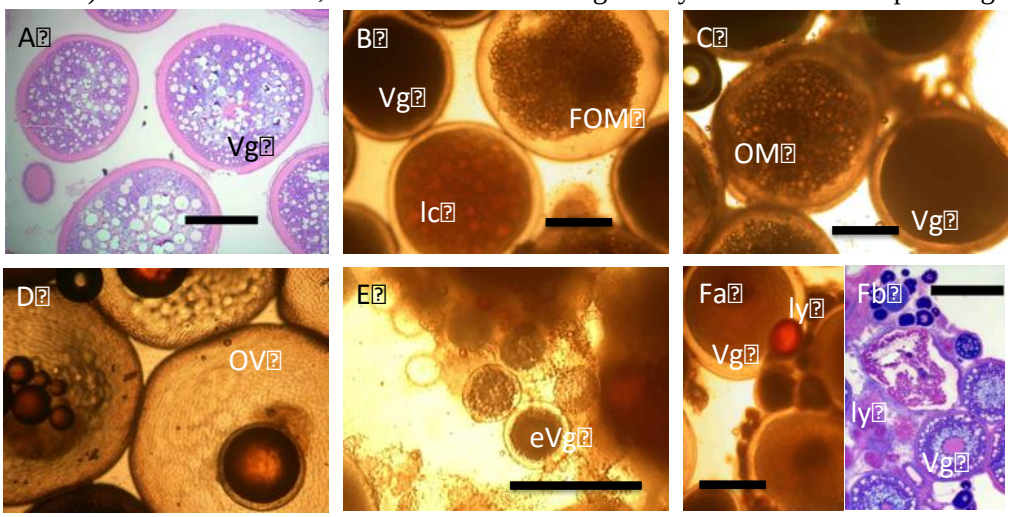


Figure 6.2.1. Microphotographs of histological sections (A, Fb) and wet mounts (B,C,D,E, Fa) from the ovary of the wreckfish maintained in P1 HCMR. A: In February 2015, in vitellogenesis (Vg). B: In March – mid April 2015, with oocytes in Vg, lipid coalescence (lc) and Final Oocyte Maturation (FOM). C,D: Female on 1/5/2015, with Vg, OM and FOM. E: In July 2015, with early Vg (eVg). Fa,b: In October 2015, with Vg and leftover yolk (ly). Bar = 500 μm.

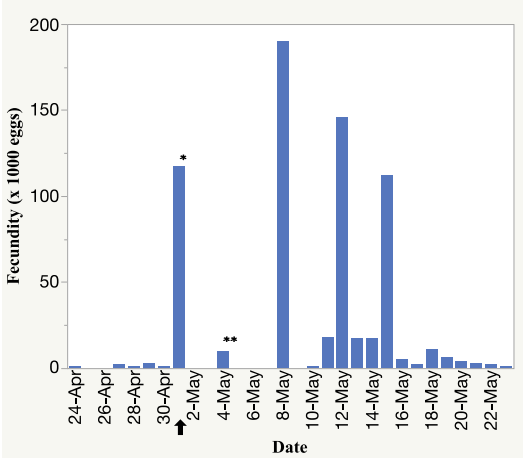


Fig. 6.2.2. Fecundity from a wreckfish maintained by P1. HCMR. The black arrow indicates the date of spawning induction during 2015. * Fecundity on 1/5/2015 was the sum of spontaneous and stripped spawns. ** eggs on 4/5/2015 were obtained with stripping.

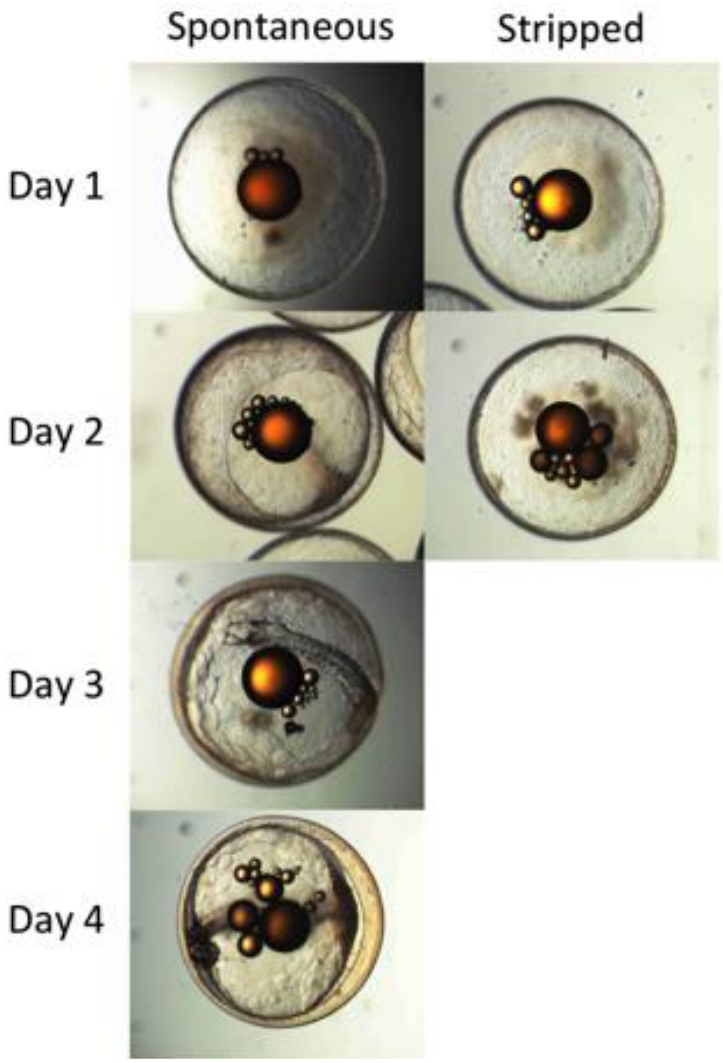


Figure 6.2.3. Development of wreckfish eggs from spontaneous or stripped spawning on 1/5/2015.

In January 2016, the female was undergoing Vg, having oocytes of 1,075 µm in diameter (**Fig. 6.2.4.A**), while the same situation was observed in March 2016, with slightly increased oocyte diameters in March (1,125 µm) and April 2016 (1,175 µm), respectively. In 9/5/2016, the fish was observed to spawn spontaneously after a GnRHa treatment on 2/5/2016 (see below in Section 6.3 for details), and the number of eggs was estimated to be ~1,000. Unfortunately, due to their small number the eggs were discarded by mistake before being evaluated. The female had Vg and post Ovulated (pOV) eggs (**Fig. 6.2.4.B**), but an effort to strip her did not result in any egg release.

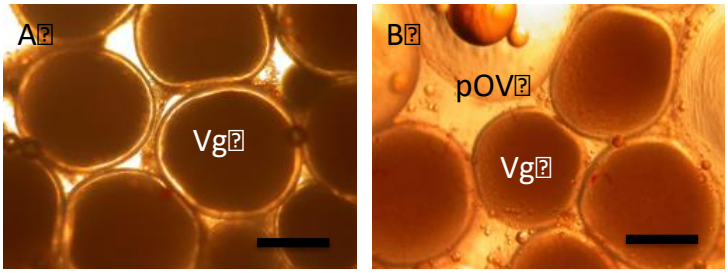
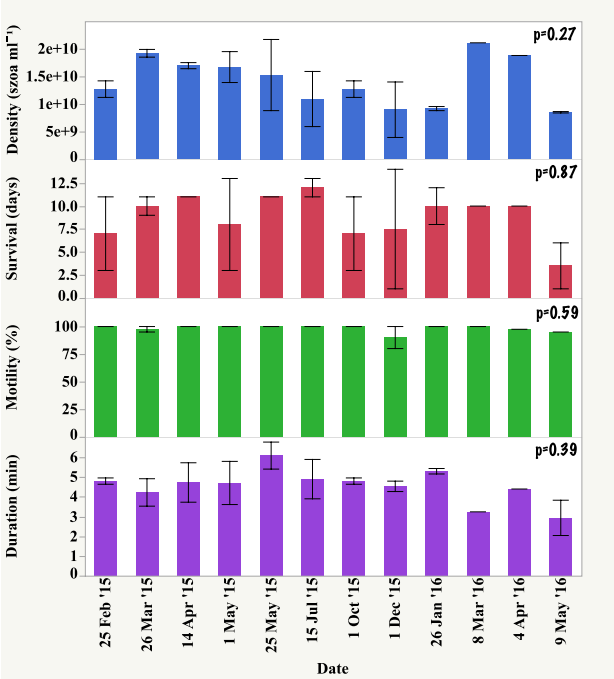


Figure 6.2.4. Microphotographs of wet mounts from the female wreckfish maintained in P1 HCMR. A: In January-April 2016, showing oocytes in vitellogenesis (Vg). B: In May 2016, with oocytes in Vg and post Ovulated eggs (pOV). Bar = 500 µm.



The two males were in full spermiation (Spermiation Index =3, copious sperm released with very gentle abdominal pressure) during the whole year, even in the summer and fall when the female was regressed. This was not the case at March – April 2016, where the produced sperm of one of the males was reduced (Spermiation index 0 or 1), probably because of an infection, which was treated with injectable antibiotic once a month for two months. Sperm quality parameters were evaluated as described in the 1st Periodic Report. Sperm quality was fairly high during the whole season and no significant variations were observed in different parameters (ANOVA, P≤0.05) (**Fig. 6.2.5**), even though the sperm from one male seemed to be a little yellowish and containing clumps (probably due to the before mentioned infection).

Figure 6.2.5. Sperm quality parameters of the wreckfish at P1. HCMR during the 2015-2016 reproductive season. No significant differences were observed. (ANOVA, P < 0.05).

Overall, from the monitoring during the season 2015 - May 2016 in Y2-3 and adding to the results of the 1st Periodic Report, it was possible to draw some conclusions as to the reproductive capacity of wreckfish in captivity:

1. Males produce large volumes of good quality sperm for a very long period of time, perhaps throughout the year, under constant 16°C of water temperature.
2. Females do undergo vitellogenesis --and they may even undergo oocyte maturation spontaneously in captivity—and remain in fully vitellogenic stage for at least 3 months (under constant 16°C water temperature).
3. Fertilized eggs could be produced both from spontaneous and stripped spawning (see later for details).
4. Spawned eggs were not of good quality, since their fertilization success was either 0 or very low, and their survival was limited to 4 days post spawning.

IEO, MC2 and CMRM:

The broodstocks were sampled every month, since February until July, and bi-monthly until December 2015 (**Fig. 6.2.6**).

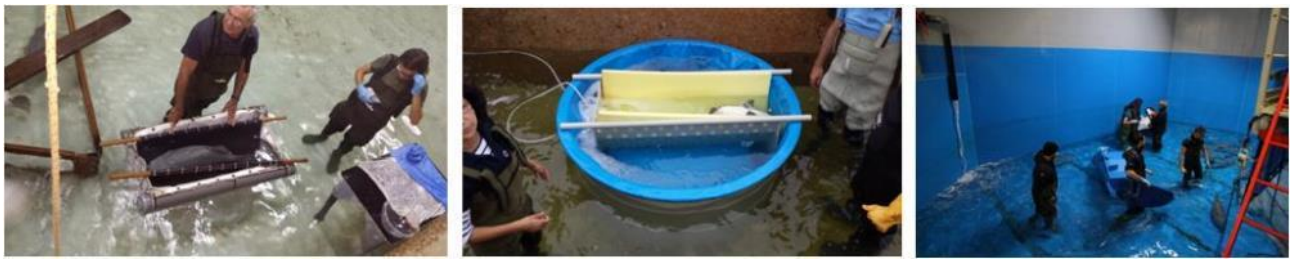


Figure 6.2.6. Broodstock sampling at the P14. IEO, P19. CMRM and P32. MC2.

This study on morphometric parameters indicates that animals stabilized their growth (size and weight), with weight increase during the reproductive season (**Fig. 6.2.7**). The feeding rate was varied between 0.2 y 0.5 % for fish fed the semi-moist diet (formulated by P14 IEO), and between 1 and 1.8 % for fish fed dry pellets (newly - formulated, WP 12). Low feeding rates were recorded during the spawning season (since March until July) and high feeding rates occurred during autumn **(Fig. 6.2.8)**. To determine the sex of the specimens from which biopsy could not be obtained, as the gonopore was completely closed, ultrasound was used (**Fig. 6.2.10**).

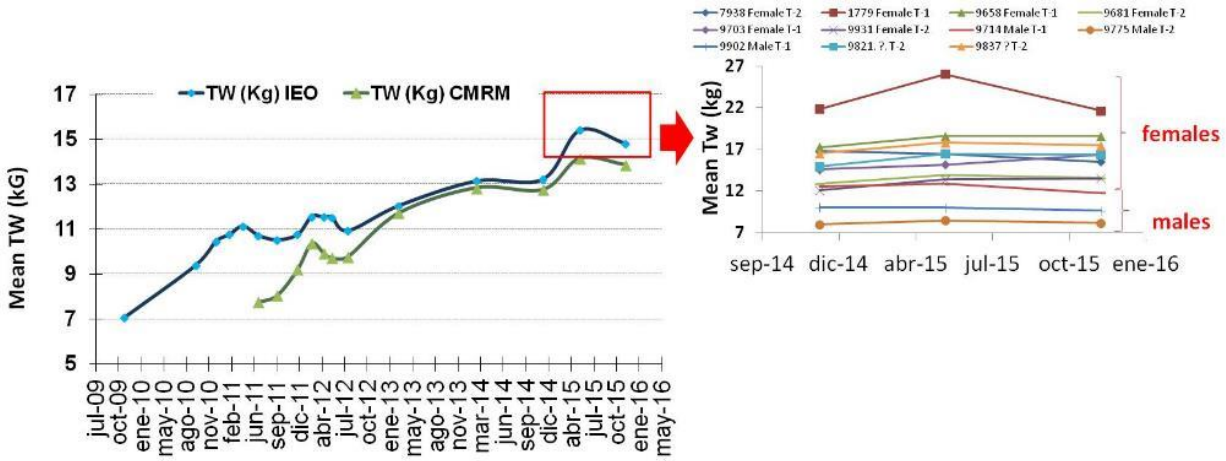


Figure 6.2.7. Size and weight changes of the P14. IEO and P19. CMRM broodstocks**,** with detailed information for 2015.

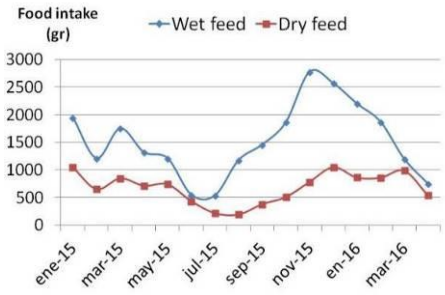


Figure 6.2.8. Food intake of the P14 IEO broodstocks between January 2015 and March 2016.

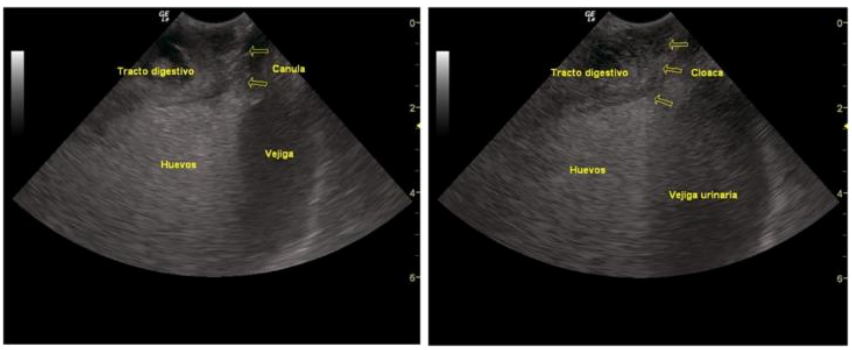


Figure 6.2.10. Gonad echography pictures of two wreckfish, to identify sex of breeders held at P32. MC2 and P19. CMRM .

During sampling, blood was extracted from some animals in each stock to determine steroid levels (P1. HCMR). The sex steroid plasma level evaluation will be done in the fall of 2016, when 2 years of sampling will have been completed from all available broodstocks. Sperm and biopsies of oocytes were also obtained to determine the stage of gametogenesis of the different fish. Average oocyte size throughout the year showed an expected variation with high values during the spawning season (**Fig. 6.2.11**). Biopsies of oocytes from immature females indicate that these females did not reach vitellogenesis. Oogenesis sequence from primary oocytes to post-maturation was described with both fresh samples and through histology **(Fig. 6.2.12**). Sperm quality parameters -concentration, motility and duration of forward spermatozoa motility- were evaluated during the period from March 2015 to May 2016. The males produced large volumes of good quality sperm during the entire year. Sperm concentration during the spawning season was between 2.64 and 16.5 x10^9 for the males in the P14. IEO broodstock.

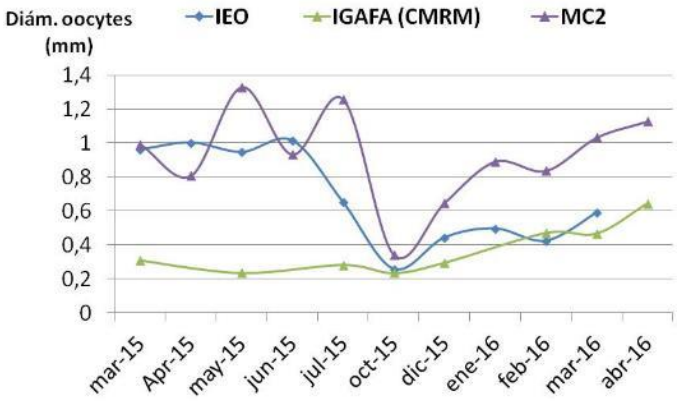


Fig. 6.2.11. Oocyte size variation from females of the three stocks throughout the year (P14 IEO, IGAFA-P19 CMRM and P32 MC2).

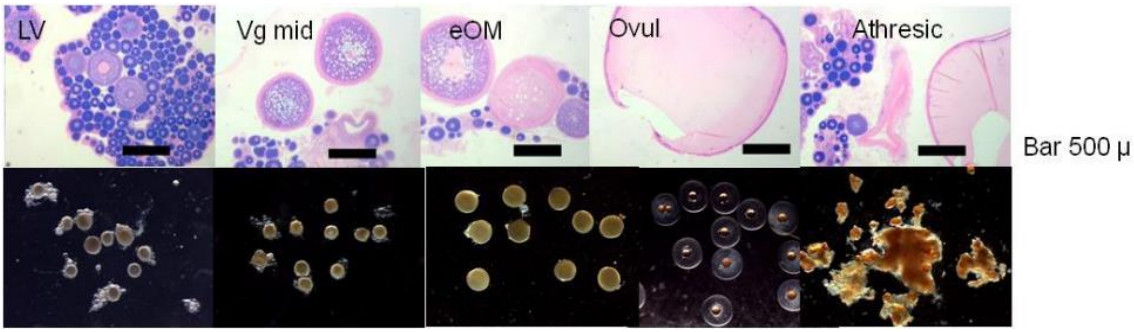


Figure 6.2.12. Oogenesis sequence: LV=lipid vesicle, Vg (mid) = mid vitellogenesis, eOM = lipid coalescence, Ovul = ovulation, Athresic = atresia.

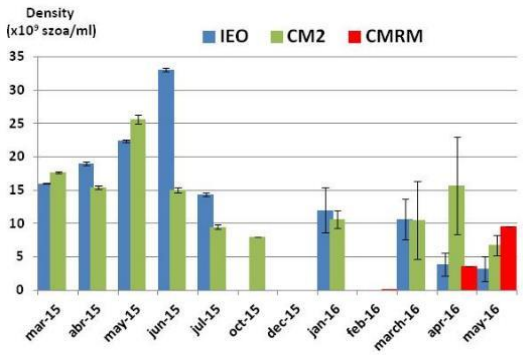


Figure 6.2.13. Sperm concentration from March 2015 to May 2016 for the males in the broodstocks held in P14. IEO and P32. CM2. The P19. CMRM male was transferred from P32. MC2 in January 2016.

Task 6.3 Development of spawning induction procedures (led by IEO, Tito Peleteiro).

HCMR Stock: As described in the 1st Periodic Report, during Y2-3 the main goal was to produce eggs using artificial insemination, based on the results so far. On 1/5/2015 (as described in Task 6.2 above), the female's ovaries contained not only Vg oocytes (1,250-1,350 μm), but also some oocytes in early OM (lipid coalescence) with diameters of 1,500-1,750 μm (**Fig. 6.2.1**), while some eggs (82,000) were also released in the tank and some (35,000) were stripped from the fish (**Fig. 6.2.2**). The female was given a GnRHa implant (600 μg) and was placed together with one of the males, which was also given a GnRHa implant (300 μg). On 4/5/2015, the female contained oocytes in OM (1,525 μm in diameter), as well as ovulated eggs (2,250 μm) (**Fig. 6.3.1**). The female was stripped –although the ovipore of the fish seemed to be blocked- and 10,000 eggs were artificially inseminated with 2 ml of freshly obtained sperm from the males. Eggs were kept in the incubator for 3 days and later the floating, viable eggs (around 6,000 eggs) were transferred to the larval rearing facility (**Fig. 6.3.2**). On 8/5/2015, 190,000 eggs were spawned spontaneously, having 12% fertilization success but the embryos survived for only 5 days (**Fig. 6.3.2**).

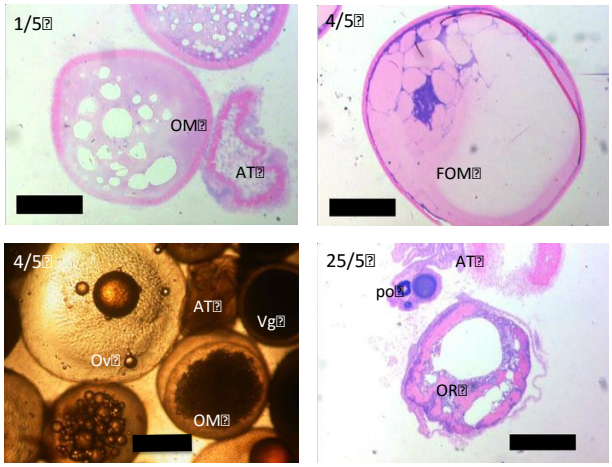


Fig. 6.3.1. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2015 reproductive season (dates on each photo). AT = atresia, Vg = vitellogenic, OM = early Oocyte Maturation, FOM = Final OM, Ov = Ovulated, po = primary oocyte OR = Overripe. Bar = 500 μm

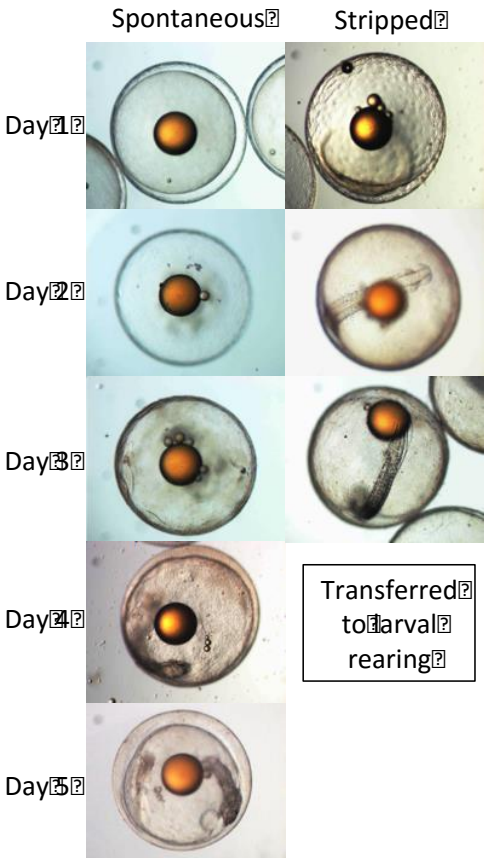


Figure 6.3.2. Floating wreckfish eggs from spontaneous or stripped spawning on 8/5/2015 or 4/5/2015, respectively, after spawning induction with GnRHa.

In 2016, a first GnRHa treatment was given on 18/4/2016 both to the female and males. At that time Vg oocytes (1,150 μm) were observed while few oocytes were in AT (**Fig. 6.3.3**). The female was almost in the same situation after 4 days, with a small increase of the diameter of the Vg oocytes (1,250 μm) (**Fig. 6.3.3**). Some signs of early maturation were observed on 2/5/2016, with oocytes undergoing lipid coalescence (lc) stage with no further change in oocyte diameters (**Fig. 6.3.3**). At that time, a second GnRHa treatment was given to the female. After 7 days (9/5/2016) the fish spawned spontaneously ~1,000 eggs (as described in Section 6.2 above).

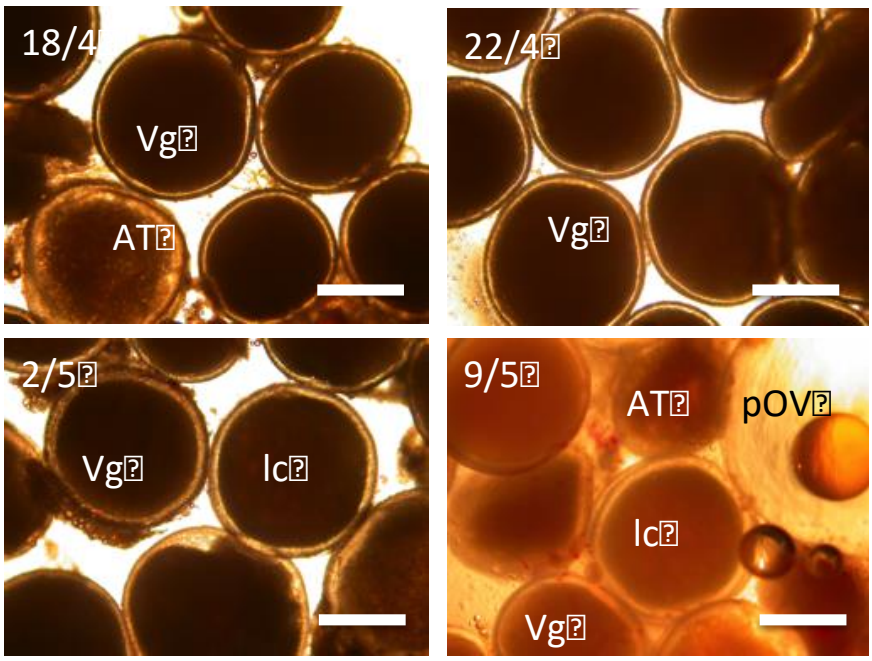


Fig. 6.3.3. Wet mount of ovarian biopsies from wreckfish during 2016 (dates on each photo). AT = atresia, Vg = vitellogenic, lc = lipid coalescence, pOV = post Ovulated eggs. Bar = 500 μm.

To sum up the work at HCMR, the results of the 2015 – May 2016 spawning induction experiments in addition to the results of Y1 of the project demonstrated that:

1. A GnRHa implant treatment of the female induced OM and ovulation consistently.
2. The exact timing of the ovulation after the hormonal treatment and the post-ovulation survival of the eggs is currently not known. Lack of this information may be the reason for the low fertilization and egg quality of the obtained eggs.
3. Spontaneously spawned fertilized eggs could be produced after spawning induction, but these are low in fecundity and fertilization success and cannot be relied upon.
4. Even though some fertilized eggs were produced during the reproductive season of 2015, an earlier induction of spawning and artificial insemination should be applied, before the spontaneous spawning starts. This plan was followed during the spawning season 2016, which is still in progress, so final results will be presented in the next periodic report.

IEO, CM2 and CMRM Stock: During the spawning season of 2015, a total of 21 spawns were obtained (10 at the P8. IEO and 11 at the P32. MC2) between March and June (**Fig. 6.3.4**). The majority of spawns were spontaneous, except one artificial at the P8. IEO (10/04/2015) and two from P32 MC2 (16 and 28/05/2015). There were no spawns from females of P19. CMRM. During 2016, since April until the end of May, 7 spontaneous spawns at the P14 IEO were obtained, and new spawns are expected. Twelve spontaneous spawns and two by stripping from P32 MC2 stock, were also obtained (**Fig. 6.3.5**). More spawns are expected during June 2016 until the end of the spawning season (July).

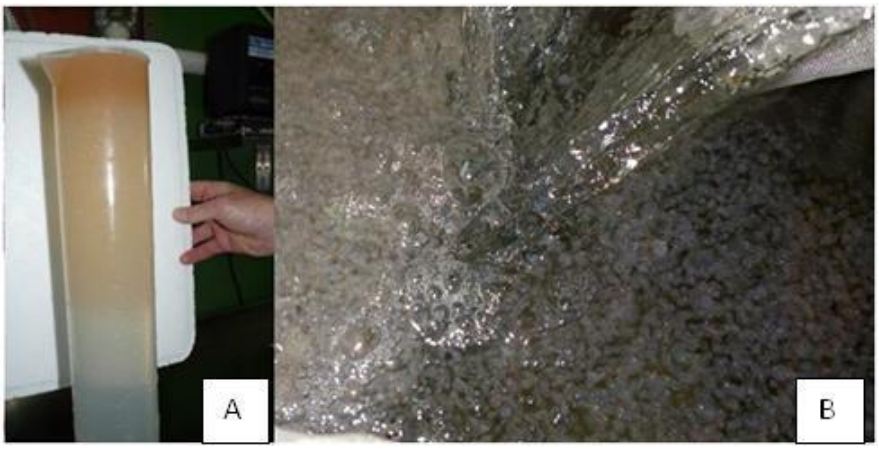
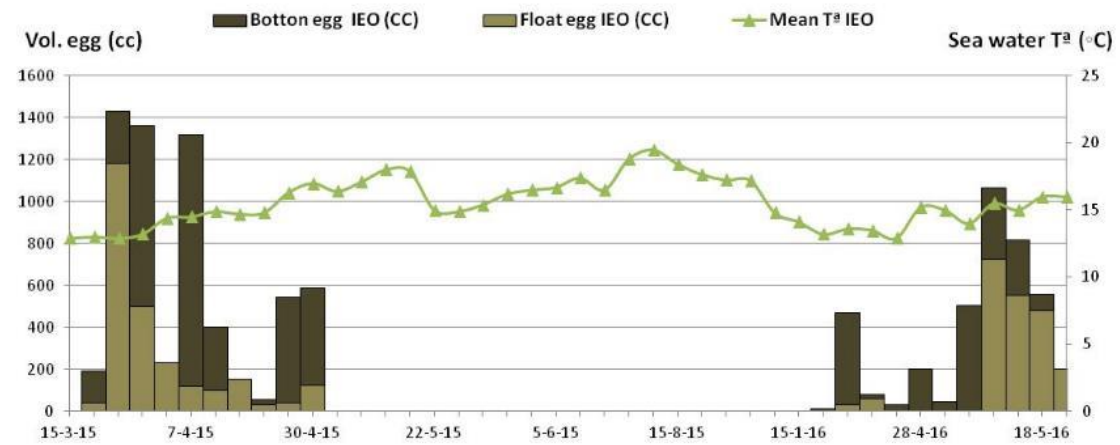
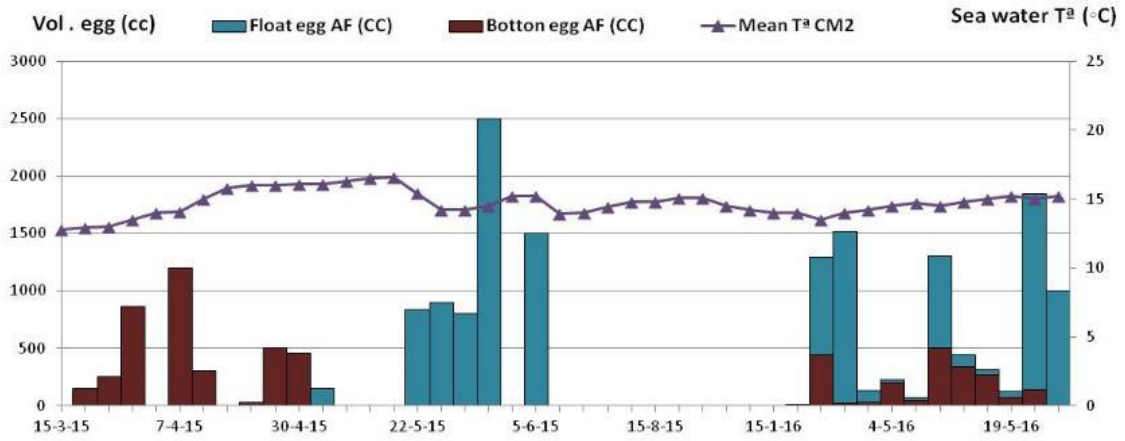


Figure 6.3.4. Spontaneous spawn (A) counted volumetrically from the egg collector and (B) in the egg collector of tank S-2 at the P14. IEO.

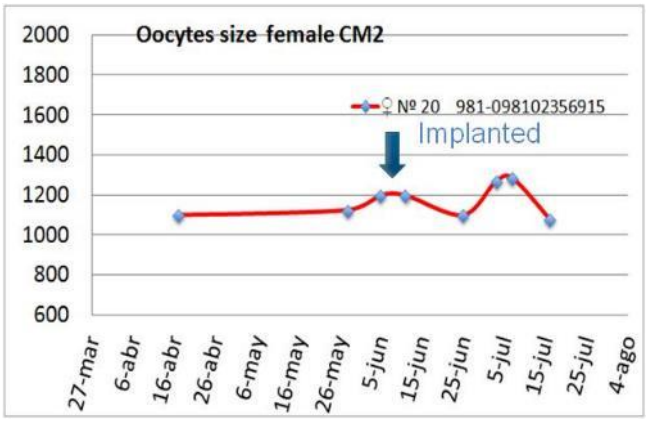


(A



(B

Figure 6.3.5. The volume of viable eggs (cubic centimetres cc) (floating) and non viable (on the bottom) obtained from spawns from wreckfish held in P14. IEO (A) and P32. MC2 (B) from March 2015 to May 2016. A number of spawns were incubated and larvae were obtained (see WP 12).



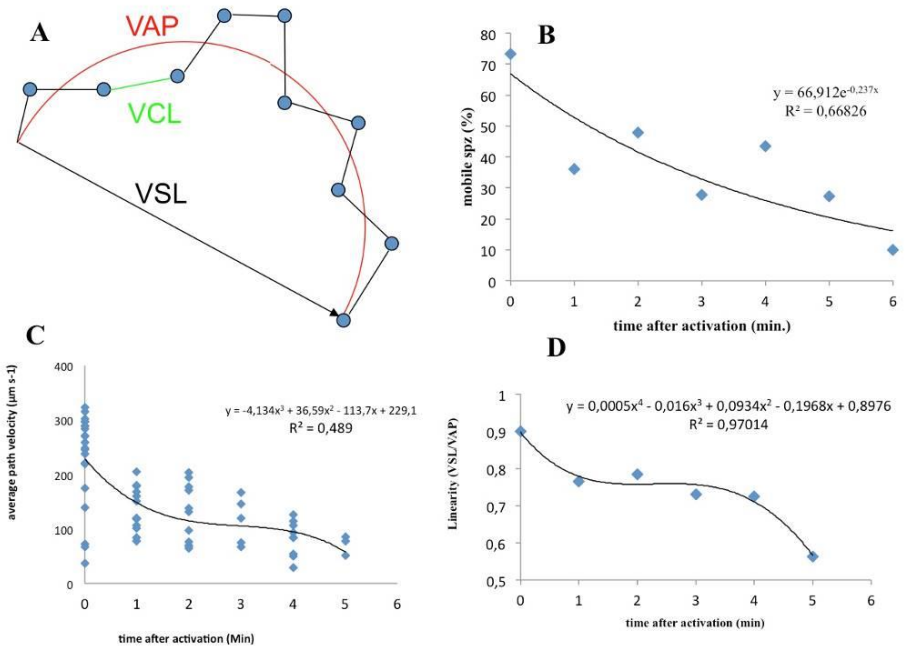
A female of 16.5 kg from P14. IEO with oocyte size of 1.3 mm was implanted in Jun 09, 2015 with 500 µg of GnRHa. No response was obtained from this treatment, possibly due to low hormone dose (less than 50 µg kg^{-1}). On June 2015, two females weighing 27.4 and 33.2 Kg were implanted with 500 µg of GnRHa. Oocyte sizes were 1.12 and 1.09 mm respectively. None of these implanted females spawned (**Fig. 6.3.7**), presumably because the oocytes had not completed vitellogenesis, which in this species occurs when the oocytes reach a diameter of >1.2 mm.

Figure 6.3.6. Oocyte size changes for an implanted female from the P32 MC2 stock

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

Sperm characteristics and CASA development

One of the objectives of this WP was to provide a tool for an objective assessment of sperm quality of male wreckfish, subject to captivity during the full reproductive season, by establishing a Computer Assisted Sperm Analysis (CASA) for the evaluation of wreckfish sperm. The best adapted CASA parameters for wreckfish sperm analyses were determined and reported to end users to optimize their abilities to check fertility potential of the semen in the course of their future spawning induction experiments. Moreover a movie describing the procedure of sperm activation and CASA was uploaded on the website of the project.



The analysis of wreckfish sperm took place in the spring of 2014 in Galicia (NW Spain) using gametes collected from three different locations and in the winter of 2015 from two locations of the same area. From 8 to 13 April 2014, sperm was collected from 6 males from P32.MC2 (Acuarium Finisterrae) facilities, from 2 males of Luso Hispana de Aquicultura (LHA) and finally from 2 males from the facilities of P8.IEO (Center of Vigo).

Figure 6.4.1. Motility parameters of wreckfish sperm: A) schematic representation of spermatozoa movement illustrating the three parameters of velocity generated by CASA; B) Variations of the percentage of swimming spermatozoa (spz) with time; C) average path velocity decrease with time after activation; D) decrease of linearity of spermatozoa trajectories after activation.

The laboratory methods of analysis were shared between researchers of the different partners and the P14. IFREMER researcher in charge of this task at the IEO laboratories. In January 2015, some of the males of P32.MC2 and P8.IEO were sampled again and a complementary transfer of know-how about sperm quality assessment was performed at the Acuarium Finisterrae facilities. Concomitantly, taking profit of regular maturation monitoring during 2014, P1. HCMR analyzed sperm quality of several wreckfish males according to its own usual routine field methods.

The fresh sperm showed a high initial percentage of mobile spermatozoa at activation, which had regularly decreased with time for 5 minutes. The mean initial VAP or mean velocity along smoothed trajectory was around 230µm per second, which progressively decreased to 0 after 5 minutes (**Fig. 6.4.1**). The velocity of spermatozoa was one of the highest reported for marine fish and the trajectories vary from straight forward at activation to progressive circling as the speed decreased as illustrated in **Fig. 6.4.2**. This illustration is corroborated by the decrease of linearity of the trajectories calculated by the ratio between the average path velocity and straight-line velocity (**Fig. 6.4.1D**).

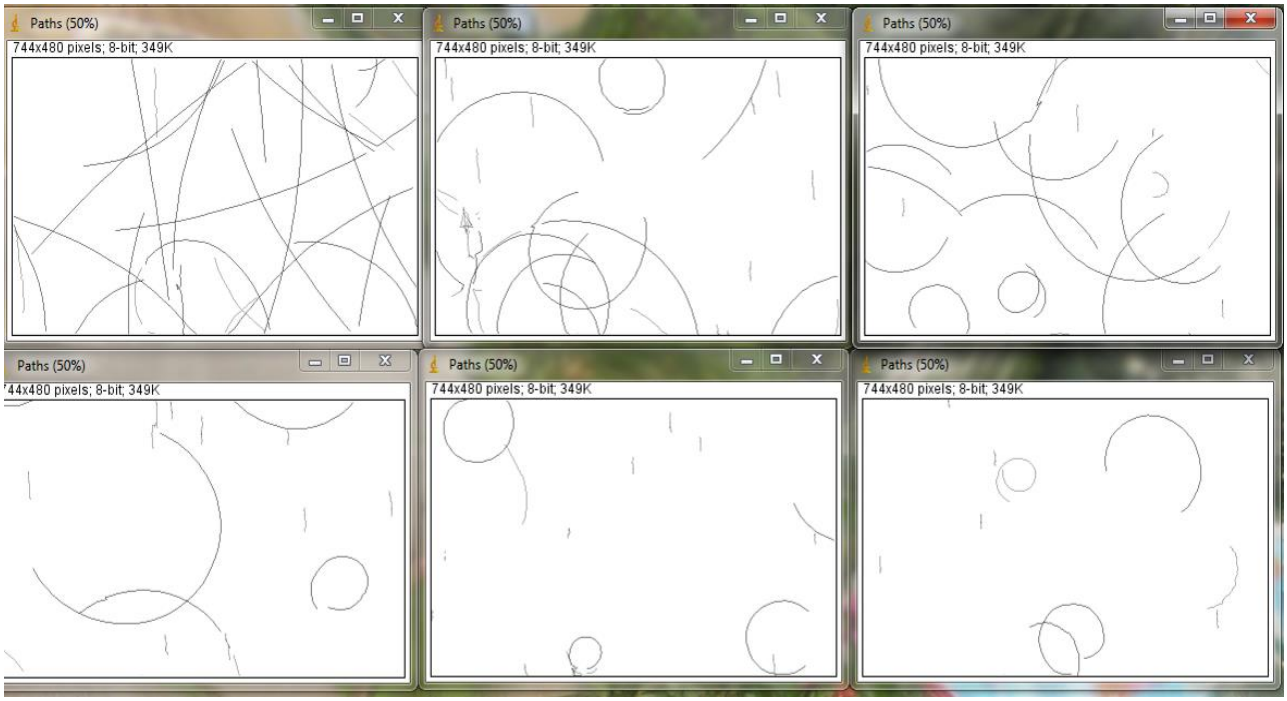


Figure 6.4.2. Spermatozoa path tracks of 2 s, generated by Computer Assisted Aperm Analysis (CASA plug-in of ImageJ) at 10 sec after activation, and at every minute for 5 min (starting from the upper left to the right) after sperm activation in wreckfish using the setting described above.

As programmed in the DOW, a method for the objective assessment of sperm quality through the analysis of motility was implemented for wreckfish that complemented and confirmed field assessment usually implemented in broodstock rearing facilities. The preliminary analyses demonstrated that sperm of captive wreckfish shares a common pattern of motility with both marine and freshwater fish, based on a general activation of all the sperm at the same time of ejaculation in activating environment, then a decrease with time down to zero in a rapid lapse of time from 30 sec to more than 20 min due to exhaustion of energetic stores badly compensated by respiration.

Wreckfish males produced a high volume of easily expressible milt with a concentration considered as medium range for marine fish and of course much higher than that of flatfish. On the top of those general features, the setup of a CASA protocol adapted to wreckfish sperm demonstrated that wreckfish sperm exhibits a high percentage of motile cells at activation and one of the highest initial speeds recorded for fish

sperm. This high speed was associated with a long swimming duration compared to other marine fish. The long duration exhibited a double trajectory shape. The first trajectory was straight (associated with the search of target eggs) and then the trajectory began bending, which was interpreted as a phase of searching for the micropyle on the egg surface. Moreover, the results obtained by CASA are in agreement with field observations obtained by human inspection under the microscope, and complement them by objective data that can be more easily statistically analyzed.

The full description of the work and results from this Task has been provided in ***Deliverable D6.1 Computer Assisted Sperm Analysis (CASA) for wreckfish sperm.***

Cryopreservation methods

The undertaken work showed the feasibility of wreckfish sperm cryopreservation, while chilled storage does not seem to be a good solution for the short-term management of sperm for artificial fertilization. The performance of frozen/thawed wreckfish sperm was overall half than of fresh sperm in terms of percentage of motile spermatozoa and duration of forward motility, while the velocity of sperm in modified Leibovitz was similar to that of fresh sperm (**Fig. 6.4.4**). Since wreckfish produce large volumes of high quality sperm in terms of concentration, velocity and duration of motility, the losses of sperm quality due to freezing may be compensated by increasing the number of spermatozoa per egg as is usually practiced in other species. The short duration of rapid movement may not be harmful since generally fertilization occurs in the first seconds of contact between gametes of both sexes. However, the current results only describe the movement of spermatozoa. They are a good index of cryopreservation coping capacity of sperm. Nevertheless, these results have to be confirmed in the future by a test of cryopreserved sperm ability to fertilize.

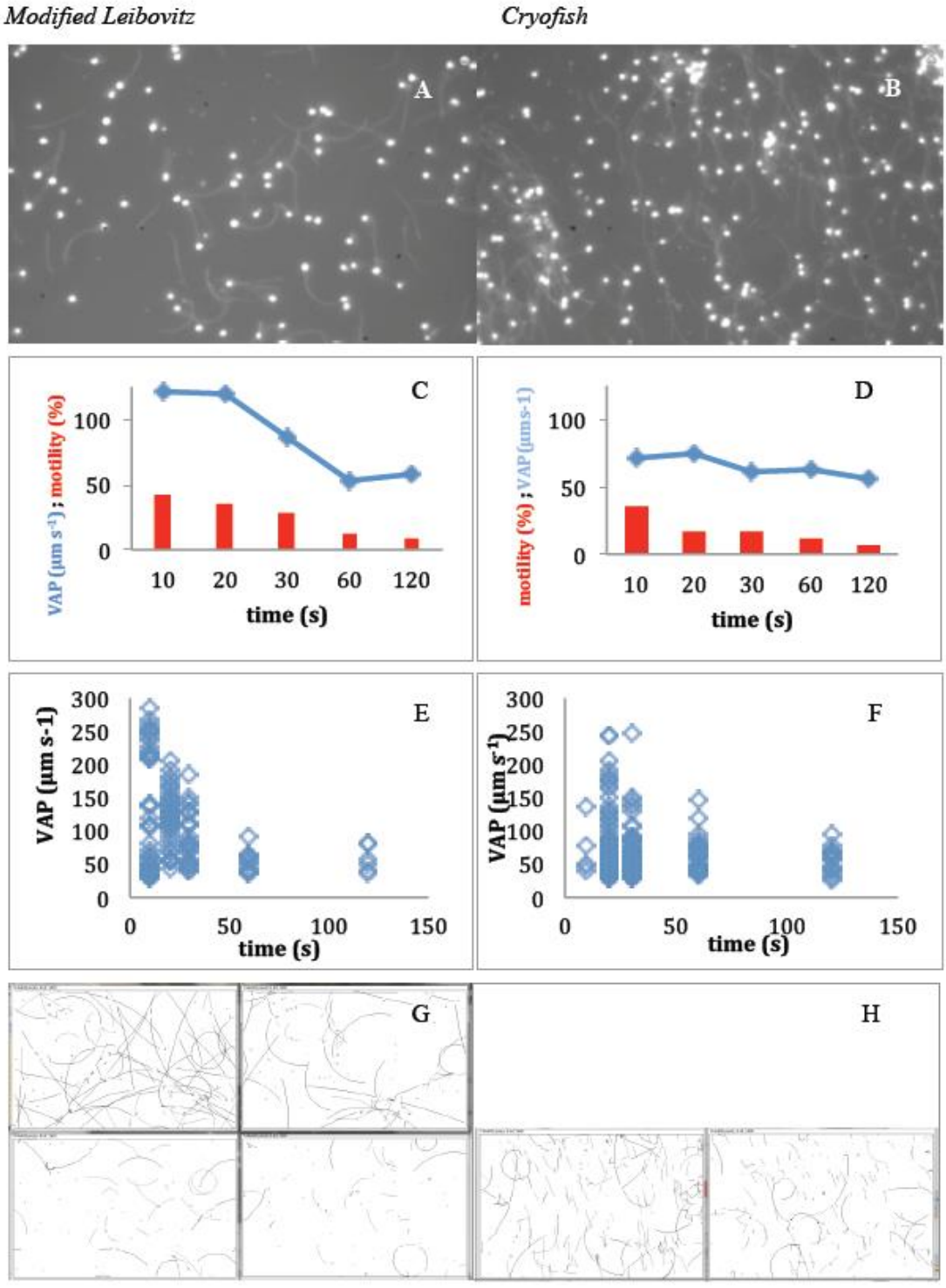
The full description of the work and results is provided in ***Deliverable D6.2 Cryopreservation method for wreckfish***.

Deviations from Annex I and their impact:

It has been so far proven more difficult to control ovulation (for *in* vitro fertilization) or spawning (for spontaneous tank fertilization) and production of fertilized eggs in response to hormonal therapy. The small number of breeders available by some partners (especially P1. HCMR) has been limiting the ability of a large number of trials during every annual reproductive season. Therefore, ***Milestones 34-36 Successful maturation and spawning of wreckfish to produce good quality eggs*** in Y1, Y2 and Y3, respectively, have been acomplisehed, but not to the complete satisfaction of the consortium. This has, in turn, limited the progress made in the WP 18 Larval husbandry – wreckfish. Nevertheless, slow progress has been made and we expect that by the end of the project we will succeed in producing a large number of viable eggs, to enable the experiments with larval rearing to be implemented.

(Next page)

Figure 6.4.4. Wreckfish sperm status after cryopreservation in modified Leibovitz and Cryofish. A and B: Pictures of the sperm diluted in the different media (extracted from video records), Cryofish samples (B) show aggregations of sperm unlike modified Leibovitz (A). C and D: Mean velocity decrease and variations of the percentage of motile sperm with time in the different media. E and F: individual velocities of spermatozoa recorded in the different media showing that modified Leibovitz (E) allows a high recovery of a larger number of spz compared to Cryofish (F). G and H: illustration of tracks generated by CASA for the spz stored in the two media: Leibovitz (G) and Cryofish (H).


Modified Leibovitz
Cryofish
A
B
C
VAP (µm s-1) ; motility (%)
100
50
0
10
20
30
60
120
time (s)
D
motility (%) ; VAP (µms-1)
100
50
0
10
20
30
60
120
time (s)
E
VAP (µm s-1)
300
250
200
150
100
50
0
0
50
100
150
time (s)
F
VAP (µm s-1)
300
250
200
150
100
50
0
0
50
100
150
time (s)
G
H

WP 7 Reproduction & Genetics – grey mullet

WP No:	7	**WP Lead beneficiary:**		P7. IOLR
WP Title (from DOW):	Reproduction and Genetics – grey mullet			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P13. UNIBA	P14. IFREMER
P15. ULL	P24. ITTICAL	P25. DOR		
Lead Scientist preparing the Report (WP leader):	Hanna Rosenfeld			
Other Scientists participating:	Constantinos Mylonas (P1), Neil Duncan (P3), Aldo Corriero (P13), Christian Fauvel (P14), Covadonga Rodriguez (P15), Fulvio Ceppolaro (P24), Hagay Sarusi (P25)			

Objectives

1. Evaluate the effectiveness of hormone-based treatments on synchronizing gonadal development and improving gamete (eggs and sperm) quality in mature grey mullet,
2. Develop hormone-based treatments for induced spawning of grey mullet,
3. Optimize a scaled-up breeding of grey mullet in captivity under natural and manipulated photo-thermal regimes,
4. 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 progress towards objectives and details for each task (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.

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.

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.

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

7.1.1 Means to evaluate grey mullet sperm quality

As programmed in the DOW, a CASA method for the objective assessment of sperm quality through the analysis of motility was implemented for grey mullet and submitted in ***Deliverable D7.1 Analysis of sperm motility: General protocol and propositions for mullet sperm quality assessment***.

Briefly, during November 2014, sperm samples were collected from sexually mature mullet males held in the P4. IOLR facilities. The sperm was diluted 1:1000 providing approximately 80 spermatozoa in the field of the microscope (X20 magnification). Following activation in seawater the spermatozoa movement was recorded (10 sec after activation; frame rate of 100 FPS). The movies were either studied *in situ* or sent by Dropbox to IFREMER (Palavas, France) in order to inter-calibrate the analysis through mail exchanges. The motility of grey mullet sperm decreases with time and the duration is limited to 50 seconds. The initial percentage of motile spermatozoa is around 50% and quickly decreases to zero after 70 seconds (**Fig. 7.1.1**).

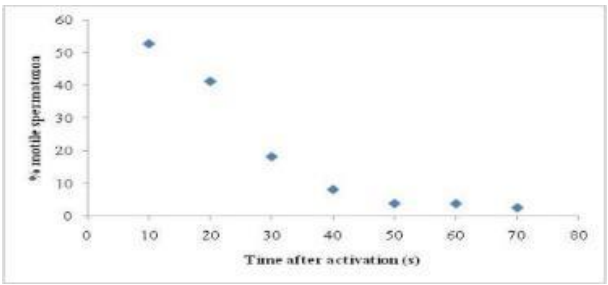


Figure. 7.1.1. Variation of grey mullet sperm motility assessed by the percentage of motile sperm with time after activation**.**

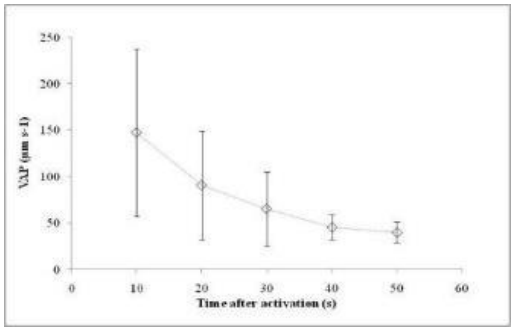


Figure. 7.1.2 Modification of Average Path Velocity (VAP) of grey mullet sperm with time; error bars illustrate the standard deviation.

The number of motile sperm decreased and the mean speed of spermatozoa also dropped with time (**Fig. 7.1.2**). The variability of velocity remained high along the studied sequence, but the higher velocities decreased strongly. The comparison of means by ANOVA revealed that the only significant difference in velocity was observed between the velocity at 10 s and all other time points. The calculated linearity objectively assessed the variations of trajectories during the spermatozoa propulsive movement. During the first 30 s after activation the majority of spermatozoa swam in straight line, then the track bend progressively as illustrated by the decrease of linearity (**Fig. 7.1.3**)

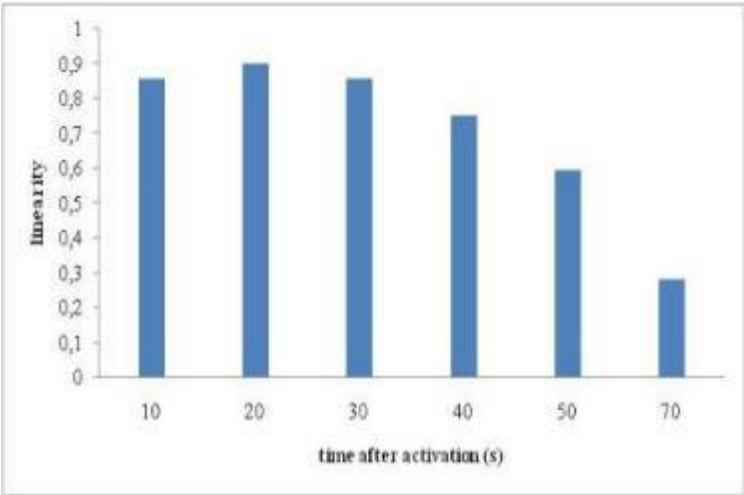
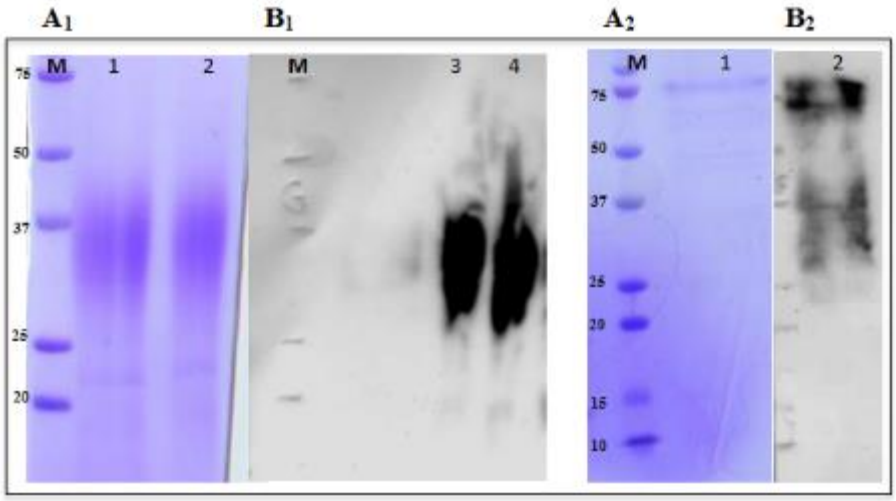


Figure. 7.1.3. Modifications of the trajectories of grey mullet sperm expressed by the linearity.

The analyses show that grey mullet sperm does not present a pattern of motility similar to that of seabass (*Dicentrarchus labrax*) or rainbow trout (*Oncorhynchus mykiss*) in terms of velocity as well as duration, which lasts less than one minute.

7.1.2 Production of recombinant bioactive LH and FSH for grey mullet

Despite the successful hormone-induced breeding of grey mullet in captive condition (Aizen et al., 2005), in most cases the treated males produced a very small volume of semen, which was highly viscous and failed to fertilize the eggs. Therefore, to further enhance grey mullet sperm quality, herein we produced and characterized fish recombinant gonadotropins, FSH and LH, the most important pituitary hormones regulating testicular physiology. This work was led by P4. IOLR and has been completed and submitted in ***Deliverable 7.2 Production of recombinant bioactive LH and FSH assay for grey mullet.***



Briefly, the recombinant gonadotropins, rLH and rFSH, were produced utilizing the yeast *Pichia pastoris* expression system, and the purity and integrity of the ~ 37 kDa recombinant proteins were verified by SDS-PAGE and Western blot analyses (**Fig. 7.1.4**).

Figure 7.1.4. Production of recombinant FSH (A) and LH (B). The integrity and purity of the yeast (*Pichia Pastoris*) produced recombinant hormones were verified by SDS PAGE (left panel) and Western blot (right panel) analyses. The immuno-detection was conducted with hormone specific polyclonal anti-FSH and anti-LH. M- Molecular size marker.

The *in vitro* bioactivity of the produced r-FSH and r-LH, was examined by their capacity to stimulate 11-ketotestosterone (11-KT) secretion from grey mullet testicular fragments (**Fig. 7.1.5**). Both r-FSH and r-LH stimulated 11-KT secretion 4-folds higher than the controls. Nevertheless, the r-FSH effective dose (0.5 ng ml^{-1}; **Fig. 7.1.5A**) was 200-folds lower than that of rLH (100 ng ml^{-1}; **Fig. 7.1.5B**), indicating higher potency of the former hormone.

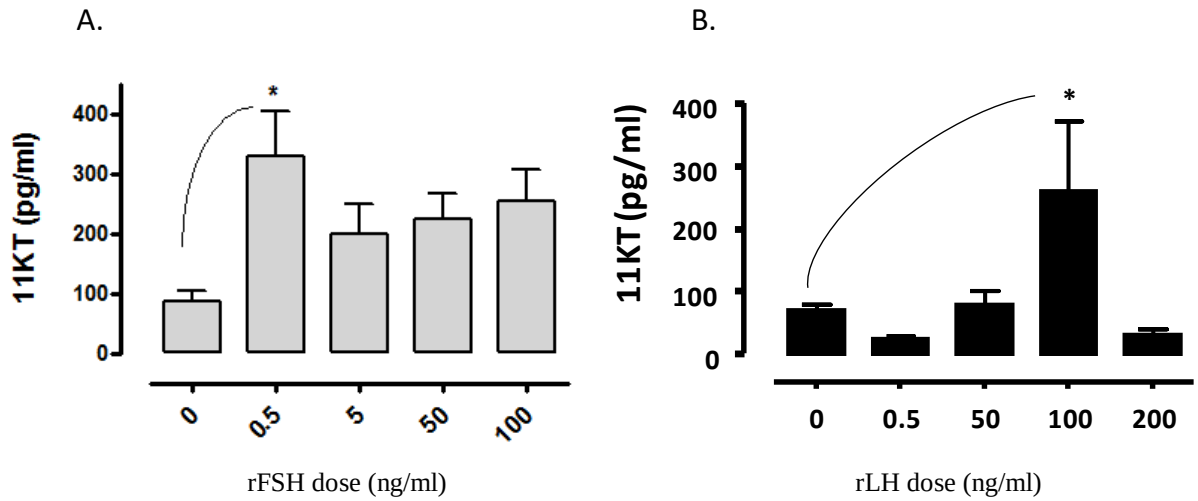


Figure 7.1.5. *In-vitro* effect of r-FSH (A) and r-LH (B) on 11-ketotestosterone secretion from testicular fragments. Results are shown as Means ± SEM (n=16). An asterisk indicates significantly different means ($P < 0.05$, Student's t-test).

7.1.3 Hormonal acceleration of gonadal development

To further improve grey mullet gamete quality in general and sperm quality in particular, the yeast produced recombinant gonadotropins (r-FSH and r-LH) were used as therapeutic agents. Over two consecutive natural spawning seasons (2014 and 2015) we evaluated the effectiveness of several hormonal treatments on gonadal development and gamete maturation in captive grey mullet broodstock. This work was led by P4. IOLR and has been completed and submitted in ***Deliverable 7.3 Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet*** .

Grey mullet breeders, consisted of P4. IOLR hatchery-produced (G1) fish that were individually tagged and maintained in 4-m^3 or 5-m^3 tanks supplied with ambient seawater at 40-ppt salinity (Gulf of Eilat, Red Sea) and subjected to natural fluctuations of light and temperature conditions (elevation to 25°C in June, 28°C in August). Fish were fed daily at the rate of 1-1.5% of their body weight using a 30% crude protein and 4% lipid commercial feed (Raanan, Israel). Sex was predicted according to vitellogenin dotblot immunoassay as described in Aizen et al. (2005), and then validated during gametogenesis when gonadal biopsies were performed.

Experiment 1 (2014) - *Effects of recombinant LH and FSH on testicular development.*

This experiment evaluated the short-term (3-weeks post treatment) effects of recombinant gonadotropins, r-LH and r-FSH (see D7.2), on testicular development. During the onset of the natural reproductive season (6^{th} of August), grey mullet males (n=12 per treatment) were injected intramuscularly with either: (i) saline (control group), (ii) r-FSH (5 µg kg^{-1} BW), or (iii) r-LH (5 µg kg^{-1} BW). Three weeks later, all fish were sampled. Body and gonad mass were recorded and the respective gonadosomatic index (GSI: gonad

weight/body weight*100) values were calculated. Blood and tissues (pituitary and gonads) were collected for further analyses, including: gonadal histology, 11-ketotestosterone (11-KT) measurements, and quantification of pituitary LHβ and FSHβ mRNA levels.

Results indicate no significant difference in BW across treatments (**Table 7.1.1**). Nonetheless, the r-FSH-treated males exhibited significantly ($P<0.05$) higher GSI values and advanced stages of spermatogenic cells (**Fig. 7.1.6**) when compared to those treated with saline only. The plasma 11-KT levels were higher in the r-FSH treated-group than in the control (90.36 ± 20.21 and 54.24 ± 8.7 pg ml^{-1}, respectively; **Fig. 7.1.7**). Lowest levels of 11-KT were measured in the r-LH treated males (43.38 ± 5.1 pg ml^{-1}).

Table 7.1.1. Treatment effect on body weight (BW) and Gonadosomatic Index (GSI). Values are expressed as mean ± SEM, (n=12). Different letters indicate significantly different means ($P < 0.05$, Student's t-test).

Treatment Group	BW (g)	GSI (%)
C	950 ± 49	0.045 ± 0.010^{a}
r-FSH	863 ± 41	0.088 ± 0.012^{b}
r-LH	890 ± 52	0.072 ± 0.014^{ab}

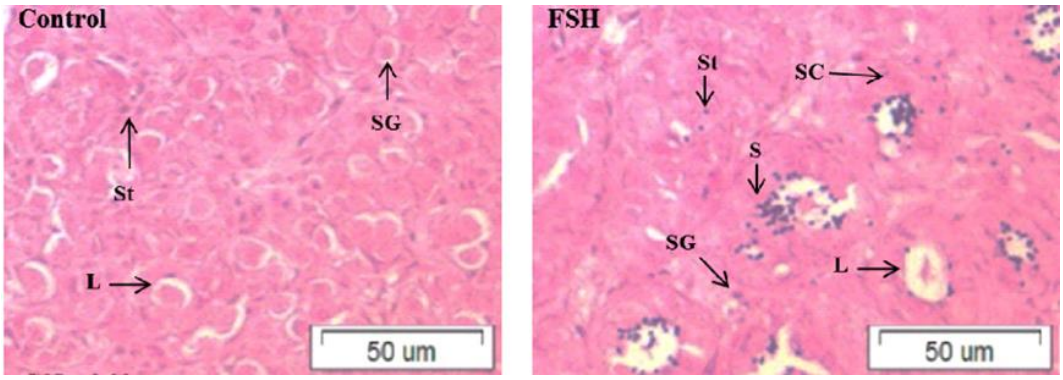


Figure 7.1.6. *In vivo* effect of rFSH on spermatogenic development in captive grey mullet. Coronal section of gonads, H&E staining. Organ is composed of mainly undifferentiated gonocytes. Black triangle indicates early features of male differentiation of the gonad as appearance of a clustered organization (spermatocysts). The holes in the images indicate early lumen (L) formation. St = Spermatids; SG= Spermatogonias; SC= Spermatocytes

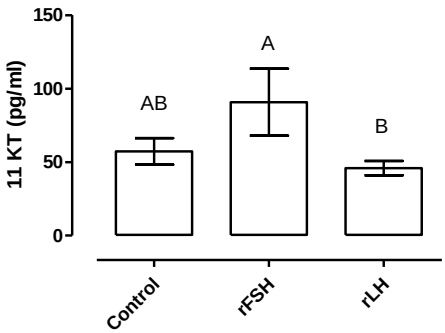


Figure 7.1.7. Plasma levels of 11-KT in control, rFSH or rLH treated grey mullet males. 11-KT Values are expressed as mean ± SEM (*n*=11). Different letters indicates significantly different means ($P < 0.05$).

Pituitary FSHβ and LHβ transcript levels in control groups sampled at the beginning of the experiment (C0; early-August, 2014) and 3-weeks later (C1; mid-September, 2014) indicate 3-fold and 5-fold increased expression over time for the FSHβ and LHβ genes, respectively (**Fig. 7.1.8**). Nonetheless, the initial levels of LHβ were approximately 3-fold lower as compared to the FSHβ ones. Treatment effect on endogenous LHβ and FSHβ transcript levels was observed only in the r-FSH-treated fish (**Fig. 7.1.9**). These fish expressed significantly ($P < 0.05$) higher LHβ mRNA levels as compared to the r-LH and saline treated groups, which did not significantly ($P > 0.05$) differ from one another.

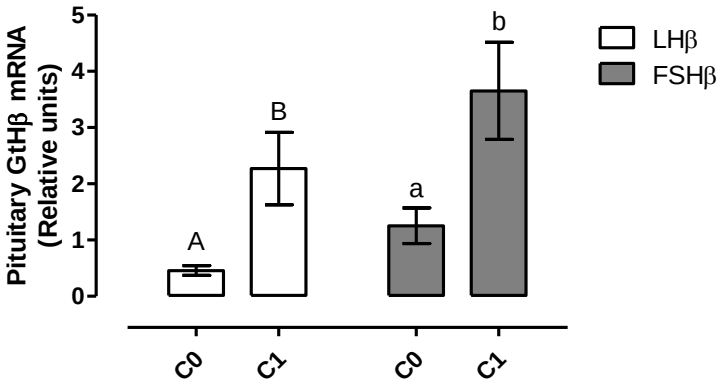


Figure 7.1.8. Pituitary expression levels of FSHβ and LHβ genes in grey mullet males at early stages of the reproductive season. The pituitary mRNA levels of *FSHβ* and *LHβ* were measured using relative Real time PCR method (RQ) at the onset of the reproductive season (C0; early August 2014) and 3-weeks later (C1; mid-September). Levels (Mean ± SEM) are expressed as relative units, normalized to the amount of 18S rRNA. Means with different letters were significantly different ($P < 0.05$).

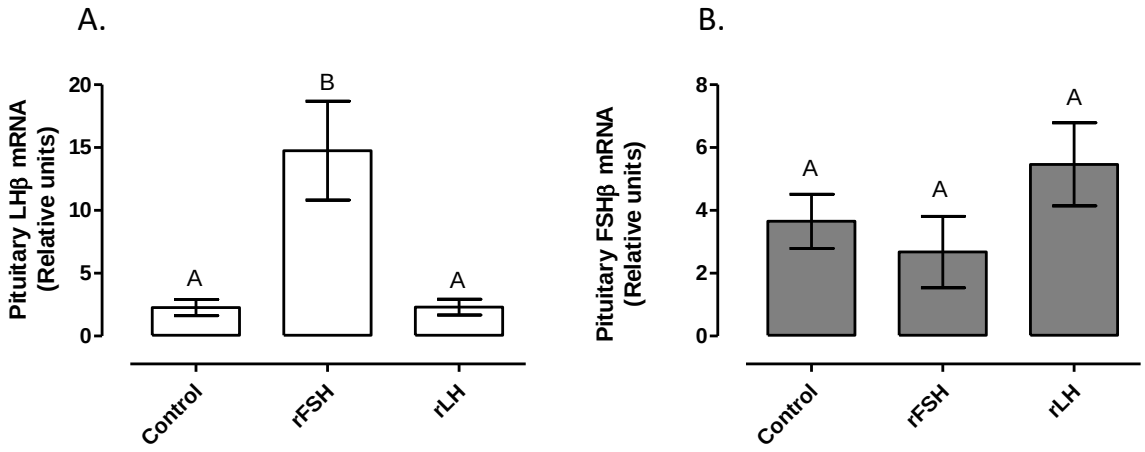


Figure 7.1.9. Effect of recombinant FSH and LH (r-FSH and r-LH, respectively) treatments on endogenous pituitary FSHβ and LHβ mRNA levels in grey mullet males. The pituitary mRNA levels of *FSHβ* and *LHβ* were measured using relative Real time PCR method (RQ). Levels (Mean ± SEM) are expressed as relative units, normalized to the amount of 18S rRNA. Means with different letters differ significantly ($P < 0.05$) from one another.

Experiment 2 (2014) - *Effects of recombinant FSH and dopamine antagonist on gonadal development in grey mullet females and males.*

In a second series of trials, grey mullet females and males (n= 186; age: 5-year old) were treated during mid-July (2014) with r-FSH (5 μg kg^{-1} BW) combined with Metoclopramide (Metoc; dopamine antagonist)

dissolved in ddH2O (15 mg kg^{-1} BW). The control fish were injected intramuscularly with saline only. The experiment was conducted in triplicates. Two weeks following the first injection (early August 2014), males received 17alpha-methyltestosterone (MT) loaded on EVAc slow-release implants, at 5 mg kg^{-1} BW produced by P1. HCMR. Upon need, additional (1 or 2) MT injections were given to enhance spermiation. The state of ovarian development, as determined by oocyte diameter, was examined, during late September and through October (natural spawning season) by obtaining ovarian biopsies using a polyethylene canula. Females were considered post-vitellogenic when mean oocyte diameter was greater than 550 µm (De Monbrison et al., 1997) and more than 50% of sampled oocytes exhibited germinal vesicle migration. In parallel, males were checked for the presence of milt by applying gentle abdominal pressure.

The relative abundance of fully mature grey mullet females and spermiating males in hormonally treated and control groups, at early- and mid-spawning season (mid-September and mid-October, respectively) are summarized in **Table 7.1.2**. The hormonal treatment consisting of r-FSH and Metoc, appears to synchronize gonadal maturation in both females and males, giving rise to consistently higher percentages of fully mature specimens as compared to control groups. The treatment effect was more pronounced in females (up to 4-fold higher frequencies of fully developed specimens compared to controls) than in males (1.2-1.3 fold higher frequencies compared to controls).

Table 7.1.2. Relative abundance of post-vitellogenic grey mullet females and spermiating males at early- and mid-spawning season (mid-September and mid-October, 2014) in control and hormonally treated fish.

	Control		Treatment	
	Mid September	Mid October	Mid September	Mid October
Fully mature females (%)	29	20	91	75
Spermiating males (%)	70	50	86	67

Experiment 3 (2015)- ***Effects of dopamine antagonist and GnRHa agonist on ovarian development.***

In a third series of trials (4 groups, each consisting of 25 specimens; age: 5-year old), grey mullet females were injected on July 29th (2015) with either Metoc (15 mg kg^{-1} BW) alone or its combination with GnRHa-EVAc (36 µg per fish) produced by P1. HCMR. Males in both treatment groups were injected with r-FSH (5 µg kg^{-1} BW). One month later half of the males received MT-EVAc implant (5 mg kg^{-1} BW). Two additional control groups were injected intramuscularly with saline only. Gonadal biopsies were carried out at two consecutive months: September 2nd, and October 7th. The relative abundance of fully mature females, and spermiating males were recorded. Sperm quality was classified into one of four categories based on its quantity, fluidity and ability to spread in the water. Additionally, treatment effects on sperm characteristics were evaluated using CASA adapted to grey mullet (see Deliverable 7.1).

The added potential of GnRHa-EVAc was tested to complement the Metoc-stimulatory effect on captive grey mullet ovarian development. During the early phase of the spawning period (September 2015) higher frequencies of females exhibiting advanced stages of vitellogenesis (*i.e.* oocyte diameter greater than 300 µm) were found in both treatment groups compared to controls (**Fig. 7.1.10A**). Nonetheless, the combined treatment, consisting of Metoc and GnRH-EVAc, appeared to be more effective than the Metoc only. The Metoc and GnRH-EVAc combined treatment synchronized and accelerated oocyte development, giving rise to over 70% vitellogenic females during September (**Fig. 7.1.10A**) and 50% post- vitellogenic females one month later, as the spawning season progressed (**Fig. 7.1.10B**). Interestingly, relatively high frequencies of vitellogenic females were observed in the control groups (up to 50%) during September (**Fig. 7.1.10A**), however, only a minority (less than 18%) reached the post-vitellogenic stage (*i.e.* oocyte diameter greater than 550 µm) during October (**Fig. 7.1.10B**).

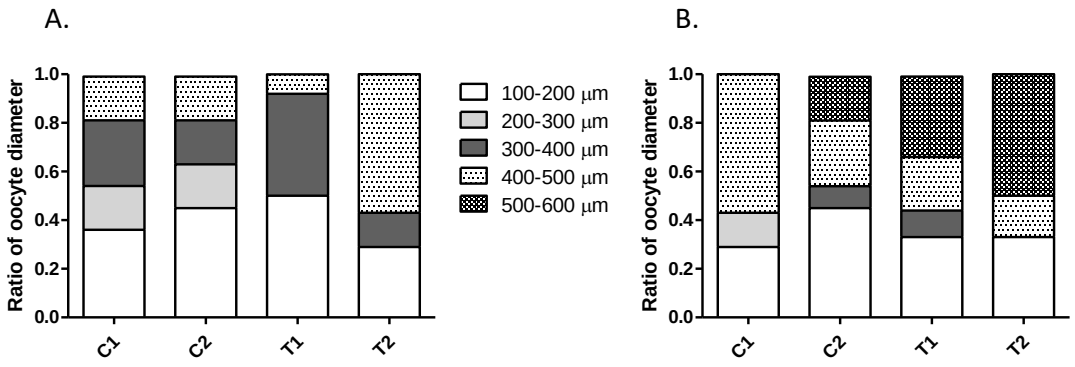


Figure 7.1.10. Effects of Metoc and GnRH-EVAct on ovarian development in captive grey mullet females (July–October 2015). Oocyte diameter profiles in control (C1, C2), Metoc (T1) and Metoc+GnRH-EVAc (T2) treatment groups (n=25), during September (A) and October (B).

Experiment 4 (2015) - *Effects of r-FSH and MT-EVAc implants on sperm production.*

The forth experiment tested the effect of a single r-FSH injection (5 µg kg^{-1} BW) vs. double r-FSH (2.5 µg kg^{-1} BW) injections given two weeks apart; August 5th and 19th) on sperm production over time. Each group consisted of 25 fish. Four weeks following the first rFSH injection, the non-spermiating specimens in each treatment group received MT-EVAc implant. The females in both treatment groups received Metoc (15 mg kg^{-1} BW) injection on August 5th. The relative abundance of fully mature females, and spermiating males were recorded, and sperm quality was evaluated as above.

This experimentation with the grey mullet males evaluated the effect of r-FSH as a sole therapeutic agent vs. r-FSH use to prime the fish prior to the administration of MT-EVAc implants. The r-FSH treatment showed no stimulatory effect on sperm production compared to controls neither during September (**Fig. 7.1.11A**) nor during October (**Fig. 7.1.11B**). However, all males that were primed with r-FSH and then subjected to MT-EVAc implantation produced sperm (**Fig. 7.1.11B**). Moreover, the latter group exhibited relatively higher percentages (66%) of fully spermiating males compared to rFSH-treated (33%) and control groups (24%).

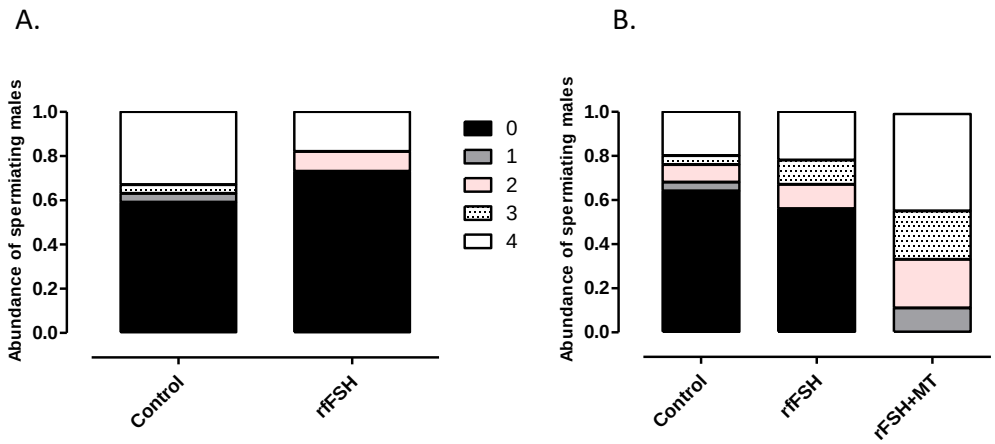


Figure 7.1.11. Effects of rFSH and MT-EVAc on sperm production in captive grey mullet males (July–October 2015). (A) Abundance of spermiating males in r-FSH treated and control groups (n=25) 4 weeks post treatment (September 2015). (B) Abundance of spermiating males in control, r-FSH, and r-FSH+MT treated groups (n=25) 4-weeks after the MT-EVAc implantation (October 2015). 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.

A CASA analysis revealed no significant (P > 0.05) treatment-effect on spermatozoa concentrations. Regardless of the treatment group, all counts ranged between 50 to 70 million cells ml^{-1} (**Fig. 7.1.12**). However, males in the rFSH-treated group exhibited sperm with relatively prolonged motility compared to those in the rFSH+MT and control groups (**Fig. 7.1.13**).

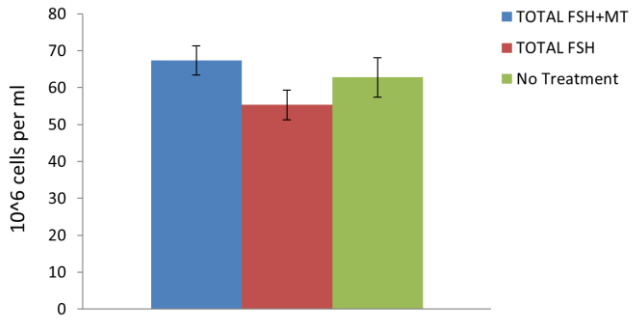


Figure 7.1.12. Effects of hormonal treatment (rFSH and rFSH+MT) on spermatozoa concentrations (n=27).

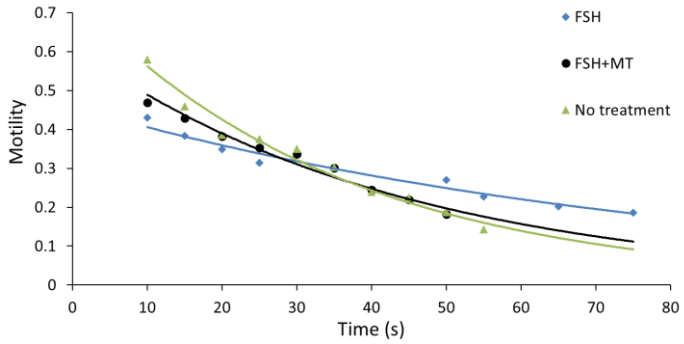


Figure 7.1.13. Effects of hormonal treatment (rFSH and rFSH+MT) on spermatozoa motility (n=27).

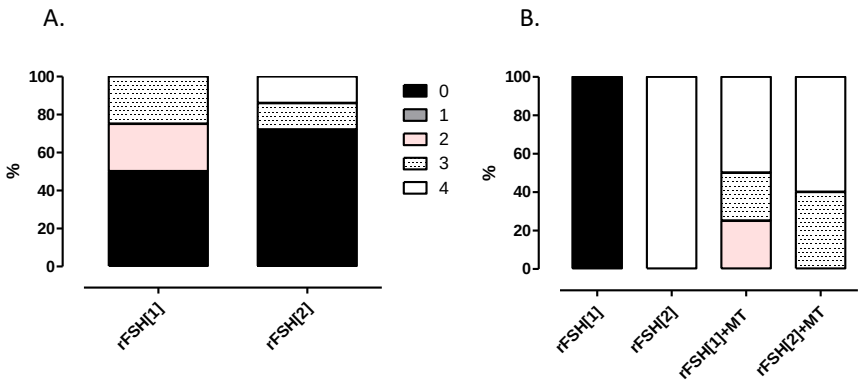


Figure 7.1.14. Effects of rFSH and MT-EVAc on sperm production in captive grey mullet males (July–October 2015). (A) Abundance of spermiating males in groups (n=13 per group) treated with a fixed rFSH dose given either via a single or double injections (r-FSH[1] and r-FSH[2], respectively) 4 weeks after first injection (September 2015). (B) Abundance of spermiating males in r-FSH, and r-FSH+MT treated groups (n= 6 per group) 4-weeks after the MT-EVAc implantation (October 2015). 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.

In addition, this study evaluated the effectiveness of a fixed r-FSH dose (5 µg kg^{-1} BW) administrated as a single injection or divided into two injections given 2-weeks apart. Although of a preliminary nature, it appears that treatment based on two- r-FSH injections is more effective giving rise to prolonged spermiation over time compared with a single injection (**Fig. 7.1.14**).

The full description of the work and results was provided in ***Deliverable D7.3 Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet***.

Conclusions

- The CASA analyses show that grey mullet sperm does not present a pattern of motility similar to that of European seabass or rainbow trout in terms of velocity as well as duration which lasts less than one minute.
- The employed methylotrophic yeast expression system produced a satisfying yield of recombinant gonadotropins.
- Both, r-FSH and r-LH, were able to stimulate grey mullet gonadal steroidogenesis *in vitro* although with different bio-potencies. In this respect, at the early stages of the reproductive season, r-FSH but not r-LH, enhanced steroidogenesis as well as somatic and germ cell proliferation in captive grey mullet males.
- The r-FSH seems to be a potent initiator of gonadal growth and germ cell proliferation/differentiation, therefore, the timing of administration may solve the observed reproductive dysfunctions in male grey mullet giving rise to successful captive breeding of this species.
- The combination of r-FSH injection and EVAc implant for sustained release of MT, was the treatment that both induced a further advance in spermatogenesis and a higher percentage of breeders to advance to spermiation among captive grey mullet males.
- Treatment combining dopamine antagonist (metoclopramide) and r-FSH enhanced and synchronized ovarian development in captive grey mullet females, giving rise to 91% post-vitellogenic females within the treatment-group.

Task 7.2 Develop hormone-based treatments for induced spawning of grey mullet (led by IOLR, Hanna Rosenfeld).

Spawning induction trials were carried out during natural (2014 and 2015) and shifted (2016) spawning season. Once identified, a reproductively mature female was stocked with either two or three spermiating males 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^{-1}; Metoc 15mg kg^{-1}) and resolving injections (GnRHa 20 µg kg^{-1} ; Metoc 15 mg kg^{-1}) given 22.5 h apart.

7.2.1 Induced-spawning trials during natural spawning season (Mid-September - November, 2014, 2015)

First series of trials (n=31) were carried out with fish primed with the combination of dopamine antagonist and r-FSH (Ex. 2; Task 7.1.3) or saline (control). Both hormonally-primed and control groups gave rise to candidates (n=14 and n=17, respectively; **Table 7.2.1**) fulfilling prerequisite criteria for the spawning induction trials. However, spawning successes was improved in the pre-treated groups compared to controls (42.9% and 29.4%, respectively). The overall successful spawns produced 42 million eggs in total. Based on numbers of floating eggs a total of 75% of the spawned eggs were considered viable.

Table 7.2.1. Summary of the spawning data obtained from hormonally induced grey mullet females during September-November 2014. Relative fecundity is expressed as means ± SEM. Spawning ratio signifies the number of females that ovulated after injection, divided by the total number of injected females. Relative fecundity stands for total number of eggs kg^{-1} body weight of treated females.

Date	Control				Treatment			
	No. of induction trials	Spawning success (%)	Fecundity (million eggs /KgBW)	Fertilization rate (%)	No. of induction trials	Spawning success (%)	Fecundity (million eggs /KgBW)	Fertilization rate (%)
10.9.14	2	0	0	0	2	0	0	0
29.9.14	1	0	0	0	3	100	2.6 ± 0.55	0-98
6.10.14	0	0	0	0	2	0	0	0
22.10.14					3	66	2.1±0.39	0-80
25.10.14					1	100	0.6	30
30-31.10.1	6	50	1.99±0.9	50-100				
5.11.14	5	0	0	0	2	0	0	0
14.11.14					1	0	0	0
21.11.14	1	100	1.64	0				
22.11.14	2	50	2.75	90				
	17	29.4			14	42.9		

Second series of trials (n=23) were carried out with hormonally-primed and control fish (Ex. 3; Task 7.1.3) during 2015. The spawning data is summarized in **Table 7.2.2**. Twenty three females were induced to spawn resulting to the production of about 35 million eggs. However, the vast majority of the eggs were non fertile. It seems that the conserved spawning induction treatments, consisting of GnRHa and Metoc were most effective in inducing final oocyte maturation and ovulation. Following the priming and to a greater extent the resolving injection, the ovulating females can be easily recognized by their swollen belly (**Fig. 7.2.1A**). However, many of the females with swollen bellies (over 40% during the 2015 spawning induction trials) did not advance to spontaneously release the eggs, and frequently (30%) died (**Fig. 7.2.1B**).

Table 7.2.2. Summary of the spawning data obtained from hormonally induced grey mullet females during September-November 2015. Priming treatment refers to hormonal treatments administered prior to or during gametogenesis to accelerate gonadal development in captive grey mullet females and males. Group structure indicates the number of female and males in each spawning unit.

Date	Priming treatment		Group structure		Remarks	Total volume of eggs (ml)	Fertilization rate	Hatching rate
	Females	Males	#Females	#Males				
9.9.2015	Metoc+GnRH	FSH + MT	1	2		640	no	
9.9.2015	Metoc+GnRH	FSH + MT	1	2	The female has died	0		
10.10.2015	Metoc	FSH + MT	1	3		930		
10.10.2015	Control	Control	1	3		650	no	
10.10.2015	Control	Control	1	3	The female was stripped	350	10%	
14.10.2015	Metoc	FSH + MT	1	4	The female has died	0		
14.10.2015	Metoc	FSH + MT	1	3		370		
14.10.2015	Metoc	FSH + MT	1	3		650	20%	11.60%
14.10.2015	Metoc	FSH + MT	4	5	2 out of 4 females spawned	520	20%	
14.10.2015	Metoc	FSH + MT	1	3		0		
21.10.2015	Metoc	FSH + MT	1	3		1930	5%	0.20%
31.10.2015	Metoc	FSH + MT	2	3		1105		
31.10.2015	Metoc	FSH + MT	3	4	1 female died and 1 spawned	1900	15%	
31.10.2015	Metoc	FSH + MT	1	3		0		
31.10.2015	Metoc	FSH + MT	2	3		710		
11.11.2015	Metoc	FSH + MT	1	1				
		Total number of induction trials	23		Total volume/ number of spawned eggs	9755 ml = 35.12 million eggs		

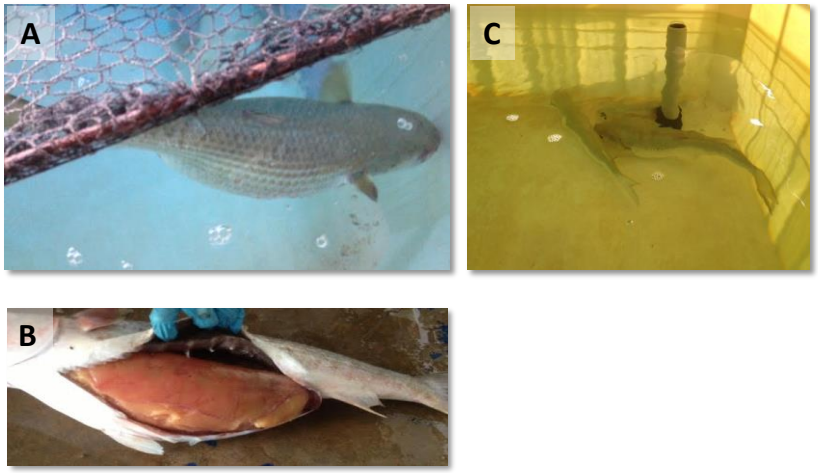


Figure 7.2.1. Spawning of captive grey mullet. (A) Hormonally-stimulated grey mullet female exhibiting swollen belly prior to egg release. (B) Hormonally-stimulated female that failed to release the eggs and died. (C) Grey mullet female and male (big and small specimens, respectively) courtship prior to spawning.

Nonetheless, despite of the successful spawning season, two major problems were highlighted: (i) female's failure to ovulate (70% and 57% in control and hormonally-treated groups, respectively) and (ii) variable fertilization rate ranging between 0 to 100%.

7.2.2 Induced-spawning trials during a shifted-spawning season (January –February, 2016)

Four-year-old grey mullet broodstock (Females: n=19, BW= 1.64 ± 0.08 kg; Males: n=16, BW=1.02±0.07 kg) were acclimated to a 4-month shifted photoperiod. During December 3rd the fish were primed with the Methoc and r-FSH combination, which appears to be the best performing protocol (see paragraph 7.1.4).

Table 7.2.3. Summary of the spawning data obtained from hormonally induced grey mullet females during January-February 2016. Group structure indicates the number of females and males in each spawning unit.

Date	Pit-tag	Sex	Weight (g)	oocyte diameter/St age -sperm	Egg volume Floating (F) vs. Sinking (S)	fertilization	Eggs(ml)/KgBW
11/01/2016	974F	F	2185	520-530	The female died		
	0343	M	750	R5			
	2585	M	885	R5			
	7563	M	815	R5			
11/01/2016	88BF	F	1240	540-560	220 ml F; 110 ml S	90%	266 ml/kgBW
	08A4	M	1540	R5			
	8C73	M	810	R5			
	3C6F	M	855	R4			
25/01/2016	8F59	F	1450	510-530	650 ml F; 160 ml S	95%	558 ml/kgBW
	8C73	M	810	R5			
	2188	M	1055	R5			
	4E16	M	1120	R5			
25/01/2016	8253	F	1705	510-530	560 ml F; 100 ml S	95%	388 ml/kgBW
	0343	M	730	R5			
	C1C6	M	1015	R5			
	3C6F	M	845	R5			
01/02/2016	28AB	F	1640	510-530	410 ml F; 180 ml S	50%	359 ml/kgBW
	C1C6	M	1015	R5			
	08A4	M	1540	R5			
	0343	M	730	R5			

The selected fish were hormonally induced to spawn using GnRHa analogue with the combination of dopamine antagonist (see Task 7.2.1). The spawning data is summarized in **Table 7.2.3**. Five females were induced to spawn, out of which one female died while the other four successfully spawned. Fertilization rate ranged between 50-95%.

Conclusions

- A spawning unit consisting of 1 female and 3 males appears to improve spawning success.
- The female´s failure to spawn the eggs and the low fertilization rate suggest that there were male to female communication problems during the spawning event.

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

No work done during this period**.**

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)

Hatchery produced (G2; spawning date at IOLR: 31.10 2014) and wild caught grey mullet fingerlings were stocked in concrete ponds (30 m^3; 20 m^2) at two different densities (0.7 and 1 kg m^{-2}). These fish will be monitored until sexual maturity. In addition, a stock of wild fingerlings obtained by P26. GEI for the Grow out experiments (WP 23) will be maintained for at least 3 years or until sexual maturity, and will be used for this task. This is done at no extra charge for the project.

Task 7.5 Establish a shipping protocol for grey mullet eggs (led by DOR, Gilad Safran)

This task is aimed at establishing procedures for handling grey mullet eggs in order to allow transport to various larval rearing facilities. The development of the protocol was based on methodology developed earlier by P4. IOLR for shipping Atlantic bluefin tuna (*Thunnus thynnus* 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 20 l cubitainers (stiff plastic 6 sided, square bottom cubitainers used in the wine industry), which is placed in a Styrofoam container (**Fig. 7.5.1A**). 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 (**Fig. 7.5.1B**).

The eggs were temperature acclimated in the tanks, pH was incrementally increased by dripping a solution of 0.1 N NaOH into the cubitainers to reach a pH of 7.5 to 8.0, the percent of dead sinking and live floating eggs was calculated and the eggs stocked in the experimental system. The following day the percent (%) hatching and survival to 1 day after hatching was high (ca 79-90 %) provided good quality eggs were sent. Based on the success of this methodology it was also tested when transporting grey mullet eggs within Israel (ca 10-11 h in transit) and a simulated transport for longer periods (23 h) was attempted. Importantly, higher grey mullet egg densities (55,000-84,000 eggs l^{-1}) were sent compared to the BFT egg (10-15,000 eggs l^{-1}) shipments.

(A) (B)

Figure 7.5.1. (A) 20 l cubitainer and (B) Styrofoam box with ice pack in cardboard, used for the shipping of fish eggs.

Experiment 1 (2014)-*Deliveries to P25. DOR Fish Farm*

During 2014, three shipments of grey mullet eggs were made to P25. DOR Fish Farm from P4. IOLR and one shipment was made from the kibbutz Ma'agan Michael to the P4. IOLR. A modified BFT protocol was employed where the final volume of the water in the 20 l cubitainer was 15 l and egg density was 55-84,000 gastrula-stage eggs l^{-1}. Pure oxygen was 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, were placed adjacent to the air-oxygen layer (not against the water layer) and the package closed. **Table 7.5.1** lists shipping conditions, as well as oxygen content and hatching results at final destination for grey mullet eggs in 2014 from the P4. IOLR facility to the P25. DOR fish farm. Shipment results of eggs sent from kibbutz Ma'agan Michael to the IOLR were also included, which has a slightly longer transit time of 11h. In contrast, the eggs shipped from the Ma'agan Michael hatchery to P4. IOLR utilized similar high egg densities (66-110,000 eggs l^{-1}), but were sent using thick plastic bags filled with pure oxygen. Domestic shipments necessitated only one internal flight and vehicle transport to the destination.

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 SELFDOTT 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	1.26×10^6	15	84000	272	9.0	85
16.8.14	2	250	0.825×10^6	15	55000	280	9.0	85
3.10.14	1	250	1.6×10^6	15	55000	330	10.5	90
3.10.14	2	250	1.6×10^b	15	55000	330	10.5	90
16.10.14	1	350	1.26×10^6	15	84000	265	9.0	85
	Eggs sent from Kibbutz Ma'agan Michael to IOLR							
14.8.14	1	500	1.65×10^6	15	110000	270	11	96
14.8.14	2	300	99000	15	66000	265	11	97

*Measured at destination

The results of **Table 7.5.1** show that despite higher egg densities in the cubitainers, compared to the BFT egg shipping protocol, the shipments arrived at their domestic destinations in 9-10 h where oxygen levels (265-339 %) and hatching rates (85-90 %) were very good. In fact, eggs that were sent in plastic bags from Ma'agan Michael to the IOLR arrived in excellent condition after 11 h and also demonstrated hatching rates of 96-97%. Taken together, these results suggest that short term shipping of gastrula stage grey mullet eggs, provided that egg quality is very good, can be readily carried out using cubitainers or strong plastic bags as long as pure oxygen is added and that the shipment does not encounter temperature extremes.

Experiment 2 (2015)- ***Testing longer transit times***

In order to test longer transit times and to monitor water quality more closely, a controlled simulation trial was set up. Two gastrula-stage egg densities (10 and 15,000 eggs l^{-1}) were tested in replicates of 4 and 3 Styrofoam containers for each treatment, respectively, over a period of 23 h (Table 7.5.2). Pure oxygen was added to the cubitainer, followed by securely closing the container. The cubitainers were then placed into Styrofoam containers (30x30x39 cm) where two icepacks, wrapped in cardboard were placed on the upper part of the container next to the air pocket. The Styrofoam boxes were placed in a temperature controlled room (24°C) for 23 h. During this period all boxes were gently shaken every few hours. A data logger was placed in Styrofoam container 7 to measure temperature variability throughout the trial (**Table 7.5.2**).

Table 7.5.2. The effect of transit simulation on water quality (pH, O_2, NH_3) in cubitainers, 23 h after trial. Poor quality eggs with low fertilization rates were used and no hatching was observed.

T0				23 h		
Box no.	pH	O_2	NH_3 (mg/l)	pH	O2	NH_3 (mg/l)
1		365		8.01	285	
2		390		8	290	
3		287		8	297	
4	8.02	314	<0.05	7.98	235	<0.05
5		257		7.96	242	
6		307		8.01	256	
7		296		8	290	

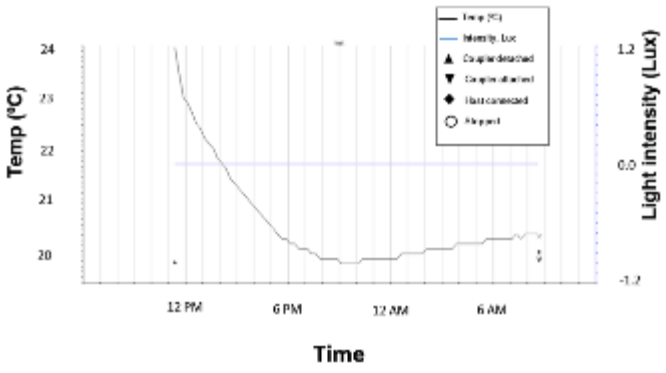


Figure 7.5.1. Temperature (^{0}C; black line) and Light intensity (Lux; blue line) measurements (data logger) in Styrofoam container 7 during the 23 h simulation of transporting grey mullet eggs. Temperature decreased from approximately 24 to 20 ^{0}C during the simulation.

Single eggs from the same batch were taken and carefully placed in each of 12 wells (3 ml) in each of 3 plastic plates to determine hatching success, after incubation in a controlled temperature incubator.

Unfortunately, this batch of eggs was poorly fertilized and hatching did not occur either in any of the 12-well plates nor in the Styrofoam containers. In fact, this study was repeated, but egg quality remained poor and no hatching was observed in the second trial either. Nevertheless, the pH, oxygen and ammonia were measured at the end of the trial in order to determine if water quality was compromised during the 23 h simulation.

Despite the failure of the eggs to hatch due to poor fertilization, **Table 7.5.2** shows that water quality was conserved, in terms of pH, O_2 and NH_3 over the 23 h test period while temperature fluctuated between 20-24 ºC (**Fig. 7.5.1**). This suggests that implementing the BFT transport protocol in sending eggs to the other partners would be sufficient at the two egg concentrations tested.

Experiment 3 (2015)- ***Nitrofurazone treatment of eggs at destination***.

A further step of reducing the bacterial load of transported eggs upon arrival was investigated in 2015. Polydine disinfectant treatment of eggs, which was the selected disinfectant with the eggs of other species, was not successful in treating grey mullet eggs as this approach frequently resulted in >70% mortality of live and highly buoyant eggs. On the other hand, no disinfection commonly leads to the development of a red bacteria on the tank walls, which has recently been identified at the IOLR as a potential cause of newly hatched larval mortality. Consequently, a number of preliminary toxicity trials were conducted with nitrofurazone, an antibiotic that appears to successfully treat this bacterial strain. In Israel, it is not legal to use nitrofurazone to treat fish during grow-out for the market, but this antibiotic can be used to treat eggs. These studies were carried out by stocking 1-2 gastrula-stage grey mullet eggs in each well of three 24 well plastic plates for the control (no antibiotic) and two nitrofurazone concentration treatments (2.5 and 5.0 mg l^{-1} of ambient 40 ‰ seawater). All nine 24 well plastic plates were placed in an incubator at 25.5 ^{0}C. After 33 h, hatching occurred where the percent (%) hatching and the survival of the pre-larvae immediately following hatching were noted. The latter parameter was measured as grey mullet larvae frequently die upon hatching. In addition, survival of the pre-larvae following a further 3 h exposure to the antibiotic was determined. Although the use of nitrofurazone is not true disinfection, as its action is only bacteriostatic, it was hypothesized that the reduced bacterial activity might be beneficial.

Exposing the eggs for 33 h to a 2.5 mg l^{-1} nitrofurazone concentration significantly ($P<0.05$) increased the hatching success of grey mullet eggs compared to the control and the 5 mg l^{-1} concentration (**Figs. 7.5.2 and 7.5.3**). In addition, there was no difference in the percent (%) of surviving larvae following hatching ($P>0.05$). On the other hand, if the pre-larvae continued to be exposed to the antibiotic for a further 3 h, mortality was total (**Fig. 7.5.4**). Nevertheless, further studies are necessary on nitrofurazone due to the use of poor quality eggs in these studies, as well as the need to examine shorter exposure times (<33 h) and other antibiotic candidates. Having said that, the preliminary results suggested that nitrofurazone may be an effective egg treatment approach that can markedly improve hatching rate after transport from the spawning tank or to another facility.

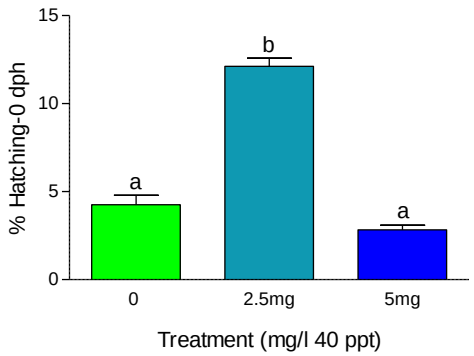


Figure 7.5.2. The effect of nitrofurazone treatment concentration on percent (%) hatching in grey mullet eggs following 33 h of immersion. Values having different letters were significantly ($P<0.05$) different.

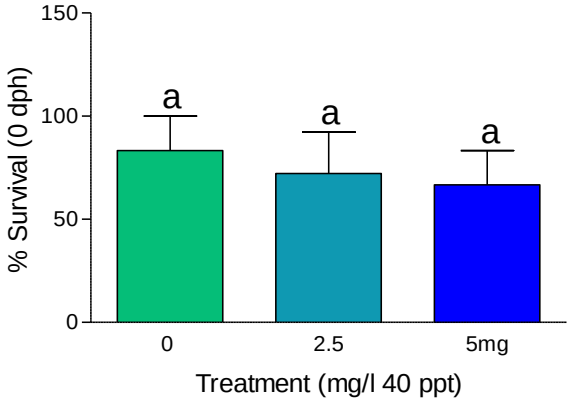


Figure 7.5.3. The effect of nitrofurazone treatment concentration on percent (%) larval survival following hatching in grey mullet eggs. Values having the same letter were not significantly (P>0.05) different.

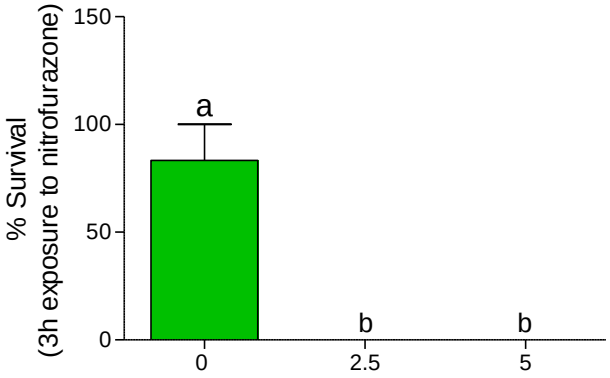


Figure 7.5.4. Survival of larvae after a further 3 h exposure to nitrofurazone following the 33 h of exposure up to hatching. Values having different letters were significantly (P<0.0%) different.

Conclusions

- Short term shipping (≤11 h) of gastrula stage grey mullet eggs, provided that egg quality is very good, 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.
- Water quality, in terms of temperature (20-24°C), pH, O_2 and NH_3,, was stable after 23 h of simulated egg transport, even when eggs of poor quality were used.
- Exposure of eggs only to nitrofurazone (2.5 mg l^{-1} sea water) is a promising treatment to reduce bacterial load and improve hatching rate. However, further studies to determine the most effective egg exposure times to this and other antibiotics are necessary.

REFERENCES

Aizen, J., Meiri; I., Tzchori; I., Levavi-Sivan, B., Rosenfeld, H., 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen. Comp. Endocrinol. 142, 212-221.

De la Gandara, F. 2012. Final Report on Project 212797: From capture based to SELF-sustained aquaculture and domestication of bluefin tuna, *Thunnus thynnus*. FP7-CP-FP

Bridges, C. 2014. Final Report on Project 311904: Translation of domestication of *Thunnus thynnus* into an innovative commercial application.

Deviations from Annex I and their impact:

Due to poor egg quality and fertilization rates in 2015, the egg shipment protocol that was submitted as ***Deliverable D7.4 Protocol for shipping grey mullet eggs***, was not thoroughly tested during the present reporting period and these studies should be repeated, and a new deliverable will be submitted. Nevertheless, the data collected so far does indicate that the SELFDOTT transport protocol can be readily adapted to shipping grey mullet eggs to the partners in DIVERSIFY. An updated version of the Deliverable will be submitted in Mo 36.

For P24. ITTICAL, it was planned to obtain a stock of wild grey mullet fingerlings (0+ year class) and maintain them until sexual maturity, to implement Task 7.4. In 5 December 2014, we were informed by the PI (Dr. Fulvio Cepollaro) that they acquired 5,000 fingerlings of 0.2 g in average size. However, upon examination of the stock maintained in the facilities of P24. ITTICAL in the Spring of 2016 by P13.UNIBA, it was found that the stock consisted of about 200 fish having a body weight ranging between 70 and 750 g, suggesting that this stock consisted of fish from different year classes (certainly no 1+ year class), and certainly not fish obtained as fingerlings in 2014 as reported by P24. ITTICAL. Therefore, this stock cannot be used for the purpose of Task 7.4 (as we do not know their real age) and contingency plans were made, by using a stock of wild fingerlings obtained by P26. GEI for the Grow out experiments (WP 23). This fish will be maintained for at least 3 years or until sexual maturity (as opposed to only 1 year to perform the WP 23 grow out study), and will be used for this task. This is done at no extra charge for the project.

Group Work Packages

Nutrition

To improve meagre larval current feeds and the optimum level of n-3 HUFA, six weaning diets containing two levels of HUFA, two of vitamin E and two of vitamin C were fed to meagre larvae. 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. The importance of supplementation of meagre weaning diets with 2.4 mg kg^{-1} vit K has been pointed out, since the absence of this vitamin markedly reduced larval survival. Meagre seemed to be very sensitive to hypervitaminosis D and 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.

Maximum growth of greater amberjack larvae was achieved in the range of dietary EPA concentrations between 8-16 %TFA, when low dietary DHA were provided. The effect of combinations of PUFA-rich lipids and carotenoids were assessed on greater amberjack larval performance, welfare and body composition. To this purpose the rotifer enrichment commercial protocol (C) was compared with three experimental emulsions. Rotifers enriched with polar rich emulsion containing a marine natural lecithin LC60 combined with 10 ppm of Naturose resulted in a significant advantage in larval growth, survival and welfare compared to rotifers enriched with other emulsions. For broodstock nutrition histidine supplementation increased the number of eggs, percentage of fertilization, hatching rate and survival of 3 days post hatching larvae.

Six experimental diets with similar levels of protein and lipid, and increasing levels of phospholipids, EPA and DHA were examined for pikeperch larvae. The combination of high phospholipid (PL) content and high DHA content improved larvae growth. Digestive enzyme activity was enhanced by dietary inclusion of PL and LC PUFAs, and trials have shown that pikeperch larvae require both high dietary inclusion levels of phospholipids in terms of soya lecithin and LC PUFAs to perform optimally. From 10 dph, larvae were fed Artemia enriched by an emulsion with high levels of n-6 fatty acids (sunflower oil), by a high level of n-3 fatty acids (rape seed oil) or by a commercial DHA enrichment medium. Three salinity levels were used (0, 5 and 10 ppt). Different patterns for EPA and DHA esterification into different lipid classes were observed, but were independent of the dietary or salinity regime. EPA was the most incorporated substrate, followed by arachidonic acid (ARA). There is also an apparent effect of salinity and dietary regime, with a decreasing incorporation of EPA with the increasing salinity for diets based on sunflower oil, and an opposite trend being observed for diets based on rapeseed oil. Adding saline water to rearing conditions did not improve growth, but changed the ability of pikeperch larvae to elongate and desaturate different fatty acids and PLs.

A protocol for weaning of Atlantic halibut at 28 days post first-feeding has been developed and almost 100% of the larvae fed Ottohime were filling up their guts with formulated feed after a five days adaptation period. A production strategy for ongrown Artemia has been established, which improves the nutritional value of Artemia with respect to protein, lipid and micronutrient contents. Growth and juvenile quality was excellent in larvae fed Artemia nauplii in this experiment and was not improved by feeding ongrown Artemia.

For wreckfish larvae, PUFA, SAFA and MUFA values (% of total fatty acids) have a little variation in the first 10 days of life. Muscle of wild fish has a large amount of proteins and low lipid levels, with DHA+EPA representing more than 30% of total fatty acids, whereas cultured fish have more lipids in muscle and liver. Diets for wreckfish broodstock should increase the amount of proteins and decreased the level of fat.

For grey mullet, a significant effect of taurine enrichment of rotifers on larval and juvenile growth from 12 to 44 dph has been described, while there appears to be no added benefit of feeding taurine enriched Artemia on larval weight. Six month juvenile grey mullet have a significant requirement for 0.5% taurine DW diet, while levels above this did not elicit further benefit. This suggests that the capability to synthesize taurine is still insufficient so this nutrient must be provided in the diet.

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):	Izquierdo, M.			
Other Scientists participating:	Lund, I. (DTU) and Rodríguez, C. (ULL)			

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 progress towards objectives and details for each task (13-30 Mo):

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

The results from this Task have been completed and submitted as ***Deliverable 8.1. Improvement of larval weaning diets.*** A brief description is provided below.

1. Optimum essential fatty acids and related micronutrients levels in weaning diets for meagre

Materials and methods

Experiment 1. A trial was conducted to test six microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco; INVE, Dendermonde, Belgium) until 14 days after hatching (dah). Meagre larvae (initial total length 4.07±0.26 mm, mean±SD; dry body weight 0.06±0.01 mg) were randomly distributed into 18 experimental tanks at a density of 2500 larvae per tank and were fed one of the experimental diets tested in triplicates for 14 days, at an average water temperature of 23.2±0.20 °C. To

avoid the nutritional contribution of Artemia with essential fatty acids and vitamins, this live prey was not added to the rearing tanks. Despite that complete weaning from 14 dah could reduce growth or survival, it was required to determine more accurately the effect of the levels of essential fatty acids and antioxidant vitamins in the weaning diets. Six isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm & 250-500 µm) were formulated using fish oil (peruvian anchovy) as source of high n-3 HUFA contents only for diets containing 3% n-3 HUFA (**Table 8.1.1**).

The desired lipid content was completed with a non-essential fatty acid source, oleic acid (Oleic acid vegetable; Merck, Darmstadt, Germany). The protein source used (squid meal) was defatted (three consecutive times with chloroform (i.e. chloroform: squid meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. Two different dietary levels of n-3 HUFAs were formulated: 0.4% (low) and 3% (high) combined with three combined levels of vitamin E+C (Vitamin E: DL-α-tocopherol acetate; Sigma-Aldrich, Madrid, Spain. Vitamin C: ROVIMIX Stay-C-35) levels vitamin E/ vitamin C: 1500/1800, 3000/1800 and 3000/3600 mg kg^{-1} (**Table 8.1.1**). Therefore six experimental diets (0.4/150/180, 0.4/300/180, 0.4/300/360, 3/150/180, 3/300/180, 3/300/360) were tested according to HUFA, vitamin E and vitamin C levels respectively. To determine larval performance and morphometry, growth was determined by measuring dry body weight and total length (Profile Projector; Nikon V-12A, Tokyo, Japan) of 30 fish per tank at the beginning, at 24 (dah) and 20 fish per tank at the end of the trial. To determine gut occupancy and digestive activity, 30 min after feeding, larvae were photographed under a binocular microscope and gut content was studied by image analysis. To determine the welfare status a stress resistance test was conducted at the end of the trial with 30 larvae that were handled out of the water in a scoop net for 30 sec. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment.

Table 8.1.1. Variable ingredients and proximate composition (g 100 g^{-1}dw) of early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels fed to meagre (*A. regius*) larvae from 14 to 28 dah.

	Diets					
	0.4/150/180	**0.4/300/180**	**0.4/300/360**	**3/150/180**	**3/300/180**	**3/300/360**
Ingredients						
Peruvian anchovy oil	0.00	0.00	0.00	10.00	10.00	10.00
Oleic acid[a]	10.00	10.00	10.00	0.00	0.00	0.00
Vitamin E*	150.00	300.00	300.00	150.00	300.00	300.00
Vitamin C*	180.00	180.00	360.00	180.00	180.00	360.00
Proximate composition						
Lipid	16.01	17.09	17.06	17.52	17.34	17.44
Protein	65.14	64.72	64.97	65.43	65.45	64.88
Moisture	10.32	10.59	9.38	9.67	9.39	9.35
Ash	5.47	5.55	5.70	5.88	5.73	5.81

Results

Gut occupancy, larval performance and morphometric parameters

The image analysis studies of the larval photographs of larvae fed the different diets denoted no significant differences in gut occupancy among fish fed the different diets. Daily weight gain in this study was ranging between 17.48±2.57% (treatment: 0.4/300/360) and 24.64±2.62% (treatment: 3/150/180); so being higher in larvae fed 3% (n-3 HUFA) (22.43±2.01%) compared to 0.4% (n-3 HUFA) larvae (18.80±1.60%). However, after only 10 days of feeding (24 dah), growth in terms of total length and dry body weight was significantly

lower in larvae fed diet 0.4/150/180 (**Table 8.1.2**), which contained the lowest HUFA, vitamin E and vitamin C levels. Larger growth was obtained in meagre fed diets 3/150/180, 3/300/180 and 3/300/360 (**Table 8.1.3**).

Table 8.1.2. Total length (mm), dry weight (mg), and survival of meagre larvae fed early weaning diets containing two levels of n-3 HUFA, vitamin E and vitamin C from 14 dah (initial total length 4.07±0.26 mm and dry body weight 0.06±0.01 mg).

	Diets					
	0.4/150/180	**0.4/300/180**	**0.4/300/360**	**3/150/180**	**3/300/180**	**3/300/360**
Total length						
24 dah	4.75±0.44^{b}	5.00±0.39^{a}	4.91±0.40^{ab}	4.96±0.45^{a}	4.96±0.48^{a}	5.06±0.38^{a}
28 dah	5.15±0.46^{ab}	5.20±0.43^{ab}	5.14±0.51^{ab}	5.29±0.44^{a}	4.97±0.31^{b}	5.34±0.59^{a}
Body weight						
24 dah	0.19±0.04^{c}	0.21±0.02^{bc}	0.20±0.03^{bc}	0.21±0.02^{bc}	0.22±0.02^{ab}	0.24±0.03^{a}
28 dah	0.23±0.02	0.21±0.04	0.21±0.03	0.27±0.05	0.23±0.05	0.24±0.04
Survival (%)	12.09±4.96	8.04±5.20	15.12±4.14	14.16±8.29	16.68±3.45	15.16±7.67

Table 8.1.3. Results of two-way ANOVA analysis on total length (mm) and dry body weight (mg) of meagre (*A. regius*) larvae fed two dietary levels of HUFA, vitamin E and vitamin C.

		Total length			Dry weight		
		24 dah	28 dah	P	24 dah	28 dah	P
HUFA	0.4	4.85±0.05	5.18±0.05	< 0.05	0.20±0.01	0.22±0.01^{b}	< 0.05
	3	4.95±0.05	5.24±0.05		0.22±0.01	0.25±0.01^{a}	
Vitamin E	1500	4.85±0.06	5.22±0.06	< 0.05	0.20±0.01	0.25±0.11	< 0.05
	3000	4.96±0.04	5.20±0.04		0.21±0.01	0.23±0.01	
Interaction		NS	NS	< 0.05	NS	NS	< 0.05
HUFA	0.4	4.89±0.05	5.19±0.05	< 0.05	0.20±0.01	0.21±0.01^{b}	< 0.05
	3	4.98±0.05	5.25±0.05		0.22±0.01	0.25±0.01^{a}	
Vitamin C	1800	4.89±0.04	5.17±0.04	< 0.05	0.20±0.01	0.24±0.01	< 0.05
	3600	4.98±0.06	5.28±0.06		0.22±0.01	0.23±0.01	
Interaction		NS	NS	< 0.05	NS	NS	< 0.05

Thus, regardless the dietary vitamin E and vitamin C levels, the increase in dietary HUFA from 0.4 to 3%, significantly (P<0.01) improved larval growth in terms of total length (4.89±0.42 and 5.00±0.43 mm for 0.4 and 3% HUFA, respectively) and dry weight (0.20±0.03 and 0.22±0.03 mg for 0.4 and 3% HUFA, respectively). Among fish fed 0.4% HUFA, elevation of dietary vitamin E from 1500 to 3000 mg kg^{-1} significantly improved total length in 24 dah larvae (P<0.01) (**Table 8.1.3**). Among fish fed 3% HUFA, increase in both vitamin E and vitamin C significantly improved body weight (P<0.05) (**Table 8.1.3**) and a significant positive linear correlation was found between dry body weight and dietary vitamin E+ vitamin C levels (y=9E-05x+0.18 R^2= 0.995). Similar trends were observed at the end of the feeding trial (28 dah). Thus, the two-way ANOVA analysis comparing the effect of dietary HUFA and vitamin E showed an improvement in growth, particularly body weight, when dietary HUFA levels were raised from 0.4 to 3%, whereas the effects of vitamin E or the interaction between both nutrients were not significant (**Table 8.1.4**).

Similarly, the two-way ANOVA analysis comparing the effect of dietary HUFA and vitamin C showed the significant positive effect of dietary HUFA on fish weight, whereas the effects of vitamin C or the interaction between both nutrients were not significant (**Table 8.1.3**).

Table 8.1.4. Main fatty acid composition (%dw) of the early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels used to fed larval meagre from 14 to 28 dah.

	Diets					
	0.4/150/180	**0.4/300/180**	**0.4/300/360**	**3/150/180**	**3/300/180**	**3/300/360**
14:0	0.09	0.09	0.08	0.60	0.63	0.63
15:0	0.01	0.01	0.01	0.08	0.09	0.08
16:0	2.41	2.17	2.00	3.29	3.84	3.26
16:1n-7	0.03	0.03	0.03	0.69	0.64	0.76
16:1n-5	0.00	0.00	0.00	0.03	0.03	0.00
16:2n-4	0.00	0.00	0.00	0.05	0.05	0.06
16:3n-1	0.01	0.01	0.01	0.01	0.01	0.02
16:4n-3	0.00	0.00	0.00	0.06	0.05	0.06
18:0	0.28	0.58	0.52	0.80	0.94	0.79
18:1n-9	9.85	10.08	10.12	3.43	3.16	3.29
18:1n-7	0.09	0.15	0.15	0.43	0.42	0.47
18:1n-5	0.00	0.00	0.00	0.02	0.02	0.02
18:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
18:2n-6	2.51	3.16	3.28	2.60	2.30	2.70
18:3n-6	0.00	0.00	0.00	0.05	0.05	0.05
18:3n-3	0.14	0.18	0.20	0.41	0.37	0.43
18:4n-3	0.00	0.00	0.00	0.15	0.14	0.16
20:0	0.04	0.03	0.03	0.05	0.06	0.05
20:1n-9	0.02	0.01	0.00	0.06	0.05	0.06
20:1n-7	0.12	0.13	0.13	0.48	0.67	0.53
20:1n-5	0.00	0.00	0.01	0.03	0.04	0.04
20:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
20:2n-6	0.00	0.01	0.01	0.06	0.05	0.06
20:3n-6	0.01	0.00	0.00	0.02	0.02	0.02
20:4n-6	0.01	0.02	0.02	0.13	0.12	0.15
20:3n-3	0.03	0.01	0.01	0.04	0.03	0.04
20:4n-3	0.00	0.00	0.00	0.08	0.07	0.08
20:5n-3	0.09	0.10	0.11	0.95	0.86	0.99
22:1n-11	0.03	0.01	0.01	0.34	0.56	0.39
22:1n-9	0.03	0.01	0.02	0.07	0.09	0.07
22:4n-6	0.00	0.02	0.00	0.02	0.02	0.02
22:5n-6	nd	0.01	0.01	0.06	0.05	0.06
22:5n-3	0.00	0.01	0.00	0.18	0.16	0.19
22:6n-3	0.17	0.22	0.27	1.64	1.52	1.67
Saturated	2.82	2.89	2.65	4.87	5.60	4.86
Monoenoic	10.18	10.42	10.47	5.59	5.70	5.66
n-3	0.45	0.52	0.60	3.54	3.22	3.64
n-6	2.54	3.22	3.32	2.94	2.62	3.06
n-9	9.91	10.10	10.14	3.58	3.33	3.44
n-3HUFA	0.29	0.34	0.39	2.89	2.64	2.97
n-6HUFA	0.02	0.06	0.04	0.29	0.26	0.31
(n-3+n-6)HUFA	0.31	0.4	0.43	3.18	2.9	3.28
18:1n-9/n-3 HUFA	5.27	5.18	4.37	0.20	0.21	0.19
n-3/n-6	0.18	0.16	0.18	1.2	1.23	1.19
EPA/ARA	1.10	1.02	1.06	1.22	1.19	1.18
DHA/EPA	0.31	0.38	0.41	0.30	0.31	0.29

Larval organ and skeleton development

Histological study of larval foregut showed that larvae fed 0.4% HUFA presented condensed enterocytes with scarce accumulation of lipid vacuoles (**Figure. 8.1.1.A and B**). However, larvae fed higher levels of dietary HUFA, such as in 3/150/180, showed enterocytes with large lipid vacuoles around the nucleus and in the basal part of the enterocyte (**Figure 8.1.1.D and E**), reflecting the higher lipid absorption activity. Similar features were observed in guts of larvae fed diets 3/300/180 and 3/300/360. Regarding the liver, larvae fed low HUFA diets showed very condensed hepatocytes with centered nucleus and marked cytoplasm staining, observing a scarce deposition of lipid reserves (**Figure 8.1.1.C**). On the contrary, larvae

fed higher HUFA levels showed hepatocytes with a higher accumulation of lipid vacuoles (**Figure 8.1.1.F**). No other alterations were found in larval organ development. Moreover, no significant differences were found in skeleton development.

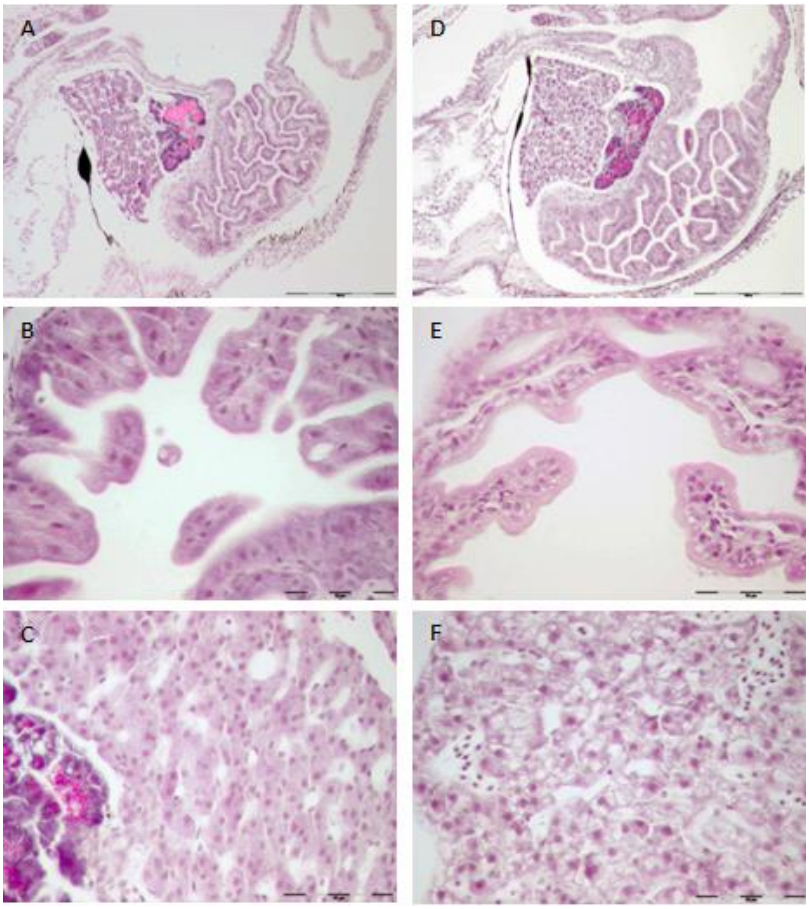


Figure 8.1.1. Sections of intestine and liver of meagre (28 dah) from different treatments. H&E.

Survival and welfare status

At the end of the feeding trial (28 dah), larval survival was not significantly different among the different groups of larvae, being in average 13.45±3.08% (mean±SD). In agreement, no significant differences were found in larval welfare status.

Biochemical composition

The diets without fish oil and containing oleic acid (0.4/150/180, 0.4/300/180 and 0.4/300/360) were characterized by a high level of monoenoic and n-9 fatty acids, particularly oleic acid (OA: 18:1n-9), as well as n-6 fatty acids, such as linoleic acid (LA) (**Table 8.1.4**). Accordingly, a high ratio OA/n-3 HUFA was obtained in these diets (**Table 8.1.4**). On the contrary, diets containing fish oil (3/150/180, 3/300/180 and 3/300/360) were high on saturated fatty acids, specially lauric (14:0), palmitic (16:0) and stearic (18:0) acids, as well as on n-3 fatty acids, including ALA, eicosatetraenoic (20:4n-3), EPA, n-3 docosapentaenoic (DPA, 22:5n-3) and DHA acids. Fish oil inclusion also raised the levels of n-6 HUFA, such as 20:2n-6, 20:6n-6, 22:5n-6 and 20:4n-6 ARA, but in a lower extend than n-3 fatty acids, and subsequently the n-3/n-6 ratio was high. All the diets kept constant proportions of the ratios among the essential fatty acids EPA/ARA and DHA/EPA (**Table 8.1.4**).

Despite dietary lipids levels were similar among diets, elevation of dietary HUFA tended to increase larval total lipid contents (**Table 8.1.5**). Moreover, lipid contents in larvae fed 3% HUFA were increased by dietary vitamin E+ vitamin C levels, and a significant positive correlation was found between the two parameters (y=0.0151x+13.26, R^2=0.91) (**Figure 8.1.2**).

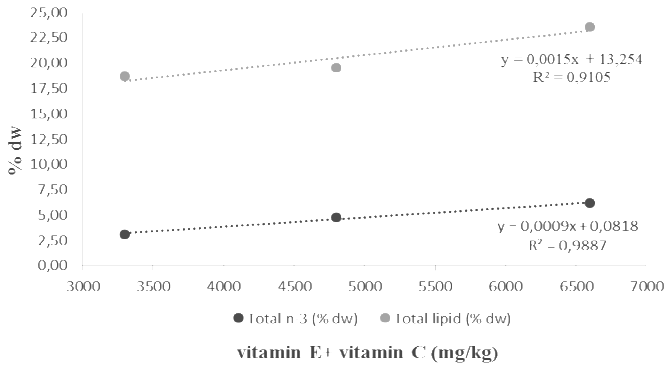


Figure 8.1.2. Effect of dietary vitamin E and C on lipid and n-3 contents (dw) in meagre (*A. regius*) larvae after 14 days of feeding 3% HUFA diets.

The n-3 HUFA contents in larvae fed diets with the low HUFA levels were even lower than those of the initial larvae, whereas feeding the high HUFA levels increased larval n-3 HUFA even over the initial levels (**Table 8.1.5**). T-student analysis showed that larval contents of n-3 HUFA were significantly ($P<0.05$) higher in larvae fed high n-3 HUFA than low n-3 HUFA. Accordingly, higher contents of DHA, EPA and ARA ($P<0.05$) were found in larvae fed high dietary n-3 HUFA. However, the total amount of saturated fatty acids was similar among larvae fed the different diets regardless dietary contents. Besides, only slightly higher values were found in larvae fed 0.4% HUFA for monounsaturated and n-6 polyunsaturated fatty acids. Despite EPA/ARA and DHA/EPA ratios were similar among the different diets, their values were higher ($P<0.05$) in larvae fed fish oil, particularly when vitamin E or vitamin E+ vitamin C were increased in the diet.

In larvae fed 3% n-3 HUFA, inclusion of vitamin E increased LA, ARA, EPA, 22:4n-6, 22:5n-6, DPA, DHA and, accordingly, the n-3, n-3 HUFA, n-6 contents and n-3/n-6 ratios (**Table 8.1.5**), regardless that similar levels were found in the respective diets (**Table 8.1.2**). Particularly, increase in dietary vitamin E+ vitamin C levels led to a significant linear increase in the DHA (y=0.008x-0.45, R^2=0.97) and n-3 fatty acid (y=0.009x+0.084, R^2=0.99) contents in the larvae. In larvae fed either 0.4 or 3% HUFA diets, the combined elevation of vitamin E and vitamin C, tended to raise larval lipid contents by increasing 14:0, 15:0, 16:0, 16:1n-7, 16:1n-5, 18:0, 18:1n-7, 20:0, 20:1n-7 and 22:1n-11, end-products of non-essential fatty acid synthesis in marine fish, as well as the levels of 20:2n-6, 20:3n-3, EPA, DPA and DHA, suggesting an antioxidant protection by these vitamins (**Table 8.1.5**).

2. Importance of dietary vitamins A, K and D in weaning diets for meagre

Materials and methods

A trial was conducted to test five microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco; INVE, Dendermonde, Belgium) until they reached 20 days after hatching (dah). Meagre larvae (initial total length 7.2±0.7 mm; dry body weight 0.5±0.1mg) were randomly distributed in 15 experimental tanks at a density of 2100 larvae per tank and were fed one of five experimental diets tested in triplicates for 14 days, at an average water temperature of 24.5±0.5 °C.

Table 8.1.5. Total lipid content (mg/g dw) and fatty acid composition (%dw) of whole body meagre, after 14 days of feeding several n-3 HUFA, vitamin E and C dietary contents.

	14 dah	28 dah					
		0.4/150/180	0.4/300/180	0.4/300/360	3/150/180	3/300/180	3/300/360
Lipids	19.49±2.81	17.54±2.12^{b}	17.63±3.79^{b}	21.11±1.12^{ab}	18.72±1.71^{b}	19.59±0.13^{ab}	23.61±0.86^{a}
14:0	0.17	0.08	0.07	0.21	0.14	0.12	0.14
15:0	0.10	0.05	0.05	0.09	0.08	0.07	0.08
16:0	4.86	3.82	3.88	5.24	4.50	4.21	4.67
16:1n-7	1.10	0.14	0.13	0.24	0.24	0.29	0.34
16:1n-5	0.12	0.04	0.04	0.05	0.05	0.05	0.06
16:2n-4	0.20	0.10	0.09	0.12	0.17	0.17	0.20
16:3n-1	0.50	0.32	0.34	0.29	0.35	0.34	0.43
16:4n-3	0.09	0.14	0.14	0.10	0.07	0.09	0.11
18:0	1.81	2.43	2.36	3.16	2.95	2.46	2.81
18:1n-9	3.85	4.87	5.00	5.54	3.01	2.58	3.12
18:1n-7	0.72	0.38	0.37	0.47	0.54	0.49	0.59
18:1n-5	0.04	0.02	0.01	0.01	0.03	0.02	0.04
18:2n-9	0.17	0.03	0.03	0.02	0.01	0.02	0.03
18:2n-6	1.46	2.45	2.55	2.65	1.70	1.83	2.31
18:3n-6	0.06	0.05	0.05	0.06	0.07	0.06	0.07
18:3n-3	0.19	0.10	0.07	0.24	0.42	0.12	0.17
18:4n-3	0.02	0.02	0.01	0.06	0.02	0.01	0.02
20:0	0.12	0.14	0.13	0.21	0.14	0.12	0.14
20:1n-9	0.04	0.01	0.01	0.01	0.02	0.02	0.03
20:1n-7	0.36	0.40	0.41	0.42	0.34	0.33	0.42
20:1n-5	0.08	0.03	0.03	0.03	0.04	0.04	0.05
20:2n-9	0.05	0.00	0.01	0.01	0.01	0.02	0.02
20:2n-6	0.13	0.11	0.11	0.17	0.13	0.12	0.16
20:3n-6	0.08	0.04	0.04	0.03	0.04	0.05	0.06
20:4n-6	0.51	0.28	0.29	0.21	0.41	0.55	0.70
20:3n-3	0.04	0.01	0.01	0.02	0.03	0.03	0.04
20:4n-3	0.05	0.03	0.01	0.05	0.05	0.04	0.06
20:5n-3	0.27	0.14	0.14	0.21	0.36	0.65	0.85
22:1n-11	0.03	0.01	0.01	0.02	0.09	0.07	0.10
22:1n-9	0.09	0.24	0.22	0.25	0.29	0.28	0.24
22:4n-6	0.04	0.02	0.02	0.02	0.03	0.04	0.06
22:5n-6	0.09	0.04	0.04	0.02	0.09	0.14	0.18
22:5n-3	0.13	0.06	0.06	0.06	0.17	0.31	0.43
22:6n-3	1.44	0.67	0.66	0.64	1.94	3.51	4.50
Saturated	7.09	6.53	6.51	8.93	7.84	6.99	7.87
Monoenoic	6.49	6.16	6.24	7.09	4.67	4.19	5.00
n-3	2.26	1.19	1.11	1.38	3.08	4.79	6.20
n-6	2.37	2.99	3.09	3.17	2.47	2.79	3.54
n-9	4.22	5.16	5.27	5.83	3.35	2.92	3.45
n-3HUFA	1.93	0.92	0.89	0.97	2.55	4.55	5.88
n-6HUFA	0.85	0.49	0.5	0.45	0.7	0.9	1.16
ı-6+n-3)HUFA	2.78	1.41	1.39	1.42	3.25	5.45	7.04
:1n-9/n-3HUFA	0.39	0.93	1.00	1.21	0.22	0.11	0.13
n-3/n-6	0.19	0.07	0.06	0.09	0.23	0.34	0.41
EPA/ARA	0.11	0.09	0.09	0.21	0.17	0.23	0.29
DHA/EPA	1.02	0.83	0.84	0.65	1.00	1.06	1.25

Five isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm & 250-500 µm) were formulated using squid powder non defatted as source of protein and lipid, and were completed with Krill-PL as source of marine phospholipids, and level of vitamin E (1.500 mg kg^{-1}), vitamin C (3.600 mg kg^{-1}), gelatin (3.0), mineral premix (4.5 g/100 g), vitamins premix (6.0 g/100g) without menadione, ergocalciferol and retinol acetate (**Table 8.1.6**). Additionally, menadione as source of vitamin K was added to the vitamin mix (175 mg kg^{-1}) in all diets except C-Vit K (diet without vitamin K supplementation diet), ergocalciferol as source of vitamin D was added to the vitamin mix (37 mg kg^{-1}) in all diets except C-Vit D (diet without vitamin D supplementation) and retinol acetate as source of vitamin A was added to the vitamin mix (3 mg kg^{-1}) in all diets except C-Vit A (diet without vitamin A supplementation). Taurine (2.000 mg kg^{-1}) was added only to C+Taurine diet (diet with taurine addition diet). The diet with vitamin K, D and A supplementation and without taurine addition was considered as a control diet (C) (**Table 8.1.6**).

Table 8.1.6. Ingredients and proximate composition of early weaning diets fed to meagre larvae from 20 to 33 dah (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

	Diets				
	C	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
Ingredients					
Taurine[i]	0.0	200.0	0.0	0.0	0.0
Vit K[j]	17.3	17.3	0.0	17.3	17.3
Vit D[k]	3.7	3.7	3.7	0.0	3.7
Vit A[l]	0.3	0.3	0.3	0.3	0.0
Proximate composition (%)					
Crude lipids	16.4	16.2	16.5	17.1	17.9
Crude protein	76.0	75.9	76.4	76.4	76.1
Moisture	13.7	13.6	13.6	13.8	13.8
Ash	6.5	6.5	6.5	6.6	6.5
Taurine[1]	4.0	5.8	4.0	4.0	4.0
Vitamin K[2]	2.4	2.4	0.0	2.6	2.2
Vitamin D[3]	28.9	29.0	30.4	2.3	27.4
Vitamin A[4]	4.2	4.3	4.2	4.3	4.1

Results

Gut occupancy, larval performance and morphometric parameters

The image analysis studies of the larval photographs of larvae fed the different diets denoted no significant differences in gut occupancy among fish fed the different diets. After only 7 days of feeding (26 dah), growth in terms of total length and dry body weight was only significantly ($P<0.05$) higher in larvae fed diet C-Vit D (8.9±1.0 mm) (mean±SD) (**Table 8.1.7**). However, at the end of the feeding trial (33 dah), the larvae feeding diets without supplementation of vitamin K (C-Vit K), vitamin D (C-Vit D) and vitamin A (C-Vit A) increased significantly ($P<0.05$) growth in terms of total length (12.8±1.6; 12.6±1.3; 12.2±1.7 mm, respectively). This same trend was found in body weight (3.2±0.2; 3.3±0.2 mg for C-Vit K and C-Vit D, respectively) except for larvae fed C-Vit A (2.5±0.3 mg) that did not show significant differences with larvae fed Control (**Table 8.1.7**).

Survival and welfare status

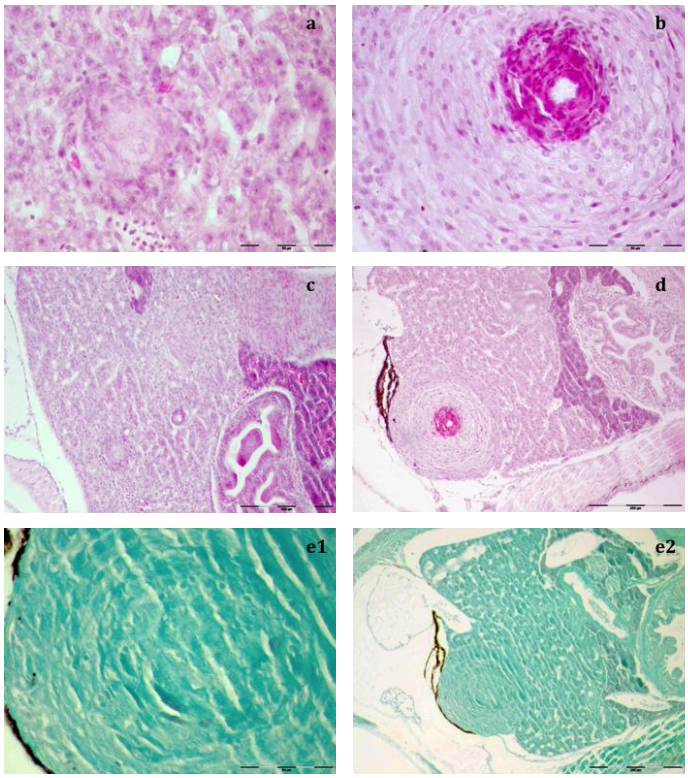
Larval survival at the end of the experiment was not significantly different among the different groups of larvae, being in average 16.6±5.1% (**Table 8.1.7**), except for the group of larvae fed C-Vit K that presented low survival (7.1%) with 100% of mortality in two tanks. No significant differences were found in larval welfare status.

Table 8.1.7. Total length (mm), dry weight (mg) and survival of meagre larvae fed early weaning diets from 20 to 33 dah (initial total length 7.2±0.7 mm and dry body weight 0.5±0.1mg). (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

		Diets				
		C	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
Total length	26 dah	8.3±1.0 [a]	8.5±0.9 [a]	8.5±1.0 [a]	8.9±1.0 [b]	8.6±1.0 [a]
	33 dah	11.5±1.7 [a]	11.7±1.3 [a]	12.8±1.6 [b*]	12.6±1.3 [b]	12.2±1.7 [b]
Body weight	26 dah	0.7±0.1 [a]	0.8±0.1 [a]	0.7±0.1 [a]	0.9±0.2 [b]	0.8±0.2 [a]
	33 dah	2.4±0.6 [a]	2.3±0.4 [a]	3.2±0.2 [b*]	3.3±0.2 [b]	2.5±0.3 [a]
Survival (%)		16.7±6.5	12.9±1.2	7.1*	17.7±12.3	19.0±0.5

Larval organ and skeleton development

By histopathological examination, granulomas were detected in the liver of some larvae of the different experimental diets. Granulomas displayed different stages of growth and morphology: initial granulomas or "Stage I", granuloma of 0.5 mm composed of a central cluster of voluminous macrophages (**Figure 8.1.3a**).

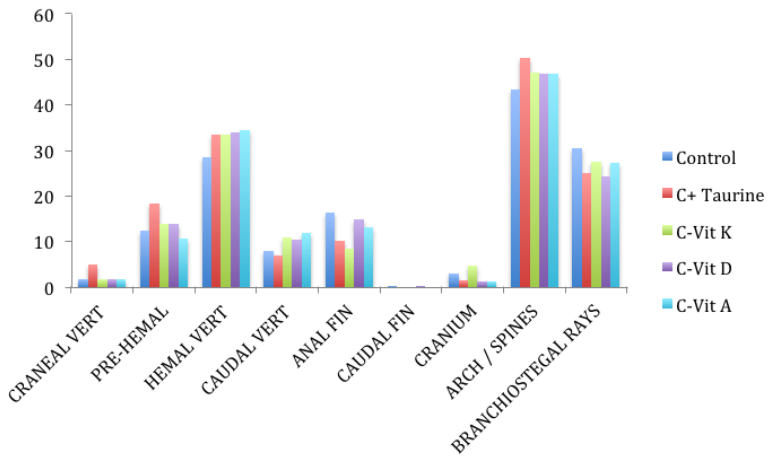


Late granulomas or "Stage II", granuloma of 1.8 mm composed of an eosinophilic necrotic central area surrounded by several concentric layers of macrophages that presented vacuolized cytoplasm, observing more flattened in the outer layers. Hepatic cells around of the granulomas were compressed (**Figure 8.1.3b**). At 33 dah high incidence of Stage I and II granulomas was found in larvae fed without supplementation of vitamin K (C-Vit K) (12.5%) (**Figure 8.1.3c and d**), followed by larvae fed without supplementation of vitamin A (C-Vit A) and without supplementation of vitamin D (8.3% and 3.3%, respectively) (**Table 8.1.8**). Larvae from control and taurine diets did not show granulomas. Larvae with granulomes were stained with Ziehl-Neelsen technique for Mycobacteria detection being negative for all cases (absence of alcohol-acid resistant bacillus, **Figure 8.1.3. e1 and e2**).

Figure 8.1.3. **a**. Initial or "Stage I" granuloma in the liver (40x). **b**. "Stage II" granuloma in the liver (40x). **c**. "Stage I" granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K) (10x). **d**. "Stage II" granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K) (10x). **e1.** (40x) and **e2.** (10x) staining with Ziehl-Neelsen technique, granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K).

Osteological study

No significant differences on frequency of severe anomalies of the total anomalies were found among the groups of larvae, accounted for 40% for larvae fed Control, C+Taurine and C-Vit A diets, 45% for larvae fed C-Vit K diet and 38% for larvae fed C-Vit D diet. The index of severe anomalies among groups varied from 1.5% for larvae fed C+Taurine diet, 1.4% for larvae fed C-Vit D diet, 1.3% for larvae fed C-Vit K and C-Vit A diets to 1.2% for Control larvae, but without significant differences among groups.



Severe anomalies of hemal vertebra were quite common among groups (32.7%), followed by pre-hemal (13.7%) and caudal vertebra (9.6%) (**Figure 8.1.4**). Anal fin (12.5%) showed higher anomalies than caudal fin (0.1%). Malformations involving spines and rays were very common in all groups of larvae, being up to 46.8% for spines of arch and 26.9% for branquiostegal rays (**Figure 8.1.4**).

Figure 8.1.4. Frequencies (%) of larvae with different anomalies in each experimental diet (33dah). (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation). Data are referred to the total of individuals of each group.

Biochemical composition

The fatty acid composition of the diets was not different among diets, being high in saturated acids (SAFA) (5.8%DW), specially lauric (14:0) (1.4%DW) and palmitic acid (16:0) (3.9%DW) acids (**Table 8.1.8**) and high in monosaturated acids (MUFA) (4.1%DW), particularly oleic acid (18:1n-9) (1.5%DW). The levels of n-3 PUFA (5.9%DW) were highest than n-6 PUFA (0.4%DW) for all diets, and subsequently the n-3/n-6 PUFA ratio was high (13.4%DW). Standing out the fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) as the n-3 fatty acids found in highest levels (2.6 and 2.6 %dw, respectively) for all diets (**Table 8.1.8**).

Despite dietary lipids levels were similar among diets, larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) tended to increase larval total lipid contents (**Table 8.1.9**), and a significant positive correlation between larvae lipids and body weight at 33 dah ($y=0.3814x-2.4621$, $R^2=0.83$) was found.

The total saturated fatty acids, specially stearic acid (18:0), total monounsaturated acids, specially oleic acid (18:1n-9) were higher in the initial larvae than those of the experimental groups (**Table 8.1.9**). Regarding the diets, total amount of SAFA, specially 16:0 was higher in the larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) (3.2%DW and 3.0%DW respectively) than those found in larvae fed C+Taurine and C-Vit A (2.5%DW), although without significant differences with the control larvae. Similarly, total amount of MUFA, especially 18:1n-9 were highest in the larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) than those of C+Taurine and C-Vit A.

Table 8.1.8. Main fatty acid composition (% dw) of the early weaning diets used to feed larval meagre from 20 to 33 dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

	Diets				
	C	C+Taurine	C-Vit K	C-Vit D	C-Vit A
14:0	1.3	1.4	1.4	1.4	1.5
16:0	3.7	3.9	3.8	3.8	4.1
18:0	0.5	0.5	0.5	0.5	0.5
Total SAFA[a]	5.7	5.4	5.7	5.9	6.2
16:1n-7	0.8	0.9	0.9	0.9	0.9
18:1n-9	1.5	1.5	1.5	1.5	1.6
20:1n-7	0.5	0.5	0.5	0.5	0.5
22:1n-9	0.1	0.2	0.1	0.1	0.2
Total MUFA[b]	3.9	4.0	4.1	4.2	4.5
18:2n-6	0.2	0.2	0.2	0.2	0.2
20:4n-6	0.1	0.1	0.1	0.1	0.1
Total n-6 PUFA[c]	0.4	0.4	0.4	0.5	0.5
18:3n-3	0.2	0.1	0.2	0.2	0.2
18:4n-3	0.4	0.4	0.4	0.5	0.5
20:3n-3	0.1	0.1	0.1	0.1	0.1
20:4n-3	0.1	0.1	0.1	0.1	0.1
20:5n-3	2.6	2.4	2.6	2.7	2.9
22:5n-3	0.1	0.1	0.1	0.1	0.1
22:6n-3	2.5	2.3	2.5	2.6	2.7
Total n-3 PUFA[d]	6.0	5.3	5.9	6.2	6.4
Total n-3 LC PUFA[e]	5.3	4.8	5.3	5.6	5.8
Total PUFA	6.3	5.8	6.3	6.6	6.9
n-3/n-6 PUFA	15.4	11.9	13.2	13.3	13.0

Table 8.1.9. Total lipid content and fatty acid composition (% dw) of whole body meagre larve (33 dah) feeding on diets (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

		Diets				
	Initial larvae	C	C+Taurine	C-Vit K	C-Vit D	C-Vit A
Lipids	14.8±2.8	13.3±2.1	12.6±0.8	15.2±0.7	14.4±0.8	12.7±1.1
14:0	0.1±0.0	0.3±0.0^{abc}	0.1±0.1^{c}	0.4±0.0^{a}	0.3±0.0^{ab}	0.2±0.0^{bc}
16:0	2.7±0.1	2.8±0.1^{ab}	2.5±0.2^{a}	3.2±0.2^{b}	3.0±0.0^{b}	2.5±0.1^{a}
18:0	1.8±0.2	1.0±0.1	1.0±0.1	1.0±0.1	0.9±0.0	0.9±0.1
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Total SAFA[a]	4.9±0.3	4.2±0.2^{ab}	3.7±0.4^{a}	4.7±0.3^{ab}	4.3±0.1^{b}	3.7±0.3^{a}
16:1n-7	0.6±0.0	0.4±0.0^{ab}	0.3±0.1^{a}	0.5±0.0^{b}	0.5±0.0^{ab}	0.3±0.1^{ab}
18:1n-9	2.2±0.1	1.3±0.0^{ab}	1.2±0.0^{a}	1.5±0.1^{c}	1.4±0.0^{bc}	1.2±0.0^{a}
20:1n-7	0.3±0.0	0.3±0.0^{ab}	0.2±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{b}	0.2±0.0^{a}
22:1n-9	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Total MUFA[b]	4.2±0.1	2.8±0.1^{ab}	2.5±0.1^{a}	3.2±0.1^{c}	3.0±0.1^{bc}	2.5±0.2^{a}
18:2n-6	1.7±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:4n-6	0.4±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Total n-6 PUFA[c]	2.5±0.0	0.5±0.1	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.0
18:3n-3	0.3±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
18:4n-3	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0
20:4n-3	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:5n-3	0.4±0.0	1.5±0.1^{a}	1.4±0.1^{a}	1.9±0.1^{b}	1.8±0.1^{b}	1.5±0.2^{ab}
22:5n-3	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
22:6n-3	1.6±0.1	3.3±0.0	3.4±0.2	3.6±0.4	3.6±0.1	3.4±0.2
Total n-3 PUFA[d]	2.5±0.2	5.3±0.1^{ab}	5.2±0.2^{a}	6.1±0.2^{b}	6.0±0.1^{ab}	5.4±0.3^{ab}
Total n-3 LC PUFA[e]	2.1±0.0	5.1±0.1^{a}	5.1±0.3^{a}	5.8±0.5^{b}	5.8±0.2^{b}	5.2±0.4^{ab}
Total PUFA	5.1±0.2	5.8±0.1^{a}	5.8±0.2^{a}	6.6±0.2^{ab}	6.5±0.1^{b}	5.9±0.3^{a}
n-3/n-6 PUFA	1.0±0.1	9.7±0.1	9.0±0.2	10.5±0.2	11.1±0.1	10.3±0.3

Total n-6 PUFA contents, especially linoleic acid (18:2n-6) were highest in the initial larvae and consequently higher than those of the experimental groups (**Table 8.1.9**). Levels were but similar among larvae fed the different diets at 33 dah. On the contrary, the total n-3 PUFA content was lowest in initial larvae (2.5% DW) mainly by reduction of levels of EPA (0.4% DW) and DHA (1.6% DW), and subsequently the n-3/n-6 PUFA ratio was lower (1.0% DW) than those of the experiment groups. Among larvae fed experimental diets, those feeding the C-Vit K and C-Vit D diets increased significantly the level of EPA (1.9% DW and 1.8% DW, respectively), and additionally these feeding groups tended to increase total n-3 PUFA, n-3 LC PUFA and n-3/n-6 PUFA ratio, although for this ratio without significant differences. Nevertheless, an increase of larvae 22:6n-3 content (≈3.5% DW) compared with the initial larval level (1.6% DW) was found regardless of the different diets.

Deviations from Annex I and their impact:

There were no deviations from the Annex I

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. HMRC	P8. IEO	P15. ULL	P20. SARC
P28. CANEXMAR				
Lead Scientist preparing the Report (WP leader):	Marisol Izquierdo			
Other Scientists participating:	Yannis Kotzamanis (P1), Jerez Salvador (P8), Covadonga Rodriguez (P15), Ramon Fontanillas (P20), Rafael Guirao (P28), Hipólito Fernández-Palacios (P2)			

Objectives

1. Improve of larval enrichment products 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 progress towards objectives and details for each task (13-30 Mo):

During the present reporting report, 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.

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

The objective of this task was to determine the optimum levels of specific nutrients that have been suggested to be particularly important for the larvae of this and other *Seriola* species such as essential fatty acids and carotenoids. The results of the task allowed to produce a list of the optimum levels and ratios of essential fatty acids and carotenoids that should be included in enrichment products for rotifers to be fed to greater amberjack larvae. During this period, the effects of essential fatty acids and carotenoids on larval performance, welfare, fatty acid analysis, lipid classes, and carotenoid profiles of enrichment products, live preys and larvae were studied. These results have been complied in Deliverable 9.1 where full description of the work and results is provided.

Sub-task 9.1.1 (FCPCT, Marisol Izquierdo)

During this reporting period the optimum levels in enrichment products for live preys for greater amberjack of eicosapentaenoic acid (EPA), an essential fatty acid, was investigated. Larvae were fed different levels of essential fatty acids and ratios prepared by P2. FCPCT, in order to determine the requirements for these nutrients during early larval development. For that purpose, larval performance in terms of survival, growth and welfare (survival to handling stress test) was studied. Proximate and fatty acid composition of enrichment products, live preys and larvae was analyzed.

At 17 days post hatching (dph), a total of 1000 larvae per tank (mean total length 6.39± 0.44 mm; mean fresh weight 2.94 ± 0.57 mg) were randomly distributed in 15 experimental tanks of 200 l capacity. Water exchange was gradually increased from 0.80 l min^{-1} at 19 dph to 1.6 l min^{-1} at 25 dph and finally to 3.3 l min^{-1} from 30 dph to the end of the feeding trial. Average seawater temperature and dissolved oxygen during this period were 24.15 ± 0.35°C and 6.55 ± 0.41 ppm. From 17 to 22 dph, there was an overlap between rotifers (unenriched) and *Artemia* with a gradual reduction in the amounts of rotifers from 5000–0 individual's l^{-1} and a progressive increase on enriched *Artemia* from 125–500 individuals l^{-1}. From 23 to 35 dph, larvae were fed exclusively with enriched *Artemia* from one of the five dietary treatments.

In order to determine the optimum dietary EPA requirement for greater amberjack larvae during *Artemia* feeding stage, five experimental emulsions, which varied in the EPA contents (0-60%), were formulated. Experimental emulsions were prepared, mixing increasing amounts of high EPA content commercial triglycerides oil (Incromega EPA 500 TG, Croda, Barcelona, Spain) containing 63% of Total Fatty Acid (TFA) as EPA, 8% as DHA and 3% as ARA; Oleic Acid oil (Sigma-Aldrich; Madrid, Spain) including 77% of TFA as oleic acid and soya lecithin (SL, Korot SL, Alcoy, Spain) containing mainly 54% of TFA as

linoleic acid (18:2n−6, LA) and trace amounts of EPA and DHA. In addition, to prevent the oxidation of high DHA levels, experimental emulsions were fortified with 3000 mg kg^{-1} vitamin E (DL-α-tocopherol acetate, Sigma-Aldrich, Madrid, Spain) and 2500 mg kg^{-1} vitamin C (L-ascorbic acid, Asc, Sigma-Aldrich) . Once prepared, emulsions were stored in a fridge at 4º C until used. Three samples of each experimental emulsion collected along the experimental test were analyzed to determine fatty acid composition (**Table 9.1.1**). For *Artemia* enrichment, 1.1 ml of each experimental emulsion was mixed with 100 ml of fresh water with a stirrer for 1 minute and added to a 10-l beaker filled with seawater and provided with aeration and oxygen supply. Enrichment time (18 hours) and density (150 indv ml^{-1}) were equal for all the experimental emulsions assayed. Temperature and salinity during enrichment were 28ºC and 37g l^{-1}.

Table 9.1.1. Selected fatty acid contents (percentage of total fatty acids, % TFA) of total lipids in the experimental emulsions.

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
	Fatty acid content (%TFA)				
Saturated	15.21	12.66	11.46	9.27	2.61
Monoenoics	74.63	58.63	50.00	36.95	7.60
n-3	2.16	20.48	30.11	45.09	79.52
n-6	7.93	7.43	7.16	6.95	7.65
n-9	72.17	56.07	47.47	34.57	5.56
Total n-3HUFA	1.37	17.35	25.60	38.74	70.62
14:00	0.02	0.12	0.20	0.27	0.35
16:00	11.45	9.21	7.99	6.01	0.95
16:1 n-7	0.76	0.81	0.88	0.89	0.87
18:00	3.14	2.54	2.25	1.72	0.41
18:1 n-9	72.13	55.84	47.13	34.06	3.94
18:1 n-7	1.40	1.46	1.33	1.16	0.94
18:2 n-6	7.80	6.14	5.16	4.05	1.96
18:3 n-3	0.67	0.65	0.64	0.66	1.18
20:1 n-9	0.02	0.01	0.01	0.02	0.41
20:4n-6 (ARA)	0.06	0.82	1.25	1.83	3.53
20:5n-3 (EPA)	0.84	14.28	21.18	31.97	60.16
22:6n-3 (DHA)	0.30	2.36	3.34	5.11	6.79
ARA/EPA	0.07	0.06	0.06	0.06	0.06
DHA/EPA	0.36	0.17	0.16	0.16	0.11
DHA/ARA	5.41	2.89	2.68	2.80	1.92
Oleic/DHA	240.20	23.67	14.13	6.66	0.58
Oleic/n-3HUFA	52.71	3.22	1.84	0.88	0.06
n-3/n-6	0.27	2.76	4.21	6.49	10.40

All larval samplings were carried out randomly from the experimental tanks. Total length was measured using a profile projector (Mitutoyo PJ-A3000, Kanagawa, Japan) and fresh body weight of 30 larvae/tank was determined initially (17 dph) and at 35 dph.

Larval survival was calculated by daily counting of dead larvae from 17 dph and by counting all the remaining alive larvae at the end of the experiment. Thirty larvae per tank at 35 dph were submitted to acute stress, handling them out of the water for 30 and 60 seconds and returning them to a bucket with aerated seawater. Survival rate was determined 24 hours later, counting all the surviving larvae. To determine the skeletal anomalies incidence, 100 larvae were collected per tank at 35 dph. Fixed larvae were stained with alizarin red and immediately photographed to evaluate skeletal anomalies occurrence. Different regions of the axial column were identified and divided. Observations were performed on the right side of the stained samples under a stereomicroscope. The numerical data set obtained was processed to calculate incidences for each descriptor (anomaly typology) and treatment.

One final sample of larvae was collected from each experimental tank at 35 dph. Besides, 5 g of each experimental emulsion of recently hatched *Artemia* and enriched *Artemia* were stored. All samples were flushed with N_2 and kept frozen at -80 °C until analysis was carried out. Total lipids were extracted and fatty acids prepared by trans-etherification. Separation and identification of the fatty acids was realized with gas chromatography (GC) (GC TERMO FINNIGAN FUCUS GC, Milan, Italia). Dry matter, ash and protein content were also calculated.

Results

Artemia enriched with different experimental emulsions (**Fig. 9.1.1**) resulted in five enriched *Artemia* treatments (**Figure 9.1.2**). The EPA content in *Artemia* was directly correlated to EPA content in the experimental emulsion ranging from 1.08 to 22.9 %; (**Table 9.1.2**), other fatty acids such as DHA (range 0.14-3.01 % TFA), ARA (range 0.39-1.72 % TFA) and total n-3 HUFA (range 3.24-29.07 % TFA) were also directly correlated with their emulsion content.

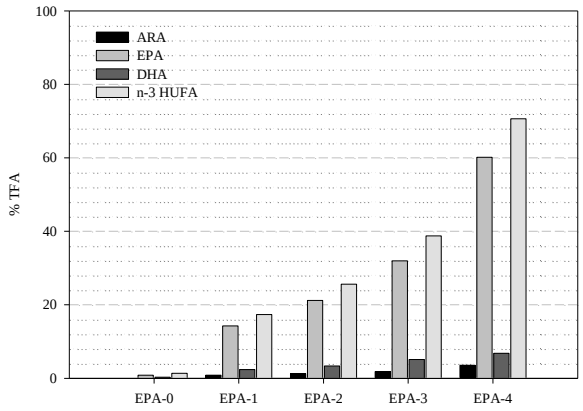


Figure 9.1.1. Fatty acids content (percentage of total fatty acids) in the experimental emulsions.

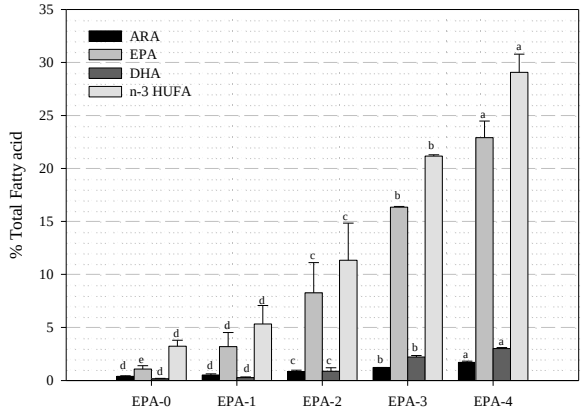
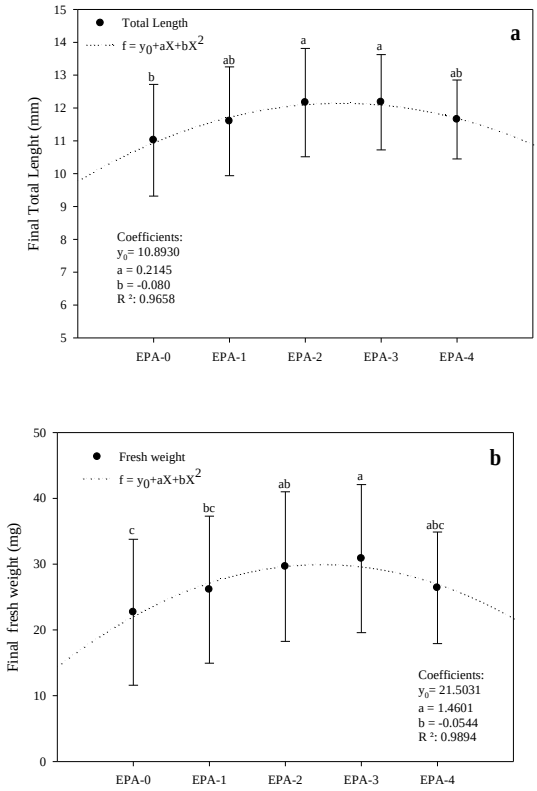


Figure 9.1.2. Increase in fatty acids composition (EPA, DHA, ARA and total n-3 HUFA; in %TFA) of *Artemia* nauplii after 18h enrichment with five experimental emulsions. Data represent means and error bars are standard deviation (n=3). Treatments means for each FA, with different superscripts indicate significant differences (ANOVA ($P \leq 0.05$); Tukey's HSD).

Table 9.1.2 Proximate (% dry matter) and fatty acids composition (%TFA) of *Artemia* nauplii after 18h enrichment with five experimental emulsions. Proximate analysis data represent means ± SD, (n=3), FA content data are presented as mean. Different superscripts within each row indicate a significant difference between DHA emulsions (ANOVA (P ≤0.05); Tukey's HSD).

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Proximate analysis (% dry matter)		
Lipids	19.72±1.04	19.45±1.97	24.29±3.16	25.36±1.06	26.31±0.68
Moisture	90.33±0.21	90.39±0.21	90.05±0.35	90.00±0.30	89.05±0.12
Ash	10.79±0.61	10.16±0.43	10.09±0.69	8.61±1.49	9.76±048
			Fatty acid content (%TFA)		
Saturated	19.07^{a}	19.93^{a}	16.18^{ab}	12.56^{bc}	10.21^{c}
Monoenoics	41.31^{a}	40.38^{a}	37.26^{a}	30.28^{b}	22.44^{c}
n-3	29.51^{b}	29.64^{b}	36.16^{b}	46.74^{a}	55.40^{a}
n-6	7.76^{b}	7.56^{b}	8.00^{b}	7.92^{b}	8.87^{a}
n-9	31.80^{a}	30.08^{a}	28.51^{a}	23.14^{b}	15.06^{c}
Total n-3HUFA	3.24^{d}	5.33^{d}	11.34^{c}	21.16^{b}	29.07^{a}
14:00	0.46^{ab}	0.57^{a}	0.40^{b}	0.32^{b}	0.34^{b}
16:00	10.66^{a}	11.11^{a}	8.83^{ab}	6.84^{b}	6.90^{b}
16:1 n-7	1.75^{ab}	1.93^{a}	1.63^{ab}	1.40^{b}	1.50^{b}
18:00	6.86^{a}	7.15^{a}	6.00^{a}	4.66^{ab}	2.23^{b}
18:1 n-9	30.70^{a}	29.00^{a}	27.44^{a}	22.07^{b}	13.77^{c}
18:1 n-7	6.39^{ab}	6.83^{a}	5.92^{b}	4.83^{c}	4.97^{c}
18:2 n-6	6.30^{a}	6.06^{a}	6.10^{a}	5.53^{b}	5.70^{b}
18:3 n-3	23.03	21.61	21.66	21.56	21.80
20:1 n-9	0.03^{a}	0.03^{a}	0.03^{a}	0.03^{a}	0.09^{b}
20:4n-6 (ARA)	0.39^{d}	0.51^{d}	0.81^{c}	1.23^{b}	1.72^{a}
20:5n-3 (EPA)	1.08^{e}	3.18^{d}	8.26^{c}	16.34^{b}	22.91^{a}
22:6n-3 (DHA)	0.14^{d}	0.27^{d}	0.87^{c}	2.22^{b}	3.01^{a}
ARA/EPA	0.38^{a}	0.17^{b}	0.11^{bc}	0.07^{c}	0.07^{c}
DHA/EPA	0.13^{ab}	0.09^{c}	0.10^{bc}	0.14^{a}	0.13^{a}
DHA/ARA	0.35^{c}	0.53^{c}	1.02^{b}	1.81^{a}	1.76^{a}
Oleic/DHA	253.82^{a}	115.64^{b}	38.41^{b}	9.98^{b}	4.58^{b}
Oleic/n-3HUFA	9.87^{a}	6.22^{ab}	2.74^{bc}	1.04^{c}	0.47^{c}
n-3/n-6	3.79^{b}	3.90^{b}	4.52^{b}	5.90^{a}	6.24^{a}

Larval growth was significantly affected by the dietary EPA levels along the feeding trial (**Figure 9.1.3**). Mean values for all the dietary treatments were 11.72±0.47 mm in total length and 27.13±3.21 mg in fresh body weight at 35 dph. Fish final total length (TL) in group EPA-0 was similar to group EPA-1 and EPA-4, but significantly lower than those in groups EPA-2 and EPA-3 (P ≤ 0.05), no differences were found among groups EPA-1, EPA-2, EPA-3 and EPA-4, respectively. Fish fed EPA-3 showed significantly higher final fresh weight (FW) (P≤ 0.05) than EPA-0 and EPA-1 but similar to EPA-2 and EPA-4 (P> 0.05), (**Figure 9.1.3 a&b**).



The relationship between final total length and *Artemia* EPA content was described by the second-order polynomial regression: $y = 10.893 + 0.214x - 0.080\ x^2$ (*y= Total length; x= dietary EPA (% TFA);* $R^2=0.965$; $P \leq 0.05$). Also, final fresh weight was described by the equation: $y = 21.503 + 1.460x - 0.0544\ x^2$ (*y= Fresh weight; x= dietary EPA (% TFA);* $R^2=0.989$; $P \leq 0.05$). Under the experimental conditions applied, both growth models suggest that maximum growth was achieved in the range of dietary EPA concentrations tested, between 8-16 %TFA with a maximum around 13-14% DHA content in *Artemia*, when low dietary DHA were provided (0.8-2.2% TFA).

Larval survival was significantly ($P \leq 0.05$) affected by dietary EPA at 35 dph, the lowest survival was recorded in those larvae receiving the lowest EPA in the *Artemia* (EPA-0) (**Figure 9.1.4a**). Hence, larval resistance to stress test, determined as the survival rate 24 hours after handling, was significantly affected by dietary EPA, the lower survival was recorded in those larvae fed the lowest EPA in the diet (EPA-0) (**Figure 9.1.4.b**).

Figure 9.1.3. Relationship between (a) total length (mm) and (b) fresh weight (mg) to dietary *Artemia* EPA (20:5n-3) content in larval greater amberjack at 35 days posthatch (mean + S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$).

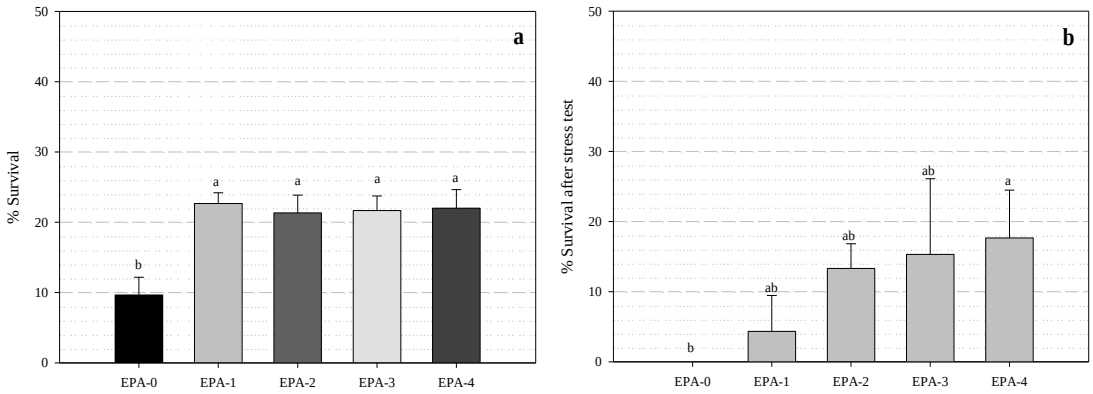


Figure 9.1.4. (a) Final survival and (b) survival after stress test in 35 dph greater amberjack larvae, fed different dietary *Artemia* EPA (20:5n-3) content. Values are mean + S.D., n=3.

The incidence of total acute skeletal deformities was not significantly affected by the dietary EPA, with an average value for all the dietary treatments of 22.63±6.25% (**Figure 9.1.5a**) Regardless of the EPA content, most of the acute skeletal deformities were major alterations in the haemal region, related with column deformities (**Figure 9.1.5b**), the most important affection was recorded as scoliosis. The relationship between total acute deformities, and *Artemia* EPA content was described by the lineal regression: $y = y_0 + ax$ *(y= Total acute deformities; x= dietary EPA (% TFA);* with a determination coefficient (R^2) of 0.85 and P> 0.05 (Fig. 5a). Also, the relationship between the sum of column deformities and *Artemia* EPA content was described by the same model stated above represented as: $y = 29.109 - 0.645x$ *(y= % column deformities; x= dietary EPA (% TFA);* $R^2=0.860$; $P \leq 0.05$) (**Figure 9.1.5.b**), suggesting a higher level of vertebral malformations when low dietary EPA was used. These results suggest that higher levels of EPA tended to intensify the apparition of this type of deformity during the live stage of development evaluated (**Figure 9.1.5b**).

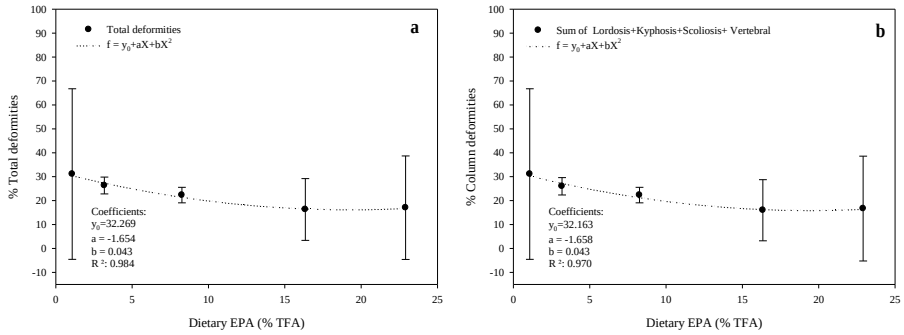


Figure 9.1.5. Relationship between (a) total deformities (%), (b) sum of column deformities to dietary *Artemia* EPA (20:5n-3) content (%TFA) in larval greater amberjack at 35 days posthatch (mean + S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$) for a, b and c data, while a linear regression analysis ($f=y_0+ax$) in d.

Results of total lipids and fatty acids analysis of greater amberjack larvae at 35 dph are shown in **Table 9.1.3**. Total lipids, ash content and moisture were equal for the larvae fed the different EPA *Artemia* treatments. Regarding FA composition, total n-3 and n-3 HUFA were significantly increased with the increase in dietary EPA. The same way, EPA level in larvae fed Diet 0–4 was significantly increased with the increase in the dietary EPA content. DHA contents in larval tissues were also affected by dietary treatments, with the lowest values in group EPA-0, which was significantly lower than those in groups EPA-4 ($P \leq 0.05$). On the other hand, ARA content was not significantly affected by dietary EPA.

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

To achieve the objectives proposed in this task a first trial of rotifer enrichment was designed (P15. ULL and P8. IEO) and performed at IEO in order to establish a good protocol for LC-PUFA enrichment according to the lipid composition of wild greater amberjack viable eggs. These preliminary assays combined different times of enrichment with different sources and levels of LC-PUFA rich lipids. The combinations of lipids were made in order to supply high polar lipids, and high LC-PUFA levels but remained DHA/EPA/ARA ratios similar to those present in amberjack wild eggs, by using a range of lipid sources. E1 was based on a polar rich emulsion containing a marine natural lecithin LC60 (PhosphoTech Laboratories, France) with up to 60% phospholipids rich in DHA. E3 was based on a mixture of different TAG sources (Incromega DHA500 TAG and cod liver oil). Finally, E2 emulsion was formulated on a blend of these three lipid sources. The three experimental emulsions were additionally supplemented with free arachidonic acid. Preliminary results showed that the best combination in terms of rotifer PL absolute contents and proportions of DHA, EPA and ARA was achieved with the marine lecithin used in treatment E1 for 3h.

Table 9.1.3. Proximate (lipid, moisture and ash content, % dry matter) and fatty acids composition (%TFA) of greater amberjack larvae 35 dah fed enriched *Artemia* with different experimental emulsions. Proximate and FA analysis data represent means ± SD, (n=3). Different superscripts within each row indicate a significant difference between DHA emulsions (ANOVA (P ≤0.05); Tukey's HSD).

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Proximate analysis (% dry matter)		
Lipids	17.27±3.85	14.71±1.70	16.08±6.94	15.00±2.75	20.10±1.39
Moisture	84.58±2.35	86.87±1.10	87.24±3.66	85.59±0.70	87.71±1.12
Ash	15.99±0.02	16.43±1.41	16.03±1.49	15.95±2.40	14.19±0.93
			Fatty acid content (%TFA)		
Saturated	27.00±1.30^{ab}	28.93±1.71^{a}	27.61±0.59^{ab}	24.11±0.82^{c}	24.93±0.27^{bc}
Monoenoics	29.07±3.23^{a}	28.75±0.81^{a}	28.61±0.27^{a}	27.98±0.37^{a}	23.00±0.24^{b}
n-3	28.78±5.25^{b}	27.83±0.56^{b}	30.31±0.21^{b}	33.37±2.16^{ab}	38.68±0.42^{a}
n-6	11.62±1.08	11.02±0.43	10.28±0.18	10.01±0.79	9.97±0.23
n-9	21.60±3.07^{b}	21.58±0.60^{b}	21.45±0.22^{b}	20.01±1.56^{ab}	15.84±0.25^{b}
Total n-3HUFA	15.59±6.24^{b}	17.31±0.59^{b}	20.09±0.23^{ab}	21.84±0.91^{ab}	26.31±0.26^{a}
14:00	0.51±0.16	0.40±0.15	0.31±0.02	0.36±0.09	0.35±0.03
16:00	14.22±0.32^{ab}	14.60±0.36^{a}	14.36±0.20^{ab}	12.50±0.83^{c}	13.24±0.23^{cb}
16:1 n-7	0.57±0.04	0.54±0.01	0.54±0.00	0.76±0.36	0.57±0.01
18:00	11.37±1.08^{ab}	13.13±1.81^{a}	12.13±0.44^{ab}	10.16±0.55^{b}	10.59±0.11^{ab}
18:1 n-9	18.83±3.29^{a}	19.16±0.41^{a}	19.28±0.18^{a}	17.23±2.56^{ab}	13.50±0.22^{b}
18:1 n-7	6.11±0.32	5.84±0.14	5.85±0.06	5.76±0.37	5.80±0.02
18:2 n-6	6.55±0.71^{a}	5.82±0.23^{ab}	5.35±0.14^{b}	4.79±0.43^{b}	5.05±0.32^{b}
18:3 n-3	11.25±1.27	8.99±0.45	8.76±0.29	9.56±1.69	10.51±0.32
20:1 n-9	0.40±0.02	0.41±0.04	0.38±0.01	0.45±0.22	0.33±0.00
20:4n-6 (ARA)	3.09±0.26	3.45±0.11	3.37±0.09	3.11±0.15	3.30±0.07
20:5n-3 (EPA)	6.65±4.48^{b}	8.50±0.29^{ab}	10.54±0.11^{ab}	11.47±1.25^{ab}	13.55±0.25^{a}
22:6n-3 (DHA)	4.81±1.64^{b}	4.51±0.28^{b}	5.11±0.22^{b}	5.73±0.27^{b}	8.02±0.19^{a}
ARA/EPA	0.59±0.29^{a}	0.41±0.01^{a}	0.32±0.01^{a}	0.27±0.04^{a}	0.24±0.01^{a}
DHA/EPA	0.82±0.22^{a}	0.53±0.02^{a}	0.49±0.02^{a}	0.50±0.06^{a}	0.59±0.02^{a}
DHA/ARA	1.56±0.53^{a}	1.31±0.09^{a}	1.52±0.06^{a}	1.85±0.13^{a}	2.43±0.06^{a}
Oleic/DHA	4.34±1.86^{a}	4.26±0.18^{a}	3.77±0.17^{a}	3.01±0.42^{a}	1.68±0.05^{a}
Oleic/n-3HUFA	1.37±0.63^{a}	1.11±0.01^{a}	0.96±0.02^{a}	0.79±0.09^{a}	0.51±0.01^{a}
n-3/n-6	2.52±0.72^{a}	2.53±0.05^{a}	2.95±0.03^{a}	3.36±0.46^{a}	3.88±0.12^{a}

In a second preliminary experiment, the lipid emulsion E1 was selected, mixed with three different proportions of carotenoids (50, 100 or 150 ppm Naturose ~2% astaxanthin), and added to the enrichment tanks at 6% concentration for evaluation of survival and rotifer total lipid and carotenoid content assessment. No significant variation in rotifers population condition was registered when E1 was combined with increasing proportions of carotenoids. Regardless of the treatment, maximum absorption of carotenoids was reached after 3h.

From these two preliminary experiments, it was concluded that rotifers enriched for short periods (3-6h) with 6% of the marine lecithin with a slight supplementation of AA (E1) in combination with a range of carotenoids well below 50 ppm, might improve species larval performance at early life stages.

The effect of these new combinations of PUFA-rich lipids and carotenoids formulated from results attained were assessed on greater amberjack larval performance, welfare and body composition. To this purpose the rotifer enrichment commercial protocol (C) was compared with three experimental emulsions (E1; E1,10 and E3,10) added at a 6% concentration for 3h to the rotifer enrichment tanks, under the same rearing conditions. E1 and E3 consisted of the same emulsions described above, with E1,10 and E3,10 consisting of these two lipid emulsions combined with 10 ppm (mg l^{-1}) of Naturose. Newly hatched larvae of greater amberjack, at a total density of 5000 larvae per tank (mean total length 3.14± 0.08 mm), were randomly distributed in 12 experimental tanks of 100 l capacity. From 3 to 11 dph, rotifers in the tanks were adjusted to 5 individuals ml^{-1} and increased to 10 individuals ml^{-1} until the end of the trial. Rotifers enriched with one of four treatments were added to the larval rearing tanks twice a day. All larval sampling at each age (1, 6, 10 and 14 dph) was carried out randomly from the experimental tanks. Total length, percentage of larvae with swim bladder inflated and eye diameter was determined. At the end of the trial (14 dph) larvae of each tank were counted and the percentage of survival calculated. Daily prey intake was also estimated by the differences between added and remaining rotifers in larval rearing tanks.

Larval growth was significantly affected by dietary treatments. Mean values for all the dietary treatments were 5.47±0.43 mm in total length at 14 dph. Total length of larvae was similar between the four enrichment treatments at 6 and 10 dph. However, the different treatments assayed showed significant differences at 14 dph. Fish total length (TL) in Control group was significantly lower at 14 dph while larvae from treatment E3,10 showed the significantly highest size but similar to treatment E1,10. Although E1 larvae were smaller than E3,10 larvae, no differences were found between groups E1,10 and E1 at the same age (P> 0.05) (**Figure 9.1.4**).

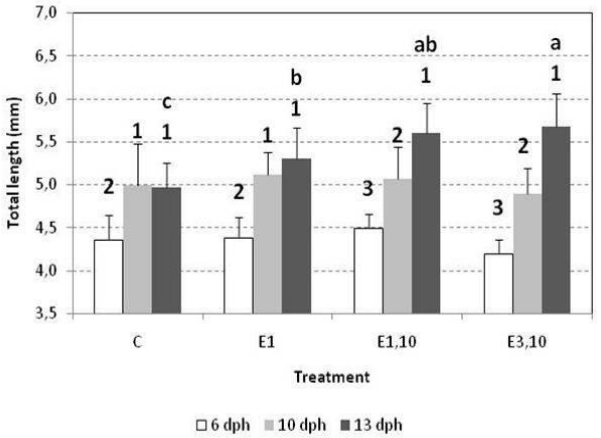


Figure 9.1.4. Total length (mm) of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions at 6, 10 and 14 dph. Values are mean ± SD, n=3. Different numbers indicate a significant difference within treatments at each age. Different letters indicate significant difference between treatments at each age (ANOVA (P ≤0.05); Tukey's HSD).

Larval survival was significantly (P≤ 0.05) affected by treatments assayed at 14 dph. The lowest survival was recorded in those larvae receiving the treatment E3,10 (**Fig. 9.1.5**) and it was significantly different to treatment E1,10, whereas no differences were found between treatments E1 and Control.

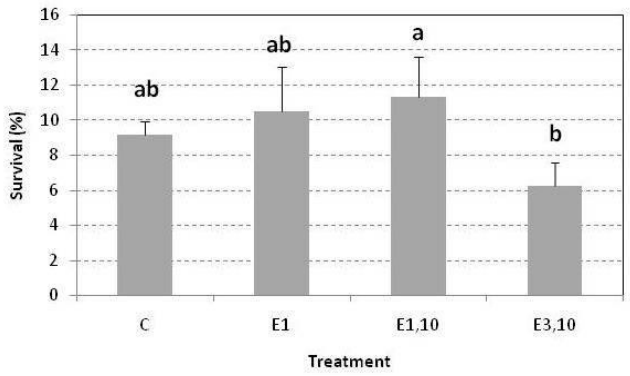


Figure 9.1.5. Final survival percentage (14 dph) of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions. Values are mean ± SD (n=3). Different letters indicate a significant difference between treatments (ANOVA (P ≤0.05); Tukey's HSD).

The eye diameter to total length ratio (%) for all treatments was 9.07±0.23 % at 14 dph. The treatment E1,10 showed the highest ratio and it was significantly different (P≤ 0.05) to treatment Control, whereas treatments E1 and E3,10 were similar between them. The percentage of larvae with the swim bladder inflated was similar for all treatments (84 ± 16 % of larvae) at 14 dph.

Elevated and significantly higher (P < 0.05) whole body cortisol levels were observed in larvae fed treatment E1 at 14 dph (**Table 9.1.4**). On the contrary the cortisol level of larvae from treatment E1,10 was the lowest one. No significant differences (P < 0.05) in whole body glucose levels were observed among the different treatments (**Table 9.1.4**) although a similar trend as with the cortisol was observed for E1,10 treatment. Whole body lactate showed higher values in Control larvae treatment when compared to the other groups, whereas whole body Na+ and K+ levels in larvae fed with different treatments were not significantly different.

Table 9.1.4. Whole body cortisol (ng mg protein -1), glucose (mg mg protein -1), lactate (mg mg protein -1), Na^+ (mg mg protein -1) and K^+ (mg mg protein -1) in 14 dph greater amberjack larvae fed with rotifers enriched with commercial (C) and experimental (E1; E1,10 and E3,10) emulsions. Values are mean ± SD (n=3). Different letters indicate a significant difference between treatments (P< 0.05)

	C	E1	E1,10	E3,10
Cortisol	6.24 ± 0.28 ab	11.36 ± 3.13 a	4.04 ± 0.80 b	5.63 ± 1.80 ab
Glucose	0.14 ± 0.05	0.10 ± 0.04	0.09 ± 0.05	0.15 ± 0.06
Lactate	1.76 ± 0.06 a	0.68 ± 0.13 b	0.65 ± 0.35 b	0.75 ± 0.14 b
Na^+	250.22 ± 26.05	160.12 ± 124.18	104.46 ± 6.09	261.78 ± 74.17
K^+	17.22 ± 3.77	11.36 ± 4.51	8.83 ± 1.41	7.69 ± 2.23

In spite of the trend of total carotenoids to increase in rotifers with dietary supplementation, the differences were not significant among experimental treatments. In addition only Control rotifers lipid contents displayed significantly higher lipid content with respect to the experimental diets (**Figure 9.1.5**). A good correlation among total carotenoid level in rotifers can be also observed in **Figure 9.1.6**, with Control and E1 larvae displaying the lowest contents and a higher value being present in both E1,10 and E3,10. The very low standard deviation of carotenoid data from E3,10, made this treatment significantly different from that of Control and E1 larvae. The high lipid contents of the commercial emulsion is not only evident in rotifers total lipids but in corresponding larvae. Although a good correlation was found among the lower lipid levels of the experimental larvae and the corresponding values in rotifers, an apparent trend for a higher incorporation of lipids in E1,10 and E3,10 larvae can be observed (**Figure 9.1.5**).

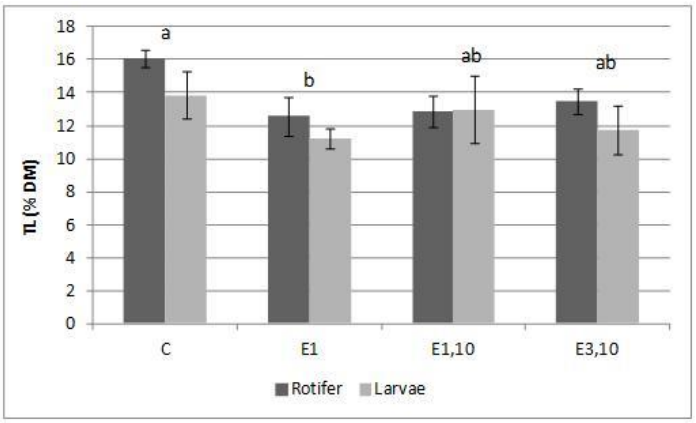
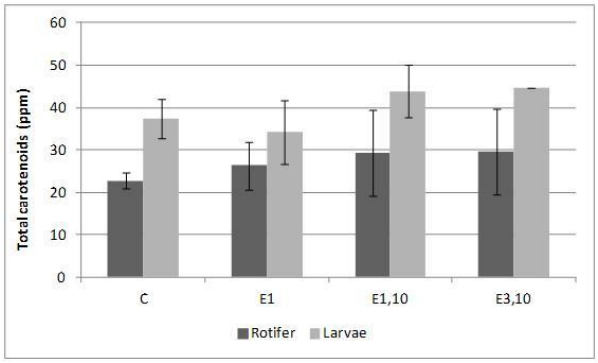


Figure 9.1.5. Total lipid content (% DM) of rotifers and larvae enriched with the control (C) or one of three experimental emulsions (E1; E1,10; E3,10).

Lipid class profiles were confirmed in rotifers sampled in present experiment (data not shown), so that higher contents of TG in Control and E3 rotifers, as well as a higher total polar lipid content of rotifers enriched with treatment E1. Due to sudden mortalities displayed by two of the Control larval replicates, only a very small sample was available for lipid analysis and its lipid profile is consistent with rotifer composition, since a higher content of TG and lower levels of TPL were present. On the contrary, independently of rotifers lipid classes' profiles, experimental larvae composition was quite similar among treatments with a significant amount of TPL compared to TG. According to the larval TL fatty acid composition, there were higher contents of DHA in both E1 and E1,10 treatments (24.8 ±1.3 and 22.0 ± 2.5%, respectively), whereas values of DHA for E3,10 was 17.8 ± 1.4 %. The same trend was even more evident for the polar lipids.



Rotifers enriched with polar rich emulsion containing a marine natural lecithin LC60 combined with 10 ppm of Naturose resulted in a significant advantage in larval growth, survival and welfare compared to rotifers enriched with other emulsions. Therefore the use of the marine phospholipids combined with carotenoids may have had a beneficial effect on greater amberjack growth and survival.

Figure 9.1.6. Total carotenoids content (ppm) of rotifers and larvae enriched with the control (C) or one of three experimental emulsions (E1; E1,10; E3,10).

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)

The work has started on 18o month as was planned. The objective of this subtask is to investigate the optimum levels of lysine in on-growing diets of amberjack juveniles, which will be based mainly on plant ingredients (low fishmeal inclusion) in favour of health, welfare status and growth of fish. Six iso-energetic (22.8 MJ kg^{-1}) extruded diets (2.5 mm pellets) containing 45% crude protein, 25% fish meal level and graded levels of crystalline lysine (1.85, 1.93, 2.01, 2.09, 2.17, and 2.25%, **Table 9.2.1)** were manufactured by Skretting and transferred to the experimental facilities of the Hellenic Centre for Marine Research (HCMR) in Ag. Kosmas, Athens, Greece.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fish meal	25.00	25.00	25.00	25.00	25.00	25.00
Wheat	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
L-Lysine	*0.00*	*0.10*	*0.21*	*0.31*	*0.41*	*0.52*
phopsphate	0.61	0.61	0.61	0.61	0.61	0.61
Mineral & Vit mix	0.50	0.50	0.50	0.50	0.50	0.50
Chemical composition						
MOIST	7.72	7.71	7.70	7.69	7.68	7.66
C PROT	45.00	45.08	45.17	45.25	45.34	45.42
C FAT	18.00	18.00	18.00	18.00	18.00	18.00
ASH	5.43	5.43	5.43	5.43	5.43	5.43
TOT LYS	18.50	19.30	20.10	20.90	21.70	22.50
CHO	29.28	29.21	29.13	29.06	28.99	28.91
Energy MJ/kg)	22.81	22.82	22.83	22.83	22.84	22.85

Table 9.2.1. Formulation, ingredients, and chemical composition of the experimental diets (%)

The diets were fed to juvenile amberjack (initial average weight 32.8 g ± 3.0) distributed in 18 experimental small cages (1.1 x 1.0 x 1.5 m), with 25 fish per cage, 3 cages per diet for a period of 60 days. 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) (**Figure 9.2.1**). Seawater was distributed in each 36 m^3 tank from 10 different pipes at 400 l h^{-1} and aerated to over 80% oxygen saturation. Water temperature followed the ambient seasonal temperature throughout the experiment with an average value of 19.4 ± 2.6ºC. The photoperiod followed the natural cycle of the season. Water quality was regularly checked and total ammonia levels were always below 0.3 mg l^{-1}. Fish were hand-fed ad libitum twice a day (09:00 and 15:00 h) to apparent satiation, six days a week with the experimental diets for a period of 60 days. Uneaten feed was collected and weighted after each meal in order to monitor daily feed consumption. Chemical analyses (body proximate composition, blood analysis, etc.) and evaluation of the amberjack growth performance are in progress.

Fig. 9.2.1. Experimental tanks in P1. HCMR's facilities and fish blood sampling from amberjack after the end of the feeding trial.

Sub-task 9.2.2 (CANEXMAR, Lara Soares)

The grow-out diet developed with the above information will be tested at an SME level, in order to assay its efficiency to maximize growth potential and enhance fillet quality. Therefore, this task cannot start until the previous one has finished.

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

Sub-task 9.3.1 (FCPCT, Hipolito Fernandez Palacios and Daniel Montero)

In the present study, 21 individuals of a group of 22 greater amberjacks, captured in May 2011 in the southeast coast of Gran Canaria (Islas Canarias, España) (**Figure 9.3.1a**) were used. The capture of the fish was done using depth pots of 3 m diameter (**Figure 9.3.1b**)

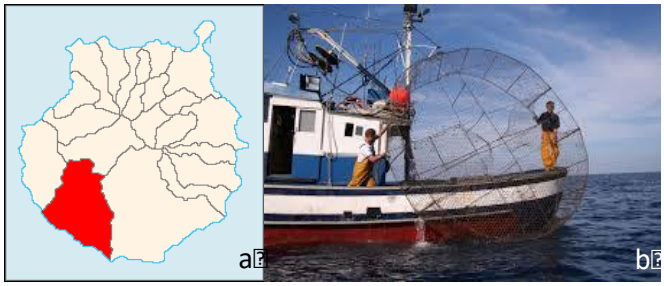


Figure 9.3.1. a) Municipality of Mogan, where wild greater amberjack were captured (Gran Canaria; Spain); b) capturing methodology

Fish, with a mean weight of 3.41±1.12 kg for females and 2.37±1.07 kg for males, were stocked at squared tanks of 10 m^3 capacity (3 m x 3 m x 1,5 m) at P2. FCPCT (**Figure 9.3.2a**). On January 2013, the fish with a mean weight of 8.27±1.11 kg for females and 8.12±1.82 kg for males, were transported to the new station of broodstock, were they were kept in round tanks of 40 m^3 (5 m x 2,35 m) (**Figure 9.3.2b**).

Figure 9.3.2. a) Tanks of 10 m^3 at P2. FCPCT facilities, b) 40 m^3 tanks

Before the start of the experiment, at the end of March of 2015, all fish were anesthetized with clove oil (Guinama SL, Valencia, Spain) at a concentration of 50 ppm and body weight was determined. The broodstock group that in the year 2014 had natural spawns, did not change, the rest were distributed in four circular tanks of 40 m^3, at a ratio of 2 females and two males in each experimental tank. Fish were weighted and growth since capture was monitored (**Figure 9.3.3**) to check that the whole population were well adapted and grew properly since their capture from the wild. The tanks were supplied with water at a circulation rate of 600% day^{-1} and salinity of 37 ‰. The photoperiod was natural, using the day period of the geographical position of (27º 59' 28" N; 15º 22'05" W). Temperature and oxygen was determined in continuous, by means of a probe system controlled by computer (Miranda, Innovaqua, Sevilla, Spain). The fish were fed twice per week with commercial pellets (Vitalis CAL, Skretting, Burgos, Spain) at a rate of 1% of the total biomass, and once a week with mackerel at a rate of 2% of the total biomass (*GIA feeding regime*).

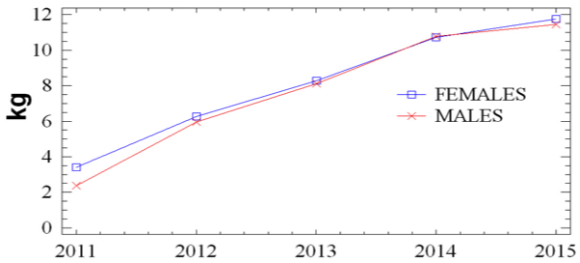


Figure 9.3.3. Evolution of the weight of the broodstock group captured in 2011.

The first natural spawn (Tank 1) was on 18 May 2015, with a temperature of 20.8ºC. The induced spawns (Tanks 2, 3 and 4) started on 2 June 2015. Spawn induction was once per week. Fish were injected intramuscularly with GnRHa (LHRHa, des-Gly^{10}, [D-Ala^{6}]-; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 µg kg^{-1}. The last natural spawn (Tank 1) was on 9 October 2015, at a temperature of 24.6ºC. Within the induced group, Tanks 3 and 4 finished spawning at 8 October 2015, whereas Tank 2 finished at 28 October 2015. The mean temperature during the spawning season ranged between 20.5±0.3ºC in May and 24.5±0.2ºC in October. The indexes of spawning quality of broodstock fed with GIA feeding regimes during May-July 2015 demonstrated that there were no differences in terms of spawning quality among the different spawning groups (**Table 9.3.1**).

Table 9.3.1. Quality of spawn indexes or broodstock fed with GIA feeding regimes *Difference superscripts in the same column indicate significant differences.

Tank	Spawn	% Fertilization	% Egg viability at 24 h	% Hatching	% 4 day live	% 8 day live
T1	Natural	71.49±27.07	83.00±28.57	80.87±29.09	84.73±8.43	7.23±5.08
T2	Induced	69.13±17.55	93.15±6.74	90.77±8.65	72.76±15.99	5.03±2.98
T3	Induced	58,81±16.78	87.36±7.83	86.16±7.76	73.19±11.58	3.36±1.78
T4	Induced	63.71±19.79	96.01±2.11	93.23±2.64	72.57±13.71	2.82±1.84

After this evaluation, fish in the different tanks were fed with three experimental diets (**Table 9.3.2**) beginning on 13 July 2015, three times per week at a daily ratio of 2% of biomass. Tank 1 with continuous natural spawns was fed with the GIA feeding regime. Tanks 2, 3 and 4 were fed the Pro, Tau and His diets, respectively. Amino acid analysis from experimental diets were also obtained (**Table 9.3.3**)

Table. 9.3.2. Raw material composition and proximate analysis of the experimental diets

	Pro	**Tau**	**His**
Raw material (%)			
Wheat	11.81	18.29	17.94
Wheat gluten	17.00	13.00	13.00
Fish meal	48.36	44.64	45.14
Squid meal	10.00	10.00	10.00
Fish oil	12.18	12.50	12.47
Taurine	0.00	0.93	0.00
Histidine HCl	0.00	0.00	0.81
Premix vit. Min.	0.64	0.64	0.64
Total	100.00	100.00	100.00
Moisture (%)	5.9	7.0	7.6
Crude protein (%)	56.1	51.5	51.3
Crude fat (%)	18.3	18.5	17.8
Ash (%)	8.4	8.4	8.4

Table. 9.3.3. Amino acid (% of feed) composition of the experimental diets

(%)	Pro	Tau	His
Arginine	2.98	2.76	2.67
Histidine	1.09	1.02	1.50
Isoleucine	2.11	1.93	1.88
Leucine	3.85	3.55	3.45
Lysine	3.21	3.09	2.90
Methionin	1.33	1.28	1.25
Cystin	0.62	0.58	0.55
Phenylalanine	2.10	2.02	1.91
Tyrosine	1.42	1.40	1.30
Threonine	2.04	1.91	1.85
Valine	2.27	2.12	2.04
Alanine	2.74	2.56	2.48
Aspartic acid	4.28	4.08	3.98
Glutamic acid	10.07	9.20	8.92
Glycine	3.11	2.91	2.86
Proline	3.15	2.93	2.93
Serine	2.36	2.21	2.13
Sum of AA- (Amm)	48.73	45.55	44.60
Taurine	0.32	1.13	0.30

The results of the spawning quality indexes obtained for a) 11 spawns of Tank 1 (natural spawns and GIA feeding regime) from 27 July 2015, b) 19 spawns of Tank 2 and Tank 3, (after 25 inductions) and c) 20 spawns of Tank 4 (after 28 inductions) are shown in **Table 9.3.4**. It can be observed that the percentage of fertilization was significantly ($P<0.05$) better in broodstock fed the GIA feeding regime (natural spawns) and those fed His diet (induced spawns), than in broodstock fed the Pro diet, with a higher protein content (induced spawns). With respect to viable eggs 24 h after hatching, best results were obtained with the His diets, being significantly higher than those fed diets Pro and Tau (all induced spawns). Results from natural spawns from broodstock fed GIA feeding regime were intermediate and not significantly different than broodstock fed diet Tau or His. Hatching rates were higher in broodstock fed His diet, without significant differences with that of fish fed GIA feeding regime (natural spawns), but being significantly higher than for fish fed Pro or Tau diets. Larval survival 3 day after hatching (dah) was higher for broodstock fed His diet, without significant differences with those fed GIA feeding regime, Pro diet or Tau diet, consecutively. Larvae survival at 5 dah was higher in broodstock fed His diets with no differences with broodstock fed GIA feeding regime, but being significantly ($P<0.05$) higher than in fish fed Pro or Tau diets. Total number of eggs, obtained during all the spawning season, was of 28.54 millions in GIA broodstock (natural spawning) and of 5.14 millions, 11.14 millions and 20.36 millions in Pro, Tau and His broodstock, respectively.

Table 9.3.4. Spawning quality indexes of broodstock fed with diet GIA feeding regime and experimental diets. *Difference superscripts in the same column indicate significant differences. Tank 1 with continuous natural spawns was fed with the GIA feeding regime. Tanks 2, 3 and 4 were fed the Pro, Tau and His diets, respectively.

Diet	Spawn	% Fertilization	% Egg viability at 24 h	% Hatching	% 3 dah larvae	% 5 dah larvae
GIA	Natural	81.22 ± 6.78^{a}	94.79 ± 2.51^{ab}	92.70 ± 3.52^{ab}	90.95 ± 8.37^{a}	8.36 ± 4.19^{ab}
Pro	Induced	56.21 ± 14.58^{b}	90.00 ± 5.45^{b}	87.60 ± 7.14^{b}	68.03 ± 9.90^{b}	4.42 ± 1.74^{bc}
Tau	"	65.81 ± 16.57^{ab}	91.99 ± 6.48^{c}	77.93 ± 8.56^{c}	53.23 ± 8.20^{c}	0.11 ± 0.33^{c}
His	"	77.85 ± 12.71^{a}	97.07 ± 3.85^{a}	96.12 ± 4.65^{a}	87.72 ± 9.83^{a}	11.49 ± 8.61^{a}

The total production of eggs during the common feeding trial with GIA feeding regime, and with experimental diets with diet GIA is shown in **Fig. 9.3.4.** Before feeding the experimental diets total number of eggs produced per female was better in the broodstock with the natural spawns (Tank 1) than in those with induced spawnings. No significant differences were found among eggs produced by hormonally induced fish (Tanks 2-4). However, when feeding the experimental diets, fish fed the His diet (Tank 4) increased the number of eggs, being closer to the numbers obtained by the natural spawning (**Fig. 9.3.5)**. **Tables 9.3.5, 9.3.6 and 9.3.7** show the amino acid (% in dm) composition of the fertilized eggs, and larvae 3 and 5 dph, respectively.

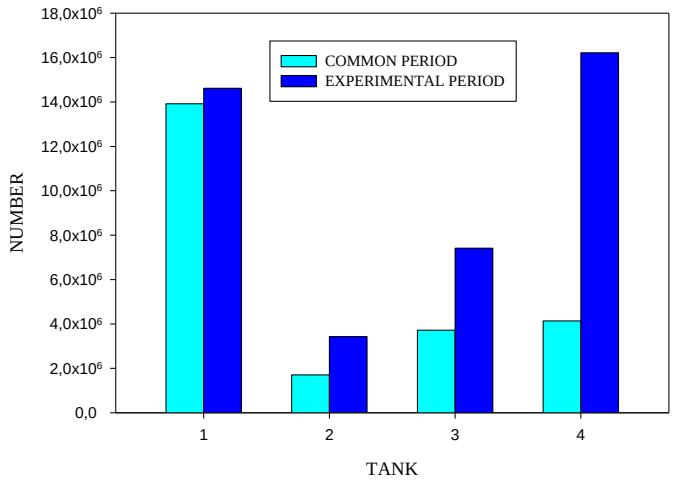


Fig. 9.3.4. Total production of eggs within the experimental tanks**.** Tank 1 with continuous natural spawns was fed with the GIA feeding regime. Tanks 2, 3 and 4 were fed the Pro, Tau and His diets, respectively.

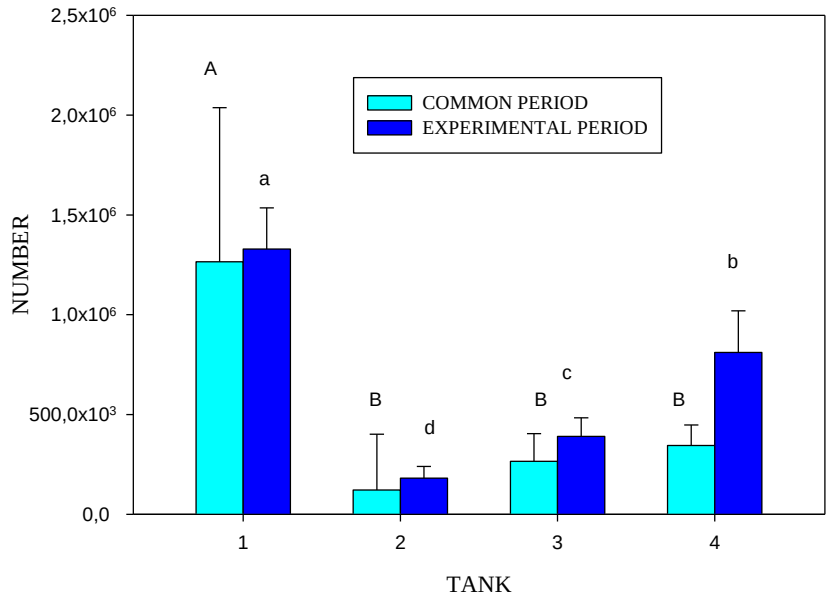


Fig. 9.3.5. Number of eggs per spawn, before and after the experimental feeding diets. Tank 1 with continuous natural spawns was fed with the GIA feeding regime. Tanks 2, 3 and 4 were fed the Pro, Tau and His diets, respectively. Different letter superscripts indicated significant differences among tanks during a specific experimental period (one-way ANOVA, $P \leq 0.05$).

Table. 9.3.5. Amino acid (% in dm) composition of the fertilized eggs.

	Diet											
	GIA			Pro			Tau			His		
Date	20/08/2015	03/09/2015	17/09/2015	26/08/2015	10/09/2015	10/10/2015	20/08/2015	03/09/2015	17/09/2015	20/08/2015	03/09/2015	17/09/2015
Amino acid												
% of dm												
Arginine	3.17	3.51	2.67	3.67	3.74	2.81	3.70	3.66	3.76	3.74	4.00	3.11
Histidine	1.69	1.96	1.43	1.91	1.94	1.54	1.90	1.91	2.02	1.95	2.00	1.65
Isoleucine	2.99	3.45	2.53	3.41	3.47	2.68	3.51	3.44	3.55	3.57	3.44	2.86
Leucine	4.64	5.15	3.91	5.13	5.21	4.08	5.32	5.13	5.30	5.41	5.19	4.28
Lysine	3.76	4.50	3.23	4.46	4.60	3.54	4.45	4.55	4.72	4.56	4.63	3.77
Methionin	1.49	1.70	1.11	1.67	1.73	1.13	1.72	1.68	1.74	1.79	1.75	1.14
Cystin	1.03	1.09	0.92	1.06	1.09	0.88	1.07	1.06	1.11	1.04	1.10	0.89
Phenylalanine	2.77	3.07	2.43	2.98	3.07	2.49	3.08	3.16	3.20	3.09	3.11	2.55
Tyrosine	1.60	1.96	<LOQ	2.34	2.42	<LOQ	2.44	2.17	1.97	2.52	2.56	<LOQ
Threonine	2.83	3.12	2.38	3.07	3.11	2.48	3.12	3.09	3.22	3.19	3.17	2.58
Valine	3.43	3.72	2.83	3.65	3.75	2.97	3.73	3.72	3.88	3.80	3.84	3.13
Alanine	4.13	4.67	3.48	4.73	4.79	3.77	4.72	4.79	4.89	4.79	4.84	3.95
Aspartic acid	4.28	4.67	3.67	4.47	4.57	3.70	4.56	4.53	4.76	4.55	4.75	3.78
Glutamic acid	7.23	7.92	6.26	7.79	7.87	6.29	7.86	7.75	8.19	7.77	8.07	6.44
Glycine	2.03	2.30	1.73	2.25	2.26	1.80	2.16	2.27	2.36	2.19	2.43	1.90
Proline	3.94	4.03	3.47	4.12	4.21	3.39	4.09	4.03	4.26	4.14	4.33	3.54
Serine	2.79	3.51	2.30	3.45	3.50	2.69	3.31	3.45	3.60	3.51	3.49	2.87
Sum of AA-(Amm)	53.80	60.33	44.35	60.16	61.33	46.24	60.74	60.39	62.53	61.61	62.70	48.44
Taurine	0.52	0.69	0.32	0.63	0.69	0.51	0.57	0.76	0.77	0.75	0.71	0.62

Table. 9.3. 6. Amino acid (% in dm) composition of the 3-dph larvae.

	DIET			
	GIA	Pro	Tau	His
Amino acid				
% of dm				
Arginine	3.35	3.38	3.60	3.34
Histidine	1.55	1.63	1.58	1.57
Isoleucine	2.40	2.34	2.30	2.38
Leucine	4.31	4.28	4.24	4.19
Lysine	4.19	4.39	4.31	4.19
Methionin	1.56	1.64	1.55	1.52
Cystin	0.80	0.81	0.78	0.82
Phenylalanine	2.45	2.64	2.34	2.51
Tyrosine	1.83	2.03	1.76	1.34
Threonine	2.51	2.58	2.48	2.41
Valine	2.65	2.70	2.62	2.58
Alanine	3.14	3.13	3.03	3.03
Aspartic acid	5.20	5.36	5.36	5.11
Glutamic acid	7.74	7.83	7.69	7.56
Glycine	2.89	2.84	2.93	2.84
Proline	2.25	2.29	2.10	2.10
Serine	2.50	2.47	2.49	2.35
Sum of AA-(Amm)	51.32	52.34	51.16	49.84
Taurine	1.13	1.02	1.27	1.11

Table. 9.3.7. Amino acid (% in dm) composition of the 5 dph larvae.

	DIET			
Amino acid	GIA	Pro	Tau	His
% of dm				
Arginine	2.47	2.75	3.38	2.32
Histidine	0.79	0.99	1.26	0.89
Isoleucine	1.73	2.12	2.18	1.71
Leucine	3.09	3.64	3.93	3.00
Lysine	2.98	3.20	3.90	2.78
Methionin	1.22	1.17	1.42	0.97
Cystin	0.69	0.78	0.76	0.59
Phenylalanine	1.66	2.16	2.17	1.62
Tyrosine	1.07	1.33	1.47	0.58
Threonine	1.96	2.22	2.30	1.76
Valine	2.08	2.44	2.43	1.90
Alanine	2.46	2.76	2.93	2.17
Aspartic acid	3.90	4.61	4.89	3.72
Glutamic acid	5.41	6.13	6.86	5.03
Glycine	2.49	2.66	2.92	2.15
Proline	1.71	2.13	2.07	1.52
Serine	1.89	2.16	2.38	1.76
Sum of AA-(Amm)	37.60	43.25	47.25	34.47
Taurine	0.93	0.97	1.35	0.90

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

With the aim of approaching the lipid and carotenoid profile of eggs released by cultured females to their wild counterparts, experimental diets with optimized Essential Fatty Acids (EFA) and carotenoid contents are being designed. Fecundity, egg quality and haematological and biochemical indicators of fish health will be studied (P8. IEO). In previous studies conducted by our research group it has been shown that cultured females displayed lower proportion 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. 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*** that has been recently submitted, 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. 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.

Taking into account these previous results, a new experimental diet has been formulated to be tested. This experimental diet will have a similar total content of proteins (52 %), crude fat (20 %) and carbohydrates (6 %), but the new formula will differ in the proportions of the above mentioned essential fatty acids. At this moment different available protein and lipid sources are being evaluated. Experimental diets will be supplemented also with Vitamin A (10.000 U.I./kg), Vitamin D3 (1.500 U.I./kg), Vitamin E (150 mg/kg) and Astaxanthin (25 ppm). Contacts have already been established with Skretting España SA for the manufacture of feeds. A diet based exclusively on mackerel (*Scomber scombrus*) will be used as reference due to the good spawning results obtained in wild broodstock so far. Moreover, a second reference diet based on mackerel supplemented with ARA (Vevodar® DSM Food Specialities) will be tested, because previous results have reported a lack of this fatty acid in cultured broodstock feed with commercial diets in contrast to an increase of linoleic acid.

There is currently a new stock of greater amberjack broodstock available for the implementation of this task at the Culture Unit of Canary Island Oceanographic Centre P8. IEO. The fish stock will be the potential broodstock group, with an average weight of 10-12 kg, and between 4 and 5 years old, above the age considered to be of first maturity for this species, to assay the experimental diets.

This diet design process shall take into account physical characteristics of diets: size particle (~ 20 mm), 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 feed three times a week
- Estimated daily ration of 1.0 -1.5 % body weight day^{-1}
- Duration of trial 6 - 12 months
- Weekly feed: 4.2 - 6.3 kg
- Monthly feed: 16.8 – 25.2 kg
- Annual feed: 201.6 – 302.4 kg

Deviations from Annex I and their impact:

There were no deviations from Annex I.

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.29 ASIALOR
Lead Scientist preparing the Report (WP leader):	Ivar Lund			
Other Scientists participating:	Marisol Izquierdo (P2); Covadonga Rodriguez (P15); Jose A. Perez (P15); Patrick Kestemont (P16); Najlae Kertaoui (P16); Manuel Gesto (P21); Tu Linh (P29)			

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 progress towards objectives and details for each task (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.

Task 10.1: Trials have shown that pikeperch larvae require both high dietary inclusion levels of phospholipids and Long Chain (LC) 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.

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.

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

One experiment has been conducted (from month 14-16) and 1 experiment started in April 2016, and was underway when this report was prepared. A first 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 (growth and organ development, tissue/liver morphology, and digestive tract development). Effects on digestive enzymatic activity, liver proteomics, candidate gene expression and skeleton morphogenesis were examined.

Fertilized eggs of pikeperch were obtained from Aquapri Innovation, Egtved, Denmark and transferred to DTU Aqua at the North Sea Research Centre, Denmark. After hatching, larvae were distributed into two 0.5 m^3 fiberglass tanks upon hatching and kept in these tanks at 19.5-20.0°C until 10 days post hatch (DPH). At 10 dph larvae were moved to a temperature controlled freshwater flow through larval system consisting of 18 conical tanks of 50 l each. Each tank was initially stocked with 16 larvae l^{-1} and reared at 20-21°C until 30 dph. The experiment was carried out in a triplicate set up with 3 tanks per feed type and 6 dry feed types (3 x 6) (**Table 10.1.1**). From 11-17 dph larvae in a triplicate setup were fed one of six experimental dry feed diets along with enriched *Artemia* from 8 a.m.- 10 p.m. During this dry feed weaning period the feeding of *Artemia* was postponed by 1 hour daily. A mixture of 2 sizes of dry feed (400-600 µm and 600-800 µm) was used for the experiment. Dry feed was administered daily by programmable automatic feeders (made by DTU Aqua), which allowed very small quantities to be fed at specific time intervals. Dry feeds were fed in surplus approximately 25% (of expected larval biomass) in the first week decreasing to 10% during the third week.

The analysis of the experimental diets confirmed the expected values **(Table 10.1.1)** with almost similar levels of protein and lipid and increasing levels of phospholipids (PL1- PL3; PL1H1- PL3H3) – and EPA and DHA (PL1H1- PL3H3). **Table 10.1.2** shows the dietary composition of FAs in % of total FA content emphasizing a significant difference between PL3 and PL3H3 for dietary DHA content and total PUFA content. In addition total n-6 content increased by increase in soy lecithin inclusion and total content of n-3 increased by addition of Algatrium DHA 70 in diet PLH1-PLH3. Consequently n-3: n-6 was highest in PLH1-PLH3 and lowest in diet PL3. EPA and DHA tissue content was especially much elevated in diet PLH2 and PLH3. The FA composition of the larvae at 30 dph reflected well the FA composition of the diets **Table 10.1.3**.

Table 10.1.1 Mean (±SEM) dietary composition and analytical content of the 6 experimental diets.

Diet Ingredients (%)	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
MicroNorse Fish Meal	45	45	45	45	45	45
CPSP 90	7	7	7	7	7	7
Squid meal	13	13	13	13	13	13
Fish gelatin	1	1	1	1	1	1
Wheat Gluten	4.4	4.4	4.4	4.4	4.4	4.4
Wheat meal	6.1	5.9	5.6	6.1	5.9	5.6
Algatrium DHA70	0.0	0.0	0.0	0.55	2.0	3.4
Olive oil	18.9	12.1	3.4	18.4	10.1	0.0
Vitamin & Mineral Premix PV01[i]	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin powder	3.0	10.0	19.0	3.0	10.0	19.0
Binder (guar gum)	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant powder (Paramega)	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant liquid (Naturox)	0.2	0.2	0.2	0.2	0.2	0.2
Analysed content (% DM)						
% dry matter (DM)						
Crude protein	54.1	54.7	55.6	54.1	55.8	55.3
Crude lipid	26.8	25.9	24.6	27.6	25.6	24.8
NFE + fibre (substracted)	3.0	3.0	2.8	2.8	3.1	3.2
Ash	9.1	9.4	10.0	9.0	9.3	10.2
HUFAs (%TFA)						
EPA	0.41	0.41	0.41	0.47	0.61	0.75
DHA	0.66	0.66	0.66	1.04	2.06	3.04
Phospholipids (% lipids)						
Phosphatidyl choline	1.40	2.61	4.31	1.42	2.68	4.29
Phosphatidyl ethanolamine	0.43	1.22	2.20	0.40	1.14	1.87
Phosphatidyl inositol	0.44	1.28	2.44	0.43	1.28	2.48
Total phosholipids	3.73	8.19	14.38	3.70	8.32	14.51

(from previous Table) [I] PVO40.01 premix for marine fish (Premix Lda, Viana do Castelo, Portugal). Vitamins (per kg diet): 100 mg DL-alpha tocopherol acetate, 25 mg sodium menadione bisulfate, 20.000 IU retinyl acetate, 2.000 IU DL-cholecalciferol, 30 mg thiamine, 30 mg riboflavin, 20 mg pyridoxine, 0.1 mg B_{12}, 200 mg nicotinic acid, 15 mg folic acid, 1.000 mg ascorbic acid, 500 mg inositol, 3 mg biotin, 100 mg calcium panthotenate, 1.000 mg choline chloride, and mg betaine, 500. Minerals (per kg diet): 2.5 mg cobalt sulfate, 1.1 mg copper sulfate, 0.2 g ferric citrate, 5 mg potassium iodide, 15 mg manganese sulfate, 0.2 mg sodium selenite, 40 mg zinc sulfate, 0.6 g magnesium hydroxide, 1.1 g potassium chloride, 0.5 g sodium chloride, and 4 g calcium carbonate.

Table 10.1.2. Mean analysed TFA content (±SEM) (mg g^{-1} d.w.) and FA composition (% of TFA) of the 6 experimental feed types**.** A different letter for each FA indicates significance (P<0.05)**.**

	PL1	PL2	PL3	PLH1	PLH2	PLH3
TFA	109.2±5.7	118.0±26.0	134.1±8.2	187.5±30.6	116.3±19.6	146.7±17.9
FA						
16:0	13.0±0.2^{a}	14.6±0.1^{c}	17.8±0.1^{e}	12.9±0.2^{a}	13.8±0.0^{b}	15.2±0.1^{d}
18:0	2.6±0.0^{bc}	2.7±0.0^{b}	2.9±0.0^{c}	2.5±0.0^{ab}	2.4±0.0^{ab}	2.3±0.0^{ab}
Total SFA	17.0±0.3^{ab}	18.9±0.1^{d}	22.6±0.1^{e}	16.8±0.1^{ab}	17.6±0.0^{c}	19.3±0.1^{d}
16:1 (n-7)	1.5±0.0	1.4±0.0	1.4±0.0	1.5±0.0	1.4±0.0	1.3±0.0
18:1 (n-9)	62.1±0.2^{e}	50.0±0.1^{d}	27.0±0.0^{b}	60.1±0.4^{e}	42.6±0.1^{c}	12.4±0.0^{a}
Total MUFAs	65.5±0.2^{e}	53.5±0.1^{d}	30.7±0.0^{b}	64.2±0.4^{e}	46.0±0.0^{c}	15.9±0.0^{a}
18:2 (n-6)	10.1±0.0^{b}	18.6±0.1^{d}	34.5±0.0^{f}	9.6±0.2^{a}	17.5±0.1^{c}	31.9±0.1^{e}
20:4 (n-6) ARA	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0
Total (n-6) PUFA	10.4±0.0^{a}	18.9±0.1^{c}	34.9±0.0^{e}	9.9±0.2^{a}	17.9±0.0^{b}	32.4±0.1^{d}
18:3 (n-3)	1.1±0.0^{a}	1.7±0.0^{b}	3.1±0.0^{c}	1.0±0.0^{a}	1.6±0.0^{b}	2.8±0.0^{c}
20:3 (n-3)	0.0±0.0	0.0±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0
20:5 (n-3) EPA	1.5±0.0^{a}	1.8±0.0^{b}	2.3±0.0^{c}	1.6±0.1^{ab}	2.5±0.1^{c}	3.6±0.0^{d}
22:6 (n-3) DHA	3.6±0.1^{a}	4.2±0.0^{b}	5.1±0.1^{c}	5.2±0.2^{d}	11.7±0.0^{e}	21.4±0.2^{f}
Total (n-3) PUFA	6.2±0.1^{a}	7.7±0.0^{b}	10.5±0.0^{c}	7.9±0.3^{b}	15.9±0.0^{d}	27.9±0.1^{e}
DHA/EPA	2.4±0.1^{a}	2.3±0.0^{a}	2.2±0.1^{a}	3.3±0.0^{b}	4.8±0.2^{c}	5.9±0.1^{d}
(n-3)/(n-6)	0.6±0.0^{b}	0.4±0.0^{a}	0.3±0.0^{a}	0.8±0.0^{b}	0.9±0.0^{c}	0.9±0.0^{d}

Table 10.1.3. Mean analysed TFA content (±SEM) in larvae at 30 dph (mg g^{-1} w.w.) and FA composition (% of TFA). A different letter for each FA (at 30 dph) indicates significance (P<0.05)

	dph 0	dph 10	dph 30 PL1	PL2	PL3	PLH1	PLH2	PLH3
TFA	109.9±27.5	139.4±18.4	41.5±7.8	69.2±36.1	64.1±9.5	73.1±19.7	69.1±14.5	38.2±15.6
16:0	11.9±0.9	13.5±0.6	13.5±0.1^{b}	14.5±1.5^{b}	18.3±0.4^{a}	13.1±0.3^{b}	13.9±0.5^{b}	14.8±0.6^{b}
18:0	3.3±0.2	7.2±0.2	4.4±0.0^{c}	4.2±0.1^{bc}	4.5±0.3^{c}	3.7±0.2^{b}	3.0±0.1^{a}	3.0±0.2^{a}
Total SFA	16.9±0.9	22.8±1.0	19.0±0.1	19.6±1.6	24.1±0.3	17.8±0.4	17.9±0.6	18.8±0.8
16:1 (n-7)	6.8±0.3	2.3±0.2	1.3±0.0	1.2±0.2	1.2±0.1	1.3±0.1	1.4±0.1	1.1±0.1
18:1 (n-9)	12.0±0.4	22.5±0.1	50.5±0.5^{e}	43.8±0.8^{d}	24.7±0.8^{b}	50.3±1.8^{e}	37.4±1.1^{c}	12.8±1.0^{a}
Total MUFAs	20.0±0.5	26.3±0.0	53.3±0.5^{e}	46.5±0.5^{d}	27.3±0.5^{b}	53.2±1.8^{e}	40.1±1.2^{c}	15.0±1.0^{a}
18:2 (n-6)	8.3±0.6	7.4±0.2	11.0±0.1^{a}	18.7±0.2^{b}	29.1±0.4^{e}	10.7±0.0^{a}	19.2±0.1^{b}	28.5±0.5^{e}
18:3 (n-6)	0.1±0.0	0.5±0.0	0.2±0.0^{ab}	0.3±0.0^{bc}	0.6±0.0^{d}	0.2±0.0^{a}	0.3±0.0^{c}	0.3±0.0^{c}
20:3 (n-6)	0.0±0.0	0.2±0.0	0.3±0.0^{b}	0.3±0.0^{b}	0.6±0.0^{d}	0.2±0.0^{c}	0.2±0.0^{c}	0.1±0.0^{a}
20:4 (n-6) ARA	1.2±0.0	0.9±0.0	0.5±0.0^{bc}	0.3±0.1^{a}	0.4±0.1^{ab}	0.4±0.0^{b}	0.4±0.0^{ab}	0.6±0.1^{c}
Total (n-6) PUFA	9.7±0.6	9.3±0.3	12.1±0.1^{a}	19.6±0.1^{b}	31.0±0.4^{e}	11.6±0.1^{a}	20.2±0.1^{c}	29.7±0.5^{d}
18:3 (n-3)	1.1±0.0	27.1±0.3	1.3±0.1^{ab}	1.3±0.0^{ab}	1.9±0.1^{c}	1.1±0.2^{a}	1.5±0.1^{b}	1.9±0.2^{c}
20:3 (n-3)	0.1±0.1	1.3±0.0	0.1±0.0^{c}	0.1±0.0^{a}	0.1±0.0^{b}	0.1±0.0^{abc}	0.1±0.0^{ab}	0.1±0.0^{ab}
20:5 (n-3) EPA	7.9±0.3	2.8±0.2	2.5±0.1^{a}	2.2±0.2^{a}	2.6±0.2^{a}	2.5±0.2^{a}	2.6±0.3^{a}	3.9±0.2^{b}
22:6 (n-3) DHA	37.1±0.3	8.5±0.8	9.4±0.3^{a}	8.6±0.8^{a}	10.6±0.4^{b}	11.2±0.9^{b}	14.6±1.1^{c}	25.4±0.8^{d}
Total (n-3) PUFA	46.1±0.5	39.7±0.7	13.3±0.3^{a}	12.2±1.0^{a}	15.3±0.7^{a}	14.8±1.2^{a}	18.7±1.4^{b}	31.2±0.8^{c}
DHA/EPA	4.7±0.1	3.1±0.0	3.8±0.0^{a}	3.9±0.2	4.2±0.0	4.5±0.0	5.7±0.3	6.6±0.2
(n-3)/(n-6)	4.8±0.3	4.3±0.1	1.1±0.0^{a}	0.6±0.1	0.5±0.0	1.3±0.1	0.9±0.1	1.1±0.0

Growth

The weight of larvae after 30 dph was significantly highest for diet (PL3H3) having the combination of high phospholipid content and high DHA content as compared with diet PL1H1; PL1 and PL2 (**Figure 10.1.1**). The lowest larval weight was observed for diet PL1 and PLH1. Results indicate the importance of inclusion of both phospholipids in terms of soya lecithin in the diets and LC PUFAs in dietary formulation of pikeperch extruded feeds.

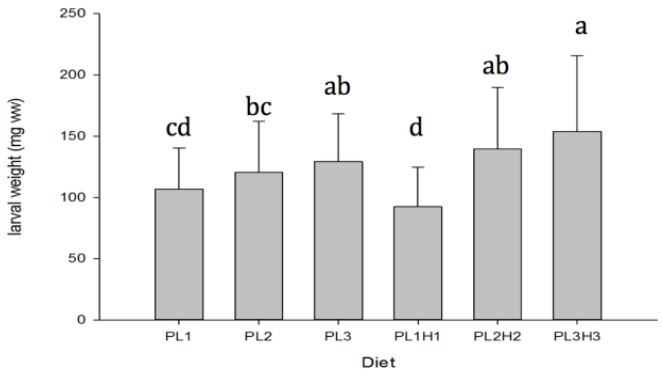


Figure 10.1.1. Mean larval weight (mg ww) (±SD) at 30 dph. A different letter on bars indicates statistically significant differences (P<0.05).

The larval enzymatic response of trypsin, peptidase (N), phosphatase (AP) and pepsin at 30 dph is detailed in **Figure 10.1.2**. Data revealed a significantly higher activity of pepsin for PL 3 and PLH3. Peptidase (N) activity also increased in diets PL3 and PLH3 while there was no clear trend in trypsin levels. Results indicate, that important digestive enzymatic activity is enhanced by dietary inclusion of PL and LC PUFAs, which may partly explain the better growth of these groups of fish larvae. Results of candidate gene expression and skeleton morphogenesis are still underway in laboratory. Certain primers are about to be made for pikeperch larvae before analysis of gene expression can be done.

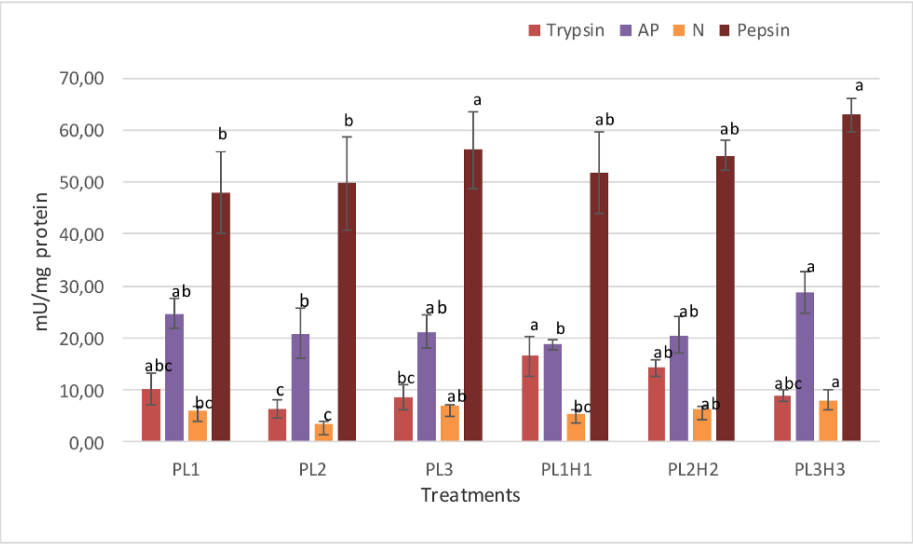


Figure 10.1.2. Mean larval enzymatic response (±SD) at 30 dph for trypsin, peptidase (N), phosphatase (AP) and pepsin. Different letters on bars with same color indicate statistically significant differences (P<0.05).

A multifactorial experiment is currently carried out **(Table 10.1.4)** to evaluate the effects of 2 levels of Ca/P, EPA+DHA, ARA, Vit E, C, A, D and Se. The experiment was carried out as a fractional factorial screening. Larvae were obtained by a Dutch producer. A total of 16 diets has been made by SPAROS (Portugal) and fabricated as cold extruded feeds in size ranges of 200-400 and 400-700 µm. The experiment was performed at P16. FUNDP involving 16 tanks. *Artemia* were fed until 18 dph and subsequently a mixture of all feed types were fed in the transition phase from live feeding to dry feed feeding. At 25 dph when larvae were weaned to dry feeds each of the 16 diets were fed to one tank of larvae for further 14 days. Survival, length, body weight and growth are measured on fry and fry are analyzed for proximate composition and FA content. Organ development, tissue morphology, digestive enzymatic activity, analyses of candidate genes expression, bone ossification and skeleton morphogenesis are analyzed. Results were evaluated by Planor software, which is able to handle multifactorial data. As for the first experiment, this work will be part of Deliverable D10.1 to be submitted in Mo 36.

Table 10.1.4. Dietary variables in multifactorial design

Dietary variable	Low level	High level
Ca/P	0.6	1.2
DHA+ EPA	1.25 %	3.5%
ARA	0.8 %	1.6 %
Vitamin E	1000 mg /kg	3000 mg/kg
Vitamin C	2000 mg /kg	3600 mg/kg
Vitamin A	8000 mg/kg	30000 mg/kg
Vitamin D	2800 IU/kg	28000 IU/kg
SE	3 mg/kg	12 mg/kg

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

This task involves the long term influence of early enrichment (*i.e.* fish marine phospholipids, DHA concentrations and n-6/n-3 studies of pathways of fish tissue lipid / FA catabolism / resynthesis, FA composition and influence on eicosanoid activity in relation to early feeding and dietary lipid composition. As pikeperch originate from brackish water, salinity may improve rearing conditions and lead to better performance, therefore salinity was introduced as an environmental cue.

Initially during 2015 several pilot studies were carried out to examine the tolerance of pikeperch larvae to various salinity gradients during early ontogeny. These experiments were conducted on pikeperch larvae obtained by AquaPri, Denmark. It has been assumed that pikeperch larvae cannot tolerate salinities higher than their own body fluids (i.e. > 10), but salinity tolerance of pikeperch seems to depend on age. Juveniles and adults can tolerate elevated salinity levels (12 ppt) with no mortality. Other studies have shown no mortalities in acute tests at 8 and 16 ppt. but full mortalities at 26 and 35 ppt. Pikeperch juveniles therefore have the ability to hypo-osmoregulate (maintain body fluid osmolality below that of the external environment) but studies with pikeperch larvae are scarce.

In the pilot series, acute salinity tolerance was tested by the transfer of 300 1dph old larvae to 15-l containers (20 larvae in each container) with water at 0, 2, 4, 8 and 12 ppt (three containers for each salinity) at 20°C, with low light intensity, all provided with aeration. This trial was performed over a period of 24h, after which survival and general behavior was evaluated. This method was repeated at 3 and 5 dph. As there was no mortality at 1 dph, salinities 16 and 20 ppt were added to the trial from 3 dph. Stepped salinity tolerance was tested by counting 300 larvae into six containers of 2L at 0 ppt, 20ºC, with aeration and low light intensity. Three containers were kept at 0 ppt (control). For the other three, the salinity was daily increased to 2, 4, 8 and 12 ppt, and survival and general behavior were evaluated.

Growth tests were carried out to observe if larvae not only could survive for some days but also grow and accept feed offered. Thus 400 larvae at 4 dph were divided by eight containers of 2L at 0, 4, 8 and 12 ppt at 20 ºC, with aeration, for 5 days until 9 dph. Larvae were fed newly hatched *Artemia* every day, from 5 dph. Survival and general behavior were evaluated every day. This trial was repeated at 9 dph. Total length (TL) of the larvae was measured several times during the experiment at different intervals, depending on the experiments. The measurements were done by taking a picture of the larvae under a microscope with a scale.

Results

The 1.3 and 5 dph larvae that were exposed to various acute saline gradients up till 20 ppt experienced some mortality observed in the high saline groups of 16 and 20 ppt with no clear effect of age. The highest mortality was at 20 ppt. Larvae laying on the bottom of the containers were displaying a lethargic behavior. At 20 ppt, the larvae that survived did not react to mechanical stimulation.

For the larvae in the stepped salinity groups some mortality occurred, but with no difference to the control larvae at 0 ppt kept in a separate tank. It seemed in general that larvae did not acclimatize well in 1-l beakers and mortality was higher than in a normal rearing tank of 300 l (1x1 m). For growth the first group was transferred at 4 dph and after 5 days (9 dph) all the larvae that at 12 ppt died, in comparison at dph 4 and dph 8 around 80% survived, but also in the control group of 0 ppt some mortality was observed until 9 dph. The total length (TL) of the larvae was 5.84±0.40. 5.65±0.12 and 5.45±0.28 mm at 0, 4 and 8 ppt, respectively, while the larvae in the control tank were 6.97±1.04 mm. Obviously larvae were growing less in the beakers. The study showed that larvae can survive and grow both by abrupt and stepped transfer to low saline levels at least up to 8 ppt and indicate that pikeperch has a hypo-osmoregulatory ability, confirming what was shown in previous studies. It has been suggested that an isosmotic environment could be beneficial for both estuarine and marine fishes, and growth rate and food conversion may be improved in that type of environment. In the present study there was no significant difference in the TL of pikeperch larvae between all the salinities used in the growth test, which may indicate that larvae can survive and grow at a salinity of 8 ppt with no obvious indicators that those salinities represented a stressful environment to the larvae.

Lipid metabolism Experiment

Based on these initial salinity tolerance pilot studies, a first experiment involved investigation of lipid metabolism, performance and stress related tolerance in pikeperch larvae by using salinity as an experimental cue.

Larvae were obtained by a commercial producer (AQUAPRI, DK) and transferred to the facilities at DTU Aqua in Denmark. After hatching larvae were distributed to 18 tanks + 1 control tank and fed un-enriched *Artemia* from 4 dph. From 10 dph larvae were fed 24 h EG Artemia enriched by an emulsion with high levels of n-6 by use of sunflower oil, by a high level of n-3 by rape seed oil by or by a commercial DHA Selco control from INVE Aquaculture. From 15 dph larvae were sampled to study the uptake, assimilation and transesterification ability.

The study involved the use of ^{14}C radio-labelled FA for checking FA assimilation of the larvae and processes of esterification into a specific lipid class, or even transformation into longer and more unsaturated FA at 3 salinities 0 ppt, 5 ppt and 10 ppt. Dietary contents of n-3 and n-6 C18-PUFA precursors, *i.e.* 18:3 n-3 and 18:2 n-6; EPA; ARA and DHA, as well as two lipid classes PC, PE were tested to determine the pathways and activities of LC-HUFA biosynthesis in pikeperch larvae, in order to elucidate potential mechanisms underpinning its FA profile.

The tissue capability to synthesize or re-esterify LC-PUFAs in larval/juveniles was examined (*i.e.* by radiotracing of ^{14}C FA metabolism, and by *in situ* hybridization techniques), studies on skeleton

morphogenesis and mineralization were carried out by staining and RT-PCR methods. Total FA incorporation rates was measured in scintillation vials and Beta-counting, whereas the amount of FA esterified in a specific lipid class or transformed by desaturation- elongation steps was be picked up by using TLC auto-radiography

The study was performed in October 2015, but after 21 days an electrical failure in the freshwater supply caused total mortality due to high salinity at the end of the trial in October 2015!! - the study was then repeated in early November 2015.

Two parameters were tested in 18 tanks: Enrichment of *Artemia* with high ALA (18:3n-3) (by enrichment with rape seed oil and enrichment with high LA (18:2n-6) by sunflower oil) against a control fed *Artemia* enriched by a commercial DHA Selco. Three salinity levels were used (0-. 5-.10 ppt), by the use of peristaltic dosage pumps to each tank. The analyses performed included:

- Metabolism of lipid classes, esterification (HPTLC, TLC)
- Growth. Enzymatic activity, gene expression. Lipid class composition and FA content
- Eicosanoid production (PGE 2 and PGE 3, by LC-MS/MS)
- Deformities, staining
- *In vivo* incubation with labelled ^{14}C FAs (ALA. LA. ARA. EPA. DHA) and ^{14}C lipid classes (PC. PE). 4 hrs incubation and 0.2 µCi (0.3 µM) of [1-^{14}C]FA, [1-^{14}C]PC or [1-^{14}C]PE: 18:2n-6. 18:3n-3. ARA. EPA or DHA
- 10 pikeperch larvae per well (SARSTEDT 6 well). 10 ml of water (0. 5. 10 ‰). 17.5 ºC. Horizontal gentle stirring.

Results

The weight of larvae during the experiment is illustrated in **Figure 10.2.1**. There was no significant difference in growth calculated as SGR (P≥ 0.25) over the experimental period from dph 14 to dph 29. The FA content of the larvae is shown in **Table 10.2.1.** (Statistics not performed so far). Levels of 18:2n-6 and 18:3n-3 varied between D1 and D2 as expected according to enrichment of *Artemia* (data not shown) with rapeseed oil (high in 18:3n-3) or sunflower (oil high in 18:2n-6). The ratio of n-3:n-6 in larvae exposed to the two different diets was 1.4 - to 3.2.

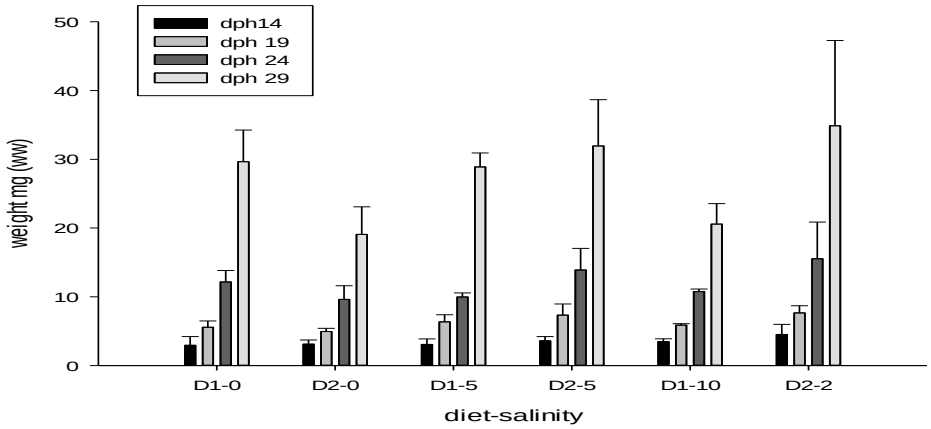


Figure 10.2.1. Weight of larvae (mg w.w.± Sd) during the experiment for the 6 treatments.

Table 10.2.1. Fatty acid content (% TFA ± SD) of larvae for the 6 treatments at 29 days post hatch (dph).

	D1-0‰	D2-0‰	D1-5‰	D2-5‰	D1-10‰	D2-10‰
C14:0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0
C16:0	14.2±0.7	13.2±0.8	13.7±0.5	14.2±0.7	14.3±0.2	14.0±0.4
C17:0	0.6±0.0	0.6±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0
C18:0	8.1±0.2	7.9±0.7	7.9±0.1	8.0±0.5	8.7±0.2	8.1±0.3
C20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
C22:0	0.6±0.0	0.6±0.1	0.6±0.0	0.6±0.1	0.6±0.0	0.6±0.0
C24:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
C16:1 cis 9	1.1±0.2	1.2±0.2	1.2±0.1	1.2±0.2	0.9±0.1	1.2±0.2
C17:1	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
C18:1 cis-9	23.9±0.9	26.2±1.4	24.7±0.5	26.1±0.5	22.9±0.6	26.4±1.2
C20:1	0.6±0.0	0.7±0.0	0.6±0.0	0.7±0.0	0.6±0.0	0.7±0.0
C24:1	1.7±0.4	1.3±0.3	1.2±0.0	1.4±0.3	1.7±0.3	1.2±0.3
C18:2 cis-9,12	15.9±0.5	8.7±0.1	16.9±0.2	8.7±0.3	15.8±0.8	8.5±0.1
C20:2	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.5±0.0	0.3±0.0
C18:3 n-6	0.6±0.0	0.5±0.0	0.7±0.0	0.5±0.0	0.6±0.1	0.5±0.0
C20:3n-6	0.6±0.0	0.5±0.0	0.7±0.0	0.5±0.1	0.7±0.1	0.5±0.1
C20:4 n-6	1.3±0.1	1.2±0.3	1.2±0.1	1.2±0.1	1.6±0.2	1.2±0.2
C18:3 cis n-3	17.1±2.0	25.9±2.2	18.9±0.5	24.1±1.3	17.5±0.9	25.2±1.1
C20:3 cis n-3	1.6±0.1	1.9±0.0	1.7±0.0	1.9±0.1	1.7±0.1	1.9±0.1
C20:5 n-3	3.0±0.3	3.0±0.5	3.1±0.2	3.2±0.3	3.7±0.2	3.2±0.3
22:6 n-3	7.1±1.8	4.8±1.0	4.4±0.3	5.1±0.8	6.0±1.2	4.3±1.1

In T**able 10.2.2** below some results are shown of esterification of C14 EPA and DHA FA in pikeperch larvae and in **Table 10.2.3** is shown total larval incorporation of LC PUFA precursors and LC PUFAs. Although statistical analyses have not been completed yet, it is evident that a different pattern for EPA and DHA esterification into different lipid classes can be observed. Independently of the dietary or salinity regime, around 14-18 % of EPA comes into PE; 10-16% into PI and 49-55% into PC. DHA was esterified at a range of 26-28% into PE; 10-18% into PI and 36-41% into PC.

Table 10.2.2. Esterification (%) of EPA (upper panel) and DHA (lower panel) into different lipid classes in pikeperch larvae at 20 dph for the two experimental diets and control diet at the three salinities tested.

	Control	D1			D2		
		0‰	5‰	10‰	0‰	5‰	10‰
TAG	6.1± 0.8	0.5 ± 0.4	0.9 ± 0.8	1.3 ± 0.1	0.2 ± 0.2	0.3 ± 0.4	0.1 ± 0.1
FFA	5.0± 0.8	3.7 ± 1.0	4.8 ± 0.1	5.4 ± 1.1	1.9 ± 0.1	3.0 ± 1.8	5.0 ± 2.2
PAG	5.5± 0.6	10.3 ± 0.8	8.3 ± 1.0	8.9 ± 0.0	10.3 ± 2.1	9.2 ± 0.6	7.6 ± 0.5
PE	14.2± 0.9	16.7 ± 0.8	17.6 ± 1.3	17.4 ± 0.4	16.5 ± 1.1	17.7 ± 0.3	18.3 ± 0.9
PG	2.0± 0.5	1.9 ± 0.3	1.4 ± 0.2	1.3 ± 0.3	1.7 ± 0.4	1.3 ± 0.0	0.6 ± 0.2
PI	10.0± 0.2	13.5 ± 0.3	13.2 ± 0.1	13.5 ± 0.6	14.1 ± 1.7	14.1 ± 0.4	16.0 ± 1.2
PS	2.7± 0.9	4.0 ± 0.3	3.9 ± 1.5	3.2 ± 0.2	3.9 ± 1.0	3.7 ± 1.0	2.5 ± 0.4
PC	54.5± 0.4	49.5 ± 1.1	49.9 ± 1.2	48.9 ± 2.0	51.6 ± 1.6	50.6 ± 2.2	50.0 ± 2.6
TNL	16.7± 1.8	14.5 ± 0.8	14.0 ± 1.6	15.7 ± 1.0	12.3 ± 2.2	12.5 ± 2.6	12.7 ± 2.7
TPL	83.3± 1.8	85.5 ± 0.8	86.0 ± 1.6	84.3 ± 1.0	87.7 ± 2.2	87.5 ± 2.6	87.4 ± 2.8

cont. in next page

	Control	D1			D2		
		0‰	5‰	10‰	0‰	5‰	10‰
TAG	8.0± 0.4	3.4 ± 0.1	4.7 ± 0.7	6.3 ± 2.1	1.7 ± 0.8	4.0 ± 0.9	3.1 ± 0.7
FFA	4.8± 0.2	5.6 ± 0.7	4.9 ± 1.1	3.5 ± 2.0	6.9 ± 0.9	4.9 ± 1.3	5.5 ± 0.3
AG	4.1± 1.2	3.2 ± 0.5	4.7 ± 0.8	4.3 ± 2.4	3.6 ± 1.6	3.6 ± 2.2	2.8 ± 0.9
PE	26.3± 0.8	27.7 ± 1.1	27.9 ± 1.5	28.0 ± 1.8	26.4 ± 1.4	27.4 ± 2.1	27.8 ± 2.0
PI	10.6± 1.3	16.3 ± 1.0	14.9 ± 1.3	16.6 ± 1.9	15.8 ± 0.6	15.4 ± 1.7	17.6 ± 1.6
PS	5.0± 0.7	5.1 ± 1.2	5.1 ± 1.7	4.8 ± 1.5	5.7 ± 1.0	5.1 ± 0.5	5.0 ± 1.8
PC	41.3± 0.6	38.7 ± 1.3	37.7 ± 1.3	36.5 ± 3.3	39.9 ± 1.4	39.5 ± 1.6	38.2 ± 1.0
TNL	16.8± 1.4	12.2 ± 0.2	14.0 ± 0.7	14.1 ± 3.1	12.2 ± 1.0	12.5 ± 2.6	11.4 ± 1.0
TPL	83.2± 1.4	87.8 ± 0.2	86.0 ± 0.7	85.9 ± 3.1	87.8 ± 1.0	87.5 ± 2.6	88.6 ± 1.0

Interestingly, among all radioactive substrates, EPA was the most incorporated one, followed by ARA, whereas the two ^{18}C FA precursors, as well as DHA were much less incorporated into the larvae. There was also an apparent effect of salinity and dietary regime, with a decreasing incorporation of EPA with the increasing salinity for diet 1, and an opposite trend being observed for diet 2. The incorporation of LC HUFA precursors and LC HUFAs for the experimental diets tested is detailed in **Table 10.2.4**. Results seem similar for the 3 diets tested, however with some indications of possible differences between salinity regimes and in incorporation of EPA.

Table 10.2.4 Incorporation of LC PUFA precursors and LC PUFAs (Pmoles/mg pp/h) for the two experimental diets and control diet at the three salinities tested.

		18:2n-6	18:3n-3	ARA	EPA	DHA
Control		7.5 ± 1.4	9.4 ± 2.0	20.6 ± 4.3	38.1 ± 13.3	6.2 ± 1.3
Diet 1	**0‰**	9.8 ± 3.8	7.8 ± 3.1	23.8 ± 3.5	57.5 ± 20.8	10.0 ± 3.0
	5‰	5.3 ± 3.8	6.6 ± 3.7	23.2 ± 20.8	41.1 ± 12.6	5.8 ± 3.4
	10‰	4.1 ± 1.0	6.9 ± 2.7	16.2 ± 7.1	31.7 ± 15.4	5.3 ± 1.8
Diet 2	**0‰**	9.3 ± 2.4	8.9 ± 1.5	24.7 ± 7.5	36.8 ± 5.4	10.5 ± 4.3
	5‰	3.6 ± 2.4	5.1 ± 1.8	26.9 ± 15.0	32.8 ± 17.6	5.3 ± 2.9
	10‰	8.9 ± 1.8	7.7 ± 4.2	13.4 ± 6.2	55.3 ± 10.3	6.4 ± 0.7

It is hypothesized, that PGE2 is involved in the stress response of fish through modulation of cortisol release, however ARA is a precursor of PGE 2 eicosanoids and therefore potentially larvae fed diets with high levels of 18:2n-6 (precursors of ARA) could be influenced based on the larval ability to esterify and elongate this FA into ARA. There was no significant difference in PGE 2 content at 30 dph, but significant higher levels of PGE 3 ($P \leq 0.037$) for treatment D1-0‰ and D2-0‰ than for treatment D1-5‰ and D1-10‰, this may be somehow related to salinity and desaturation ability, but needs further data evaluation.

Table 10.2.5 Prostaglandin PGE 2 and PGE 3 content (Pg g ww^{-1}) in larvae (DPH 30) by use of 2 diets and 3 salinity levels. Different letters within a column indicate significant differences (P<0.05).

	PGE 2 (Pg g ww^{-1})	PGE 3 (Pg g ww^{-1})
D1-0‰	5912.2±1587.6	$1211.5±322.6^{a}$
D2-0‰	5754.6±476.4	$1127.7±244.0^{a}$
D1-5‰	4758.6±2504.7	$634.4±84.8^{b}$
D2-5‰	5747.6±1442.5	$997.2±113.9^{ab}$
D1-10‰	3661.3±410.9	$677.9±120.2^{b}$
D2-10‰	3810.3±1247.3	$617.5±228.9^{ab}$

The digestive enzymatic response at 29 dph for trypsin, phosphatase (AP), peptidase (N), pepsin and lipase at 29 dph is detailed in **Figure 10.2.2**. Pepsin values were significantly lower (P<0.01) for treatment D2-10, and pepsin activity was detected at 29 dph for larvae fed D1-5, D2-5 and D1-10. Peptidase (N) activity increased (P<0.01) in treatments D1-10 and D2-10, as did trypsin activity (P<0.05) in larvae fed diet 1 (treatments: D1-0, D1-5 and D1-10) but there was no salinity effect on trypsin levels. Data also reveal a significantly higher (P<0.05) activity of alkaline phosphatase (AP) for treatment D1-10 and no significant effect was found on lipase levels. Regardless of diets, amylase activity decreased (P<0.05) in larvae exposed to 5 and 10 salinity as shown in **Figure 10.2.3**

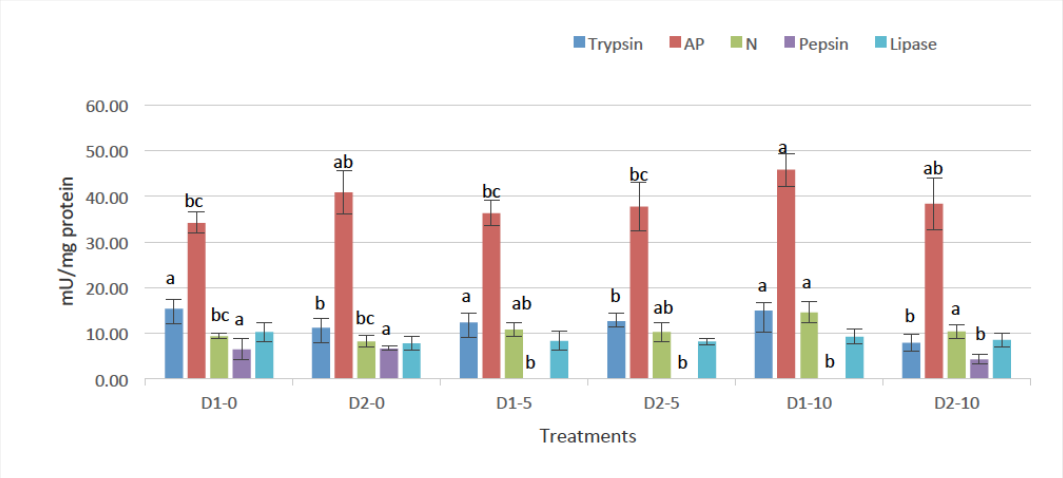


Figure 10.2.2. Mean larval digestive enzymatic response (±SD) at 29 dph for trypsin, phosphatase (AP), peptidase (N), pepsin and lipase. Different letters on bar with same color indicates significance (P<0.05).

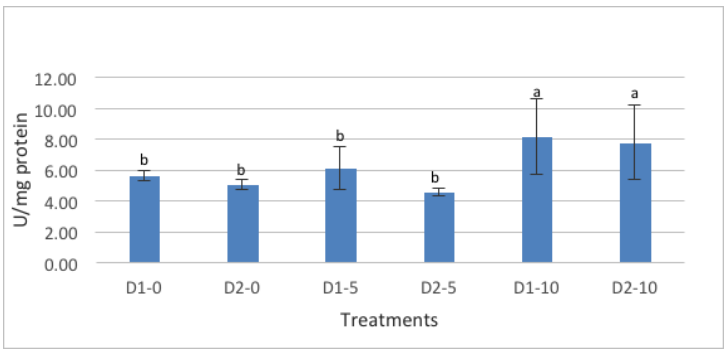


Figure 10.2.3. Larval digestive enzymatic response (±SD) at 29 dph for amylase. Different letters on bars indicate significance (P<0.05).

At the end of the trial all remaining larvae were gently siphoned from the rearing tanks without air exposure and collected on a 20x20x2 cm plate at +100 lux, and observed for 5 minutes and then mortality was registered**.** As indicated in **Figure 10.2.4** there was a high mortality in all diets except for the control group. Fish larvae reacted by erratic behaviour and most died within 30 seconds. In the control group larvae were affected but erratic movements were less and mortality much lower than for the other dietary treatments even after 5 minutes of exposure. This is most likely related to dietary HUFA level given a DHA Selco enrichment, as DHA has previously positively affected stress related mortality in pikeperch larvae. Mortality by exposing app. 100 larvae to confinement stress is shown in **Figure 10.2.4.** Analyses of gene expression and deformities are still underway.

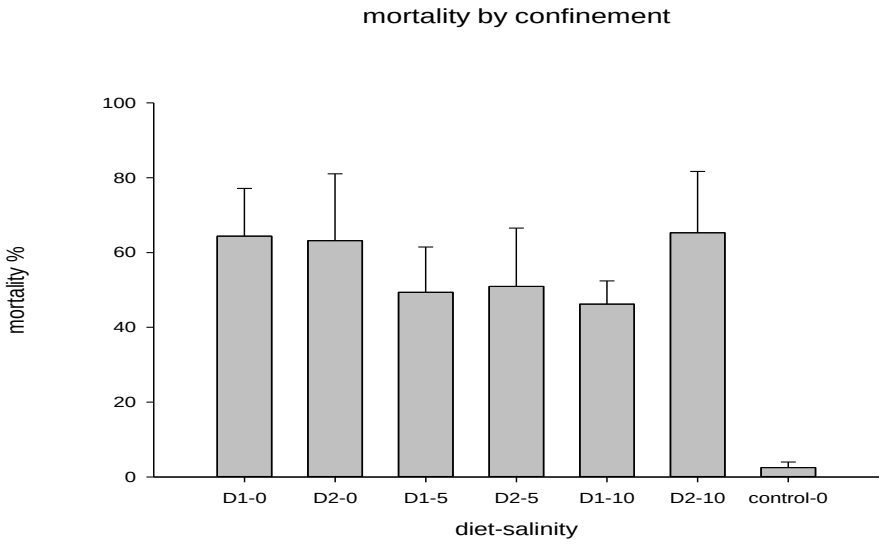


Figure 10.2.4. Mortality of pike perch larvae after confinement stress.

Another study has been started at end of April 2016 to investigate the influence of phospholipids. LC PUFAs and n-3/n-6 ratios *on* ***c****ompromised* behaviors risk taking, escape response, response to stress tests, cortisol levels, brain serotonin levels, glucocorticoid/corticotrophin releasing receptors, serotonergic receptors as well as dietary metabolic costs and hypoxia tolerance measured as oxygen consumption. Effects of pikeperch early FA nutrition on long-term stress sensitivity (led by DTU)

Both trials will be part of Deliverable D10.2 to be submitted in Mo 36.

Deviations from Annex I and their impact:

There have been some delays within the period due mainly to some technical failures (1 experiment) or high cannibalism of larvae (2 experiments) meaning that 3 trials had to be repeated within the time frame of the 2nd Reporting Period. These trials have been repeated, so we have managed to keep on track in terms of planning and future deliverables. The use of resources and planned man months are higher than expected due to repetition of expensive trials, but within the framework of the budget.

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:	Torstein Harboe (P7), Covadonga Rodriquez (P15).			

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 progress towards objectives and details for each task (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.

Task 11.1 Early Weaning of Atlantic halibut (led by IMR, Torstein Harboe)

Introduction

Atlantic halibut larvae are approximately 12 mm in standard length (SL) at first-feeding, and because of their relatively large larval size they are first-fed on *Artemia*. The main constrains for Atlantic halibut hatcheries are (1) slow growth during the late larval stages and (2) high mortalities caused by opportunistic bacteria and 3) slow growth after weaning. The slow growth in late larval stages may be overcome by early weaning. Most often, weaning of Atlantic halibut occurs only at 60 days post first-feeding (dpff), but attempts have

been made to introduce formulated diets from 20 and 50 dpff, with varying results. The first problem arising is that the larvae refuse to eat formulated feed (Harboe, Hamre and Erstad, unpublished results). It has been observed frequently, however, that they ingest inert particles such as *Artemia* cysts and pollen from pinewood, the main similarity being that both particles have neutral buoyancy and a bright color. Previous experiments have also shown better feed ingestion with floating compared to sinking feed particles. Furthermore, the structure of the visual system of Atlantic halibut larvae indicates that they hunt prey in the horizontal plane (Helvik pers. com.), favoring feed intake when particles stay in the same position in the water column for some time. Additionally, the type of feed could also affect digestive capacity determined as proteases, carbohydrases and lipases activities (Caruso et al., 2009) or even ATPase activity, which is essential to ensure the ion gradient necessary for nutrient uptake in the gut.

In this experiment we have chosen three candidate feeds (AgloNorse, Otohime and Gemma micro) based on their chemical content and earlier experience, and tested them on larvae 28 dpff and for 5 d.

Materials and methods

2015 experiment:

Approximately 5000 Atlantic halibut larvae were transferred from a yolk sac incubator (silo) to a standard 1.5-m diameter 0.8-m deep first feeding tank. The larvae were fed *Artemia* nauplii from 1 until day 28 dpff and then transferred to 50-l tanks (**Figure 11.1.1**). In the experiment, the larvae were fed either Gemma micro, AgloNorse or Otohime in triplicate tanks for 5 d. Larvae in one tank were fed *Artemia* to have a quality control. Each tank had continuous water supply of 10 h^{-1}, 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, 28 larvae were examined for gut content using a strong light. The categories used for gut fullness were full, partly full and empty.

2016 experiment:

We use the same setup as in 2015. The aim was to wean the larvae using the best feed from the previous year (Otohime) at days 14, 21 and 28 after first feeding. This experiment was running at the moment the report was prepared.

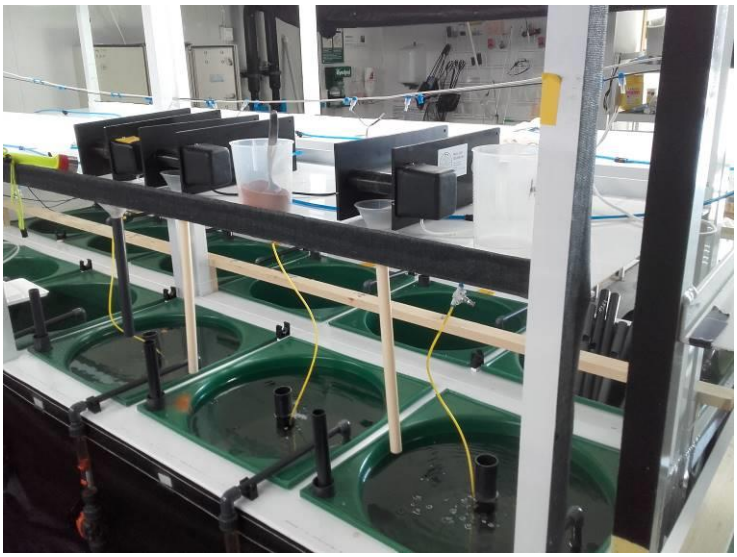


Figure 11.1.1. Tanks used for early weaning of Atlantic halibut larvae.

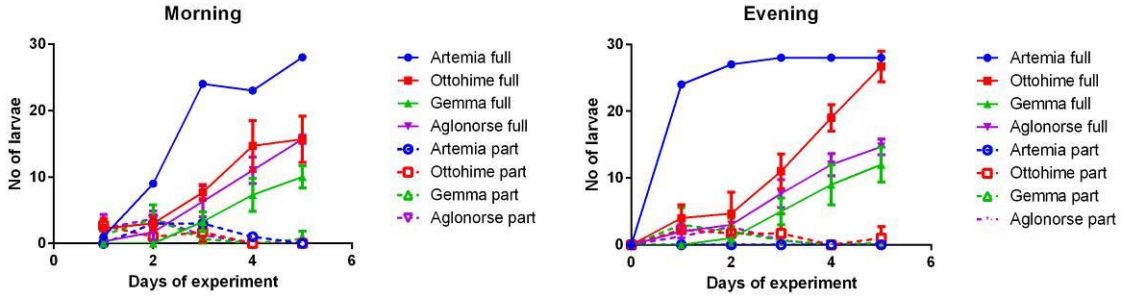


Figure 11.1.2 Gut filling in Atlantic halibut larvae fed different diets for 5 days from 28 dpff. The experiment was run in triplicate tanks and 28 larvae were examined for gut filling morning and evening each day. The categories for gut filling were full, partly full and empty.

Results and Discussion

Gut fullness was lower in the morning than in the evening, possibly because the larvae were measured before and after hand feeding and clay addition in the morning and in the evening, respectively (**Figure 11.1.2**). According to the evening measurements, larvae fed *Artemia* were almost full after one day and stayed full for the rest of the experiment. Larvae fed Ottohime showed increasing fullness over the whole period and on day 5 almost 100% of the larvae were full in the evening. The fraction of larvae with food in their gut increased more slowly on Gemma and Aglonorse. On the evening of day 5, 12.0±0.6 and 14.7±1.2 larvae, respectively, out of 28 had filled guts, while 0-0.3 larvae had partly filled guts on these diets.

The trial concluded that Ottohime is the best of these three diets for weaning of Atlantic halibut larvae. In 2015, this diet was used in weaning-trials with larvae that were younger than 28 dpff.

Task 11.2 Development of a production strategy for on-grown *Artemia* (led by IMR, Torstein Harboe).

The full description of the work and results from this Task has been provided in ***Deliverable 11.1*** **Report on the nutrient profile of *Artemia* nauplii and on-grown *Artemia***.

Experiments were performed at P7. IMR and P22. SWH. Briefly, *Artemia* were hatched and either enriched directly with Larviva Multigain (Biomar, Denmark) or grown for 3-4 days on OriGreen (IMR) or Ori-One (SWH) and then enriched with Larviva Multigain. The growth and survival of *Artemia* grown for 4 days is indicated in **Figures 11.2.1** and **11.2.2.** First, an experiment to determine the optimal growth period was performed at IMR. Based on the development of protein and lipid content in the *Artemia* (**Figure 11.2.3**) combined with labor costs it was concluded that the optimum growth period is 3 days. In a second experiment performed both at P7. IMR and P22. SWH, *Artemia* were grown for 3 days and then enriched. The nutrient composition of the on-grown *Artemia* was superior to the nauplii in many respects (**Table 11.2.1**).

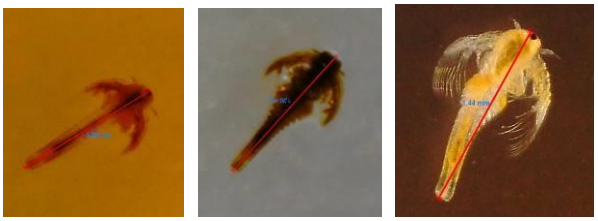


Figure 11.2.1. *Artemia* grown from nauplii for 2, 3 and 4 days. Length: 1.06, 1.2 and 1.4 mm respectively.

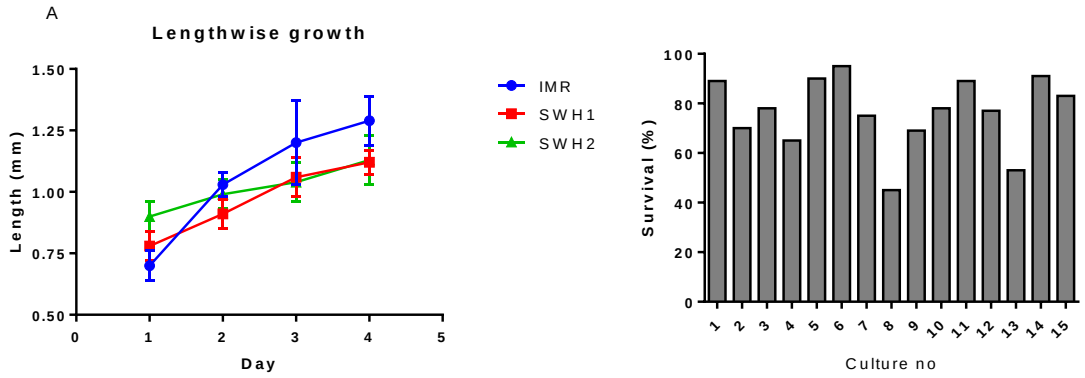


Figure 11.2.2. A. Growth of *Artemia* cultured for four days (mean±SD). B. Survival in 15 batches of on-grown *Artemia* cultured for three days. Numbers are based on numbers of *Artemia* ml^{-1} at start and end of the 3-day culture period

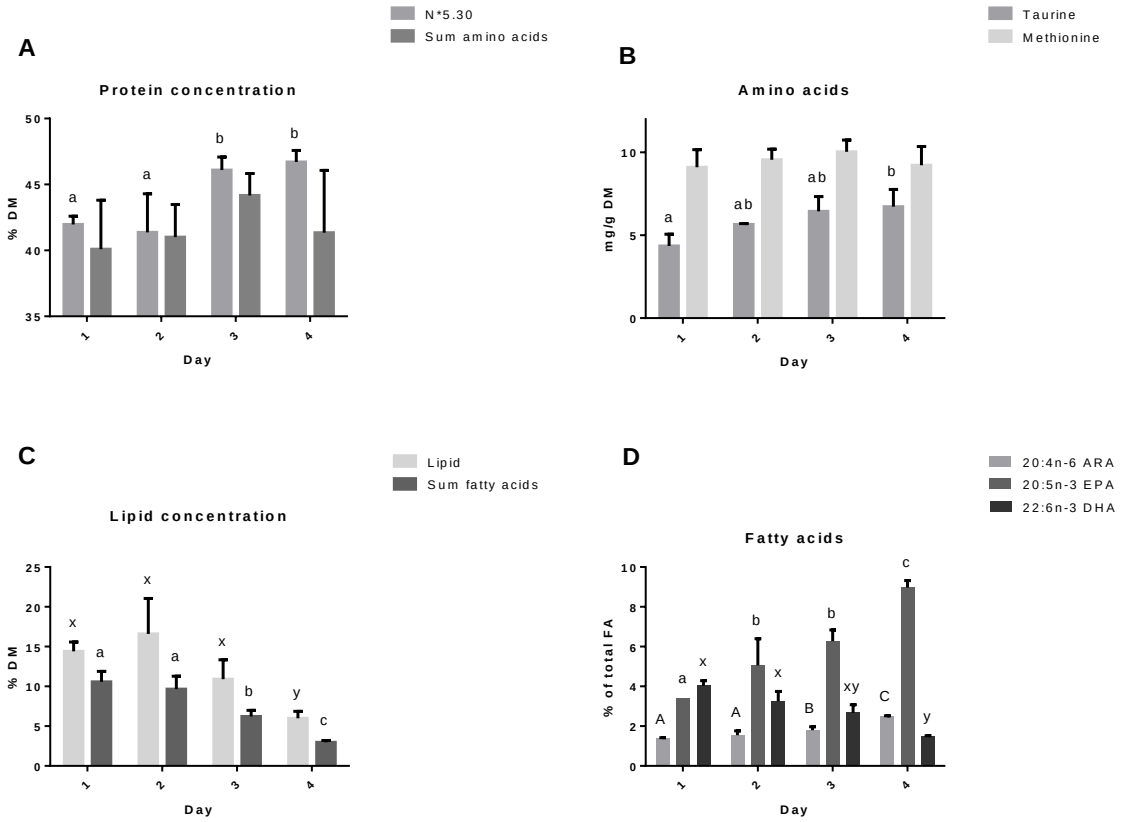


Figure 11.2.3. Nutrient concentrations in *Artemia* grown for 4 days on OriGreen in Experiment 1. A) Protein concentration (% of dry matter) expressed as the sum of amino acids, cysteine and cysteine excluded, or as nitrogen (N) * 5.30 (the calculated protein to nitrogen factor for *Artemia* according to Hamre et al., 2013). B) The amino acid methionine and the aminosulfonic acid taurine (mg g^{-1} dry matter). C) Lipid concentration measured as the sum of fatty acids or as total lipid after acid hydrolyses. D) Arachidonic (ARA), eicopentaenoic acid (EPA) and docosahexaenoic acid (DHA) in % of total fatty acids. Data were analyzed with one-way ANOVA and differences between days are indicated with different letters ($p<0.05$).

Table 11.2.1. Nutrient composition of *Artemia* from IMR; nauplii, on-grown, enriched nauplii and enriched on-grown *Artemia*. Data are on dry matter. *Artemia* were grown on OriGreen for three days in triplicate and both nauplii and on-grown *Artemia* were enriched with Multigain. Only one sample of the unenriched *Artemia* types were taken, therefore these samples were not included in the statistical analyses. Differences between enriched nauplii and on-grown *Artemia* (Day 1-3) were analyzed by t-tests. Means were assumed to be different at $p<0.05$ (labeled in red).

	Unit	Nauplii unenriched	On-grown unenriched	Nauplii enriched	On-grown enriched	*P* Day 1-3
Dry matter	g $100g^{-1}$	10.3	10.2	8.4±0.6	8.3±0.4	0.884
Protein Nx5.30	g $100g^{-1}$	53	50	46±1	51±2	0.026
TAA	g kg^{-1}	443.8	-	411±10	452±10	0.008
Protein/TAA		0.119	-	0.11±0.01	0.11±0.004	0.908
FAA	g kg^{-1}	62	-	70±4	92±13	0.044
Taurine	g kg^{-1}	4.15	-	4.4±0.2	5.5±0.6	0.040
Glycogen	g kg^{-1}	-	-	25±3	7.1±3.2	0.002
Lipid	g $100g^{-1}$	20	-	17±1	11±1	0.004
TFA	g kg^{-1}	168	93	147±6	75±8	0.000
PL	% TL	25.2	21	24±3	34±3	0.013
ARA	% TFA	1.6	1.8	2.4±0.1	2.1±0.1	0.016
EPA	% TFA	1.5	6.3	4.1±0.2	6.0±0.7	0.010
DHA	% TFA	<0.1	2.7	5.9±0.6	17±2	0.001
Thiamine	mg kg^{-1}	10	12	10.8±0.8	12.5±1.1	0.096
Vitamin C	mg kg^{-1}	824	307	1037±336	1401±66	0.168
Vitamin D3	mg kg^{-1}	0.10	0.29	0.12±0.01	0.24±0.01	0.000
Vitamin E	mg kg^{-1}	129	775	580±27	890±224	0.076
MK4	µg kg^{-1}	1.1	-	1040±137	102±37	0.000
Phylloquinone (K1)	µg kg^{-1}	7.6	-	13±1	281±131	0.024
MK6	µg kg^{-1}	0.0	-	nd	15±7	0.024
MK7	µg kg^{-1}	4.8	-	6.7±0.7	75±37	0.033
MK8	µg kg^{-1}	0.0	-	nd	242±111	0.020
MK9	µg kg^{-1}	0.0	-	nd	22±11	0.026
MK10	µg kg^{-1}	0.0	-	nd	41±22	0.031
Total vitamin K	µg kg^{-1}	13.5	-	1073±124	778±340	0.231
Iodine	mg kg^{-1}	2.2	3.1	5.2±0.5	8.2±0.5	0.002
Ca	g kg^{-1}	2.3	3.9	3.4±0.5	3.1±0.5	0.460
K	g kg^{-1}	14.6	12.7	15±1	14±0.1	0.152
Mg	g kg^{-1}	6.6	7.7	8.2±0.8	7.1±0.9	0.165
P	g kg^{-1}	12.6	9.8	11.1±0.9	10.9±0.4	0.420

Task 11.3 Nutrient retention and digestive physiology of Atlantic halibut juveniles fed *Artemia* nauplii or on-grown *Artemia* (led by NIFES, Kristin Hamre).

Introduction

A possible strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality (**Figure 11.3.1**) 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 Atlantic halibut larvae fed on-grown *Artemia* develop into juveniles with better pigmentation and eye migration than Atlantic halibut fed *Artemia* nauplii. This was verified in a feeding experiment performed in 2005 (Hamre and Harboe, unpublished). The industry is considering implementing this knowledge in their production line, but will need further documentation.

A holistic understanding of feeding and digestive functions is important for designing diets for fish larvae and the adaptation of rearing conditions to meet requirements for the best presentation of prey and microdiets, and their optimal ingestion, digestion and absorption (Ronnestad et al., 2013). In this sense, it is

obvious that a better knowledge of larval digestive ontogeny and its physiology when using different scientific approaches and techniques such as that of feeding Atlantic halibut larvae with *Artemia* nauplii or on-grown *Artemia*, will contribute to the optimization of diets and rearing conditions. The analysis of main digestive enzymes under these two different rearing sceneries may help to this understanding of functions and limitations in processing capacity of the digestive system of a species such as Atlantic halibut, that is, the plasticity of their digestive processes to deliver nutrients to the rapidly growing larval tissues under changeable feeding and environmental conditions.

Materials and methods

Atlantic halibut larvae were fed *Artemia* nauplii from 1 until 14 dpff. Then one group of larvae was fed *Artemia* nauplii, and the other group on-grown *Artemia* (2+ out of 3 meals) in triplicate tanks until 28 dpff. There was no difference in larval performance. Both groups showed good growth and survival, 100% normal pigmentation and good eye migration (score: more than 2.5/3, See Task 17.4 for details on larval rearing and performance). Samples were taken for nutrient analyses (NIFES) and analyses of digestive capacity (ULL) after end of feeding ongrown *Artemia*. The nutrients were analyzed by ISO accredited methods at NIFES (**Table 11.3.1**).

Samples of larvae were collected for comparisons of main digestive enzyme activities. Larvae at day 15 (start) and day 28 from each stock (*Artemia* nauplii or on-grown *Artemia* fed larvae) were pooled according to their age-size after previous dissection and discard of heads and tails. The samples were completely homogenized (Ultra-Turrax T8, IKA©-Werke, Germany), in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4ºC, the supernatant removed for enzyme quantification and kept at -80ºC until further analysis. Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were based on methods performed and described by Dr. Gisbert (P3. IRTA) (see Gisbert et al. 2009). In brief, total alkaline proteases were measured using azocasein (0.5%) as substrate in Tris-HCl 50 nmol.l-1, and pH 9. Alkaline protease activity is measured in nmoles azo dye per minute and per ml of tissue homogenate at 366 nm. Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity was defined as the amount of starch (mg) hydrolyzed during 30 min per ml of tissue homogenate at 37ºC at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6080 x g and 4ºC and the increase in absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity was defined as the amount (nmol) of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Finally pepsin activity was defined as the nmol of tyrosine liberated per min at 37ºC per ml of tissue homogenate at 280 nm (Worthington, 1991).

All enzymatic activities were expressed as specific activity defined as units per milligram of larvae or protein. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Beckman Coulter DU800, Fullerton, CA).

Figure 11.3.1 Atlantic halibut juvenile quality (Photo: Øystein Sæle)

Table 11.3.1. Analytical methods: Principles and references

Analyte	Principle	Reference
Protein	N x 6.25 Leco N Analyzer	(Hamre and Mangor-Jensen, 2006)
Free amino acids	HPLC and post column derivatization	(Srivastava *et al.*, 2006)
Fatty acids	Transmethylation extraction and GC/FID	(Lie and Lambertsen, 1991)
Glycogen	Hydrolysis and spectrometric detection	(Hemre *et al.*, 1989)
Thiamine	HPLC	(CEN, 2003b)
Vitamin C	HPLC	(Mæland and Waagbø, 1998)
Vitamin A	HPLC	(Moren *et al.*, 2002)
Vitamin D	HPLC	(CEN, 1999)
Vitamin E	HPLC	(Hamre *et al.*, 2010)
Sum vitamin K[3]	HPLC	(CEN, 2003a)
Iodine	ICPMS	(Julshamn *et al.*, 2001)

Statistic analysis

Nutrient concentrations are given (mean±SD) on wet weight or as % of total FAs. Differences between larvae fed *Artemia* nauplii and on-grown *Artemia* were identified using a t-test after checking for homogenous variances by Levenes test, software Statistica (ver 12, Statsoft Inc, Tulsa, OK).

Enzyme activities are expressed as mean ± SD. Normal distribution was checked for all data with the one-sample Kolmogorov–Smirnoff test and homogeneity of the variances with the Levene test. The group data were statistically tested using one-way ANOVA followed by the Tukey test. When variances were not homogeneous, a non-parametric Kruskal-Wallis test was applied. The significance level for all the analysis was set at 5%. All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com).

Results and discussion

In the industry, the routine larval rearing method is to feed *Artemia* nauplii, and it is quite common to produce large fractions of Atlantic Atlantic halibut larvae with abnormal pigmentation and lack of eye migration, allthough the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. The nutrient concentrations of Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff were similar, except that the on-grown group had a slightly lower level of EPA than larvae fed nauplii, a difference that is probably biologically insignificant (**Table 11.3.2**). This is another possible explanation of the lack of differences in growth and larval performance between the two treatments. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on-grown group had to be fed nauplii to get enough food. As the fish grow, more feed is needed and due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as was planned. These are all possible reasons that no differences were detected between the groups.

The average enzymatic activities measured per mg larvae are shown in **Table 11.3.2**. The activity of amylase, alkaline protease and pepsin, but not lipase, were higher in larvae fed nauplii and sampled at 28 dpff than in larvae sampled at 15 dpff. The activity of amylase and alkaline protease was lower in larvae fed

the on-grown *Artemia* than in those fed nauplii and a similar tendency was seen for pepsin activity. Lipase activity did not change from 15 to 28 dpff, and was similar in the two larval groups at the end of the experiment.

Taking into consideration expression data from the study performed by Murray et al. (2006), and enzyme activity data from Gawlicka et al. (2000), it seems that Atlantic halibut have the capacity to digest both proteins and lipids from as early as 45 dph, approximately at first feeding. However, their potential for gastric protein digestion is limited until stomach development and gastric gland formation is completed at 80 dph (35 dpff). Therefore, the increment in the pepsin activity displayed by the older larvae is probably insufficient for efficient gastric digestion of proteins. Activities of amylase and protein digestive enzymes were generally higher in larvae fed nauplii compared to those fed on-grown *Artemia*. The reason for this is not known, but a possible explanation may be that nauplii are less digestible and that a higher digestive capacity is needed in these larvae.

Table 11.3.2. Nutrient concentrations in Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff. Significant differences are marked with red (t-test, $p<0.05$).

On wet wt		Start	Nauplii	Ongrown
Protein	%	-	11.4±0.4	11.5±0.6
Sum FAA	mg/g	3.5	4.4±1.1	4.8±0.3
Taurine	mg/g	1.4	1.8±0.2	1.8±0.1
Glycogen	mg/g	0.95	1.6±0.3	1.4±0.3
20:4n-6 %	%TFA	5.2	6.2±0.1	6.2±0.2
20:5n-3 EPA %	%TFA	8.4	7.8±0.3	6.9±0.2
22:6n-3 DHA %	%TFA	14	9.6±0.9	8.4±0.7
Total FA	mg/g	16	15±3	19±2
Thiamin	mg/kg	2.6	2.1±0.2	2.2±0.2
Vitamin C	mg/kg	158	155±31	136±12
Vitamin-D3	mg/kg	0.02	0.01±0.00	0.01±0.00
Vitamin E	mg/kg	37	25±2	23±1
Vitamin A1	mg/kg	0.7	1.2±0.1	1.1±0.1
Iodine	mg/kg	0.26	0.28±0.02	0.26±0.01

Table 11.3.2. Activities of digestive enzymes in Atlantic halibut larvae (U mg larvae^{-1}) fed *Artemia* nauplii or on-grown *Artemia* from 15 (Start) until 28 dpff. Values are expressed as mean ± SD (n=3).

	Start	Artemia naupli	Ongrown Artemia
Amylase	11.2 ± 0.4^{a}	20 ± 1^{c}	14.2 ± 0.5^{b}
Alkaline Protease	132 ±33^{a}	293 ±24^{b}	120 ±53^{a}
Lipase	57 ±12	36 ± 8	48 ± 4
Pepsin	28 ±24^{a}	100 ±28^{b}	51 ±11^{ab}

Comparisons among groups were performed by oneway ANOVA followed by Tukey´s test. Different letters within a row indicate significant differences ($P < 0.05$). Amylase (U) = mg starch hidrolyzed during 30 min at 37oC (580 nm); Alkaline protease (U) = nmol azodye per min (366 nm); Lipase (U) = nmol myristate per min (405 nm); Pepsin (U) = nmol tyrosine per min at 37oC (280 nm).

Task 11.4 Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS (led by NIFES, Kristin Hamre).

The experiment of this task was running at the time this report was prepared and samples for nutrient retention analyses and digestive physiology will be taken in early June 2016 (Mo 31). See task 17.1 for details on larval rearing.

Task 11.5 Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles (led by NIFES, Kristin Hamre).

The experiment of this task will run in August 2016 (Mo 33). At the moment we are in the process of deciding the feed formulation and ordering the feed from P20. SARC.

References

Bradford M.M., 1979. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry, 72:248-254.

CEN (1999) Comitè Europèen de Normalisation: Foodstuffs - Determination of vitamin D by high performance liquid chromatography -Measurement of cholecalciferol (D_3) and ergocalciferol (D_2), prEN12821.

CEN (2003a) Foodstuffs - Determination of vitamin K_1 by HPLC. In *EN*, Vol. 14148.

CEN, C.-E.-N. (2003b) Foodstuffs -Determination of Vitamin B1 by HPLC. EN14122: 2003., TC 275 WI 002750053N134.

Gawlicka A., Parent B., Horn M.H., Ross N., Opstad I. and Torrissen O.J. (2000) Activity of digestive enzymes in yolk-sac larvae of Atlantic halibut (Hippoglossus hippoglossus): indication of readiness for first feeding. Aquaculture, 184: 303–314.

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y and Estevez. A. 2009. Development of digestive enzymes in common dentex Dentex dentex during early ontogeny. Aquaculture 287 (3): 381-387.

Hamre, K. and Mangor-Jensen, A. (2006) A multivariate approach to optimization of macronutrient composition in weaning diets for cod (Gadus morhua). *Aquaculture Nutrition*, **12**, 15-24.

Hamre, K., Kolås, K. and Sandnes, K. (2010) Protection of fish feed, made directly from marine raw materials, with natural antioxidants. *Food Chemistry*, **119**, 270-278.

Hemre, G.I., Lie, Ø., Lied, E. and Lambertsen, G. (1989) Starch as an energy source in feed for cod (Gadus morha): Digestibility and retention. *Aquaculture*, **80**, 261-271.

Iijima, N., Tanaka, S. and Oka, Y. 1998. Purification and characterization of bile-salt activated lipase from the heaptopancreas of red sea bream Pagrus major. Fish Physiol. Biochem. 18 :59-69.Kamler, E., 2002. Ontogeny of yolk-feeding fish: an ecological perspective. Reviews in Fish Biology and Fisheries 12, 79-103.

Julshamn, K., Dahl, L. and Eckhoff, K. (2001) Determination of iodine in seafood by inductively coupled plasma/mass spectrometry. *Journal of Aoac International*, **84**, 1976-1983.

Lie, Ø. and Lambertsen, G. (1991) Fatty acid composition of glycerophospholipids in seven tissues of cod (Gadus morhua), determined by a combined HPLC/GC method. *J. Chromatogr.*, **565**, 119-129.

Mæland, A. and Waagbø, R. (1998) Examination of the qualitative ability of some cold water marine teleosts to synthesise ascorbic acid. *Comparative Biochemistry and Physiology Part A*, **121**, 249-255.

Métais, P. and Bieth, J. 1968. Détermination de l´α-amylase. Ann. Biol. Clin. 26:133-142.

Moren, M., Naess, T. and Hamre, K. (2002) Conversion of beta-carotene, canthaxanthin and astaxanthin to vitamin A in Atlantic halibut (Hippoglossus hippoglossus L.) juveniles. *Fish Physiology and Biochemistry*, **27**, 71-80.

Murray, H.M., Gallant J.W., Johnson S.C, and Douglas, S.E. 2006. Cloning and expression analysis of three digestive enzymes from Atlantic halibut (Hippoglossus hippoglossus) during early development: Predicting gastrointestinal functionality. Aquaculture 252: 394-408.

Olsen, A.I., Attramadal, I., Jensen, A. and Olsen, Y. (1999) Influence of size and nutritional value of *Artemia fransiscana* on growth and quality of halibut larvae (*Hippoglossus hippoglossus*) during the live feed period. *Aquaculture*, **179**, 475-487.

Rønnestad, I., Yufera, M., Überschär, B., Ribeiro, L., Sæle, Ø. and Boglione, C. (2013) Feeding behaviour and digestive physiology in larval fish: current knowledge and gaps and bottlenecks in research. *Reviews in Aquaculture*, **5**, S59-S98.

Rønnestad, I., Yúfera, M., Ueberschár B., Ribeiro, L., Sæle Ø. and Boglione, C. 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Reviews in Aquaculture 1: 559-598.

Sarasquete, M.C., Polo, A., Yúfera, M., 1995.Histology and histochemistry of the development of the digestive system of larval gilthead seabream, Sparus aurata L. Aquaculture 130, 79-92.

Srivastava, A., Hamre, K., Stoss, J., Chakrabarti, R. and Tonheim, S.K. (2006) Protein content and amino acid composition of the live feed rotifer (Brachionus plicatilis): With emphasis on the water soluble fraction. *Aquaculture*, **254**, 534-543.

Worthington Biochemical Coorporation. 1972. Worthington Enzyme manual: Enzymes, Enzyme Reagents, Related Biochemicals. Worthington Biochemical Coorp., Freehold, N.J.

Deviations from Annex I and their impact:

There were deviations from the Annex I in this Reporting Period.

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), J. Benito Peleteiro (P8), Rosa Cal (IEO P8), Gema Pazos (P19) and Marisol Izquierdo (P2)			

Objectives

The objective of this WP is to test the effectiveness of live prey and influence of enrichment on wreckfish larvae and determine the influence of broodstock feeds on fecundity and spawning quality.

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 progress towards objectives and details for each task (13-30 Mo):

Task 12.1. Live preys and enrichments for wreckfish larvae.

The influence of different enrichments products for live food in wreckfish larvae could not be tested because the amount and the survival of larvae obtained was not sufficient to perform the experiments. Samples of wreckfish eggs were sent from P19. CMRM to P2. FCPCT for biochemical analysis to obtain data for developing some live food enrichment products for larval wreckfish.

Some wreckfish larvae from different spawnings of P8. IEO and P32. MC2 broodstocks were obtained with a low survival at hatching and mortality was 100% at 20 days after hatch. The enrichment of live food was based on microalgae and to maintain the quality of live preys the green water technique was used. Some samples of larvae were taken out at days 0, 5 and 10 post hatching (dph), to obtain the fatty acid profile of wreckfish larvae (**Figures 12.1.1, 12.1.2 and 12.1.3**). The first results show that PUFA, SAFA and MUFA values (% of total fatty acids) have a little variation in the first 10 days of life. PUFA values are between 44-49%, SAFA between 22-29% and MUFA 22-33%. n-3 PUFA represent 40-45% of the total fatty acids and n-6 PUFA the 2-7% and EPA, DHA and ARA have values of 8- 9%, 26-31% and 2% respectively. Furthermore, only little differences were observed between wreckfish larvae of 5DPH and 10DPH from different spawnings of the same female (Stock P32. MC2) and from different females of stocks P32. MC2 and P8. IEO.

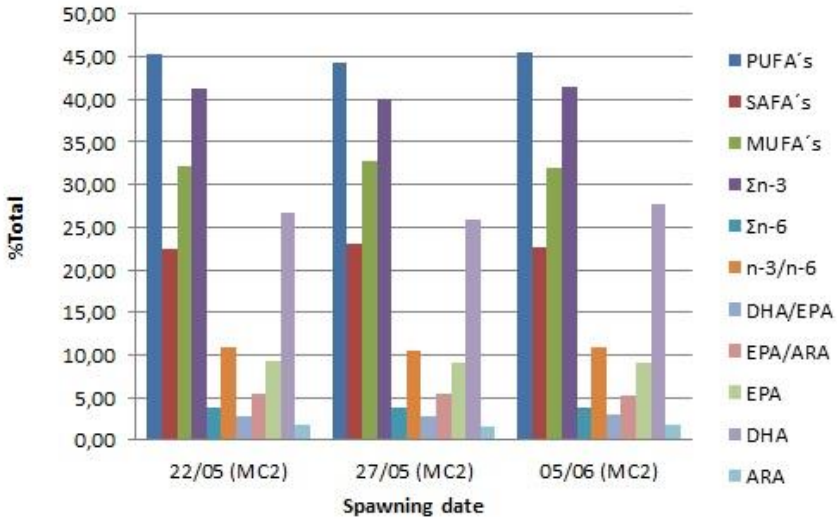


Figure 12.1.1. Fatty acids of wreckfish larvae at hatching from three different spawnings of one female (P32. MC2)

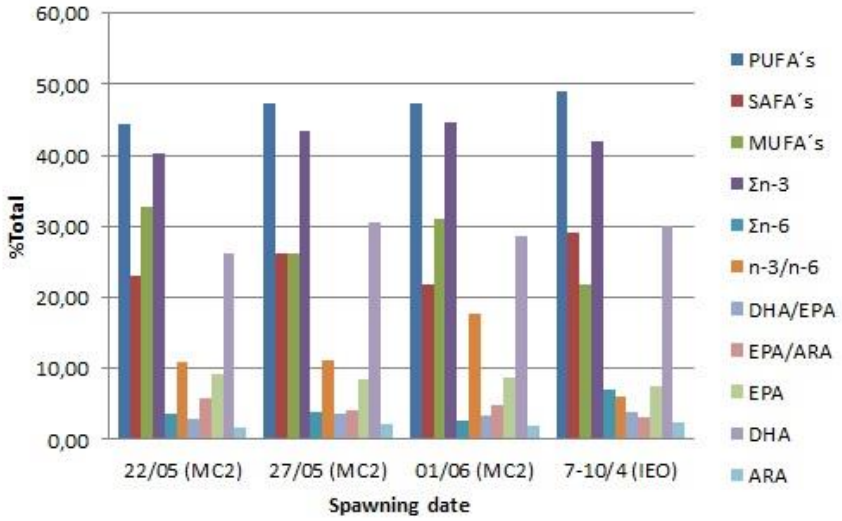


Figure 12.1.2. Fatty acids of wreckfish larvae at 5 dph from three different spawnings from two different females (P32. MC2 and P8. IEO)

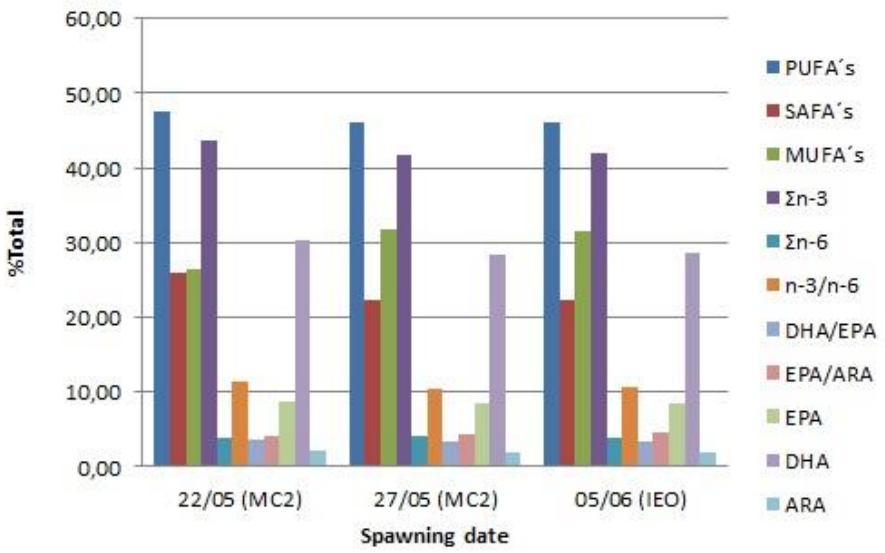


Figure.12.1.3. Fatty acids of wreckfish larvae at 10 dph from different spawnings and from two different females (P32. MC2 and P8. IEO)

Task 12.2. Influence of broodstock feeding regimes for fecundity and spawn quality.

The activities included in this task are related to the composition of wild fish and the feeding of wreckfish broodstock. Samplings of wild wreckfish from October 2014 to April 2015 were carried out to complete the data obtained last year. A total of 91 fish were sampled from the Azores (North Atlantic, Portugal). Total length (cm), perimeter (cm), and total and eviscerated weight (kg) were recorded for each fish. Perivisceral fat was collected and its percentage of total weight was determined. Gonads, liver, stomach and intestine were also collected from each fish and weighed, to determine the respective indexes. The stage of the reproductive development was checked (data shown in WP6). Sample collection of muscle, liver and gonads were carried out for biochemical analysis to know the nutritional status of wild fish. A total of 24 samples of muscle and 58 of liver and gonads were processed by P19. CMRM, stored at -80ºC and freeze-dried to perform biochemical analysis.

Biochemical analysis (**Table 12.2.1**) confirmed data obtained last year (1st Periodic Report) showing that wild wreckfish have a large amount of protein in the muscle (84% DW) and low level of lipids (7%). In the liver, protein and lipid content was 38 and 40%, and in the gonad it was 39 and 35%, respectively, with a high variability among individuals. Regarding fatty acid composition, in muscle PUFA (polyunsaturated fatty acids), SFA (saturated fatty acids) and MUFA (monounsaturated fatty acids) fractions have average values of 39, 29 and 32% of total fatty acids respectively. DHA presents a very high content (26% of total fatty acids) and DHA+EPA represent more than 30% in the muscle of wild wreckfish. Additionally, some samples from cultured fish (4 females from different broodstock, which died: 1 from P8. IEO, 2 from P19. CMRM and 1 from Aquarium O Grove, which is not an official partner) were collected and analyzed to compare with the results obtained in wild fish. The first results (**Table 12.2.2**) showed that fish maintained in culture had more lipids in the muscle and liver 27.5% (DW) and 62%, respectively, than those obtained in wild fish, which had 7% in the muscle and 40% in the liver. Protein content was also higher in the muscle of wild wreckfish (84%) than in cultured fish (76%). Some differences were also observed in the values of fatty acids (% total fatty acids). Values of HUFA and Σn-3 were higher in wild wreckfish than in cultured fish. However MUFA values were higher in cultured fish than in wild fish. The DHA value represented 11% in cultured fish and 26% in wild fish, and ARA 1% in cultured fish and 3% in wild fish.

Table 12.2.1. Biochemical composition, proteins, lipids and fatty acids (means±SD) of muscle, liver and gonad of wild wreckfish.

	Muscle	Liver	Gonads
Proteins(%DW)	84.41±7.34	37.94±13.66	38.96±18.21
Lipids(%DW)	6.92±3.39	40.19±15.25	35.05±22.27
Fatty acids (% total)			
SAFA´s	28.83±1.28	26.11±3.51	28.12±2.54
MUFA´s	32.09±5.43	56.23±8.80	44.00±7.99
ARA	3.11±0.79	1.55±0.88	3.05±2.20
EPA	4.55±0.70	3.09±1.37	4.37±1.34
DHA	26.38±3.33	9.31±5.05	15.29±4.37
PUFA´s	39.08±4.41	17.66±8.19	27.87±7.58
Σn-3	34.51±3.75	14.93±7.01	23.45±6.01
Σn-6	4.08±0.81	2.55±1.23	4.07±2.18
n-3/n-6	8.50±1.18	5.79±1.42	6.52±2.58
DHA/EPA	5.69±1.23	2.99±0.91	3.49±0.77
EPA/ARA	1.54±0.37	2.13±0.60	1.95±1.04

Table 12.2.2. Biochemical composition, proteins, lipids and fatty acids (means±SD) of muscle, liver and gonad of cultured wreckfish.

	Muscle	Liver	Gonads
Proteins(%DW)	84.41±7.34	37.94±13.66	38.96±18.21
Lipids(%DW)	6.92±3.39	40.19±15.25	35.05±22.27
Fatty acids (% total)			
SAFA´s	28.83±1.28	26.11±3.51	28.12±2.54
MUFA´s	32.09±5.43	56.23±8.80	44.00±7.99
ARA	3.11±0.79	1.55±0.88	3.05±2.20
EPA	4.55±0.70	3.09±1.37	4.37±1.34
DHA	26.38±3.33	9.31±5.05	15.29±4.37
PUFA´s	39.08±4.41	17.66±8.19	27.87±7.58
Σn-3	34.51±3.75	14.93±7.01	23.45±6.01
Σn-6	4.08±0.81	2.55±1.23	4.07±2.18
n-3/n-6	8.50±1.18	5.79±1.42	6.52±2.58
DHA/EPA	5.69±1.23	2.99±0.91	3.49±0.77
EPA/ARA	1.54±0.37	2.13±0.60	1.95±1.04

Regarding the influence of the feeding of wreckfish broodstock on fecundity and spawn quality, a new dry food was formulated (P2. FCPCT and Sparos S.A., Portugal) based on the results of composition of wild fish and a bibliographic review. From December 2014 to December 2015, two different diets -Semi-moist diet and dry food 1 (new) with a specific formulation were supplied to two different batches of the P8. IEO broodstock. In tank S1, 5 wreckfish (3 females and 2 males) were fed with semi-moist diet, and in Tank S2, 6 wreckfish (3 females, 1 male and 2 undetermined) were fed with dry food 1.

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 are shown in **Table 12.2.3** and consisting of 50% fish meal, 12.5% squid meal, 6% krill etc. The feeding rate was 0.2-0.5% with semi-moist diet (Tank S1) and 1-1,8% with dry food 1 (Tank S2). Crude protein was 60.3% in dry food and the lipid content was 17.3% in

both diets. The fatty acid profile of both diets (**Table 12.2.4**) showed that dry food 1 had a higher HUFA content (45% of total fatty acids) than semi-moist diet (38,5%) and this is because the n-6 HUFA content is higher in dry food 1 (17%) than in semi-moist diet (8%). The main difference between both diets was found in ARA content with values of 9% in dry food 1 and 1% in semi-moist diet.

Table 12.2.3. Ingredients of dry food 1 formulated specifically for wreckfish broodstock.

Ingredients	Dry food 1
	%
Fishmeal 70 LT FF Skagen	50.000
CPSP 90	7.500
Squid meal	12.500
Krill meal (Aker Biomarine)	6.000
Wheat Gluten	6.000
Wheat meal	4.940
Tuna oil	2.000
Incromega DHA 500TG	2.000
VEVODAR	3.000
Vit & Min Premix PV01	2.000
Lutavit E50	0.060
Soy lecithin - Powder	2.000
Macroalgae mix	1.000
Antioxidant powder (Paramega)	0.200
Antioxidant liquid (Naturox)	0.200
SelPlex - Se yeast	0.020
Carophyll Pink 10% - astaxanthin	0.050
Nucleotides (Nucleoforce)	0.030
L-Taurine	0.500
Total	**100.000**

Table 12.2.4. Fatty acid profile (%total) of semi-moist diet and dry food 1.

Fatty acid	Semimoisture diet	Dry food 1
14:0	4.81±0.59	3.92±0.10
16:0	19.51±0.93	14.88±0.10
17:0	1.01±0.18	0.78±0.05
18:0	4.05±0.30	4.47±0.04
ΣSFAs	**29.96±1.19**	**24.44±0.22**
16:1n-7	4.79±0.51	3.05±0.02
18:1n-9	12.21±1.46	14.48±0.09
18:1n-7	3.82±0.35	2.17±0.04
20:1n-9	3.55±0.64	3.79±0.03
22:1n-11	3.53±1.03	4.86±0.22
ΣMUFAs	**31.55±0.97**	**30.83±0.15**
18:2n-6	7.03±0.64	7.55±0.01
18:3n-3	1.16±0.14	1.23±0.02
18:4n-3	1.66±0.26	1.49±0.11
ARA	**1.25±0.28**	**9.06±0.06**
EPA	**8.84±0.48**	**6.61±0.02**
DPA	**1.29±0.24**	**0.99±0.15**
DHA	**15.91±1.16**	**16.67±0.32**
ΣPUFAs	**38.49±1.65**	**44.72±0.37**
Σn-3	**29.40±1.57**	**27.61±0.43**
Σn-6	**8.28±0.56**	**16.62±0.07**
n-3/n-6	**3.56±0.23**	**1.66±0.03**
DHA/EPA	**1.43±0.66**	**2.52±0.04**
EPA/ARA	**7.45±1.86**	**0.73±0.01**

Fish were sampled monthly and some samples of oocytes obtained by gonadal biopsies of females and sperm of the males were taken out to be characterized (size of oocytes, velocity and motility of sperm) and to

perform the fatty acid profiles. The fatty acid profile of oocytes from Tank S1 fed with semi-moist diet and females from Tank S2 fed with dry food 1 (**Table 12.2.5**) showed the main differences in fatty acid composition between the two diets were in n-6 HUFA content, which reached 8% in the oocytes from Tank S2 and 5.6% in oocytes from females in Tank S1.

Table 12.2.5. Fatty acid profile of oocytes from two different females fed with semi-moist diet (S1) and the dry food 1 (S2).

Fatty acid	Oocytes S1	Oocytes S2
14:0	1.47±0.07	1.42±0.02
16:0	15.35±0.65	13.750.55
17:0	1.02±0.20	0.91±0.19
18:0	4.92±0.28	4.70±0.19
ΣSFAs	**23.12±0.86**	**21.09±0.76**
16:1n-9	1.34±0.14	0.91±0.03
16:1n-7	4.11±0.13	3.45±0.17
18:1n-9	17.54±0.97	17.45±1.01
18:1n-7	4.94±0.46	4.64±0.51
20:1n-9	1.74±0.05	2.27±0.18
ΣMUFAs	**32.41±0.86**	**31.69±0.46**
18:2n-6	3.87±0.18	4.14±0.48
ARA	**1.76±0.16**	**3.72±0.22**
EPA	**7.70±0.32**	**6.61±0.31**
DPA	**2.63±0.18**	**2.70±0.15**
DHA	**26.33±1.07**	**27.92±1.06**
ΣPUFAs	**44.47±1.62**	**47.23±1.14**
Σn-3	**38.62±1.57**	**39.18±1.16**
Σn-6	**5.63±0.20**	**7.96±0.59**
n-3/n-6	**6.86±0.32**	**5.01±0.44**
DHA/EPA	**3.42±0.08**	**4.23±0.27**
EPA/ARA	**4.40±0.44**	**1.78±0.11**

The results obtained in the first experiment of wreckfish broodstock feeding have shown that the amount of fat of dry food 1 could be too high to feed wreckfish broodstock and a new dry food (dry food 2) was formulated to be used during 2016. Since February 2016 a new experiment has been underway in two batches of P8. IEO broodstock, with the same scheme that was carried out in 2015. The semi-moist diet was supplied to Tank S1 and the new dry food 2 was supplied to Tank S2 (**Table 12.2.6**). The comparison in some components between dry food 1 and dry food 2 are shown in **Table 12.2.7.**

Table 12.2.6. 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 (Naturox)	0.200
SelPlex - Se yeast	0.020
Carophyll Pink 10% - astaxanthin	0.050
Nucleotides (Nucleoforce)	0.030
L-Taurine	0.500
Total	**100.000**

Table 12.2.7. Comparison between the developed dry food 1 and dry food 2.

As fed basis	Dry food 1	Dry food 2
Crude protein	60.3	68.2
Crude fat	16.4	12.5
ARA	1.1	**0.5**
EPA	0.9	**0.7**
EPA/ARA	0.8	**1.5**
DHA	2.3	**1.7**
DHA/EPA	2.5	**2.3**
PC	1.6	1.6
PE	0.4	0.4
PI	0.5	0.4
TPL	3.3	2.7
Σn-3 (% total fat)		**27.6**
ARA (% total fat)		**3.9**

Food samples and oocytes, sperm, eggs and larvae produced from breeders in the two tanks will be analyzed during 2016 (Mo 31-37). In the other hand, the feeding regime of P19. CMRM broodstock was commercial dry food from Skretting, consisting of Vitalis Repro during the resting period and Vitalis Cal from November 2014 to the end of March 2015. These diets have total lipids of 16.3% DW (Vitalis Repro) and 19.8% (Vitalis Cal) and the fatty acid profile is shown in **Table 12.2.8**. On 26 March 2015 a high amount of fat (more than 10% of perivisceral fat and around the heart, air bladder. etc) was found in a female from this broodstock, and the feeding was changed to squid to avoid problems caused by the excessive fat of the fish. The feeding was ad libitum.

Table 12.2.8. Fatty acid profile (% total) of Vitalis Repro and Vitalis Cal (Skretting, Spain).

Fatty acid	Vitalis Repro	Vitalis Cal
14:0	4.93±0.06	6.66±0.13
16:0	18.40±0.13	19.26±0.13
17:0	0.99±0.04	0.79±0.03
18:0	4.58±0.10	4.66±0.08
ΣSFAs	**29.44±0.29**	**31.86±0.34**
16:1n-7	5.87±0.05	7.56±0.06
18:1n-9	17.15±0.13	12.46±0.06
18:1n-7	3.66±0.08	3.47±0.01
20:1n-9	2.54±0.01	1.22±0.01
22:1n-11	1.84±0.05	0.83±0.01
ΣMUFAs	**33.64±0.10**	**27.27±0.03**
18:2n-6	8.88±0.08	6.50±0.01
18:3n-3	1.74±0.02	1.18±0.01
18:4n-3	1.52±0.05	2.12±0.01
ARA	**1.16±0.04**	**0.95±0.03**
EPA	**9.42±0.20**	**13.09±0.20**
DPA	**1.40±0.02**	**1.57±0.03**
DHA	**10.61±0.25**	**12.05±0.20**
ΣPUFAs	**36.92±0.36**	**40.87±0.36**
Σn-3	**25.37±0.40**	**30.67±0.38**
Σn-6	**10.04±0.12**	**7.45±0.02**
n-3/n-6	**2.53±0.07**	**4.11±0.05**
DHA/EPA	**1.13±0.02**	**0.92±0.00**
EPA/ARA	**8.11±0.41**	**13.78±0.57**

Additionally, samples of oocytes were taken out from some females of the different wreckfish broodstocks at different times, being at different stages of development. Results indicated that there is a relationship between oocyte development and fatty acid content. Oocytes with diameters between 200-400 µm present a lower amount of all fatty acids (ng μDW^{-1}) than oocytes with diameters between 700-1000 µm. In percentage of total fatty acids (**Table 12.2.9**), there were some significant differences in fatty acid profile, being MUFA and n-3 PUFA content higher in oocytes with larger diameter, while SAFA´s and n-6 HUFA were lower than in oocytes with a smaller diameter. The DHA represented 26% of total in the largest oocytes and 18% in the smallest, while EPA did not vary with the different sizes and ARA content was 4% in the smallest and 2% in the largest.

Table 12.2.9. Fatty acid profile of oocytes with different diameter size (200-400 µm and 700-1000 µm).

Fatty acid	Oocytes 200-400µ	Oocytes 700-2000µ
14:0	0.81±0.46	1.58±0.17
16:0	9.12±4.63	15.07±1.91
17:0	0.52±0.27	0.89±0.17
18:0	4.03±1.71	4.56±0.30
ΣSFAs	14.71±6.52	22.44±1.88
16:1n-9	0.51±0.30	1.05±0.18
16:1n-7	1.95±1.28	4.11±0.44
18:1n-9	9.94±6.88	18.05±1.42
18:1n-7	3.49±3.42	4.53±0.42
20:1n-9	1.04±0.64	1.82±0.28
ΣMUFAs	18.34±11.88	32.35±1.69
18:2n-6	4.04±4.99	3.75±0.66
ARA	4.20±2.07	2.20±0.82
EPA	6.64±4.85	8.01±0.93
DPA	2.11±1.38	2.70±0.12
DHA	17.99±9.86	25.98±1.54
ΣPUFAs	36.04±21.01	45.21±1.55
Σn-3	27.69±15.68	39.04±1.40
Σn-6	8.24±6.56	5.95±1.21
n-3/n-6	3.71±1.32	6.84±1.52
DHA/EPA	3.26±1.24	3.30±0.55
EPA/ARA	1.85±1.22	4.08±1.26

Summarizing up the results of the period 2015 – May 2016 in addition to the results of Y1 of the project, the following conclusions can be made:

1. Important differences in perivisceral fat were observed among wild caught fish, which are not related to capture season or sex. Biochemical composition of different tissues of wild wreckfish has shown that in muscle they have a large amount of proteins (84% DW) and low lipid levels (7%). In the liver, protein and lipids have values of 38 and 40% and in gonads 39 and 35%, respectively with a high variability among individuals. The DHA presents a very high content (26% of total fatty acids) and DHA+EPA represent more than 30% in muscle of wild wreckfish. Results were very useful to formulate a specific feed for wreckfish broodstock.

2. The first results of fatty acid profile of wreckfish larvae show that HUFA, SAFA and MUFA content (% of total fatty acids) have a little variation in the first 10 days of life.
3. Comparisons of wild and cultured wreckfish composition showed that cultured fish have more lipids in the 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 cultured fish and some differences were also observed in the fatty acid profile with higher values of HUFA and n-3 HUFA in wild than in cultured wreckfish. The DHA values represent 11% in cultured fish and 26% in wild fish.
4. 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 we should increase the amount of proteins and decrease the level of fat. Furthermore, dry food for wreckfish must contain a large amount of n-3 HUFA and the EPA/ARA ratio must be around 1.5, similar to that obtained previously in wild wreckfish.
5. A clear relationship between fatty acid profile of oocytes and broodstock diet was found. Samples of oocytes were obtained from females fed with semi-moist 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 oocyte development.

Deviations from Annex I and their impact:

There were deviations from the Annex I in this Reporting Period.

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 progress towards objectives and details for each task (13-30 Mo):

Task 13.1 Improvement of larval performance (led by IOLR, Bill Koven).

Sub-task 13.1.1

The effect of DHA/EPA ratio on larval and juvenile performance during rotifer and *Artemia* feeding was planned for the autumn of 2015 and was scheduled to conclude in June 2016. However, due to poor and very few grey mullet spawns, this sub-task was postponed to the autumn of 2016. However, about 400 F2 juvenile grey mullet were produced from the 2015 season and these fish will be used to test the effect of dietary DHA/EPA ratio on older juveniles in June 2016 (Mo 31). Nevertheless, the larval and juvenile taurine studies were successfully carried out and they are reported here.

In 2016, further analyses of the previous year's taurine experiment were 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 (**Figure 13.1.1a, b, c, d**). As un-enriched *Artemia* have considerable levels of taurine, there appears to be no added benefit of feeding taurine enriched *Artemia* on larval weight (**Figure 13.1.2a, b, c**). 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 (**Figure 13.1.2d)**. 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 (**Figure 13.1.3).**

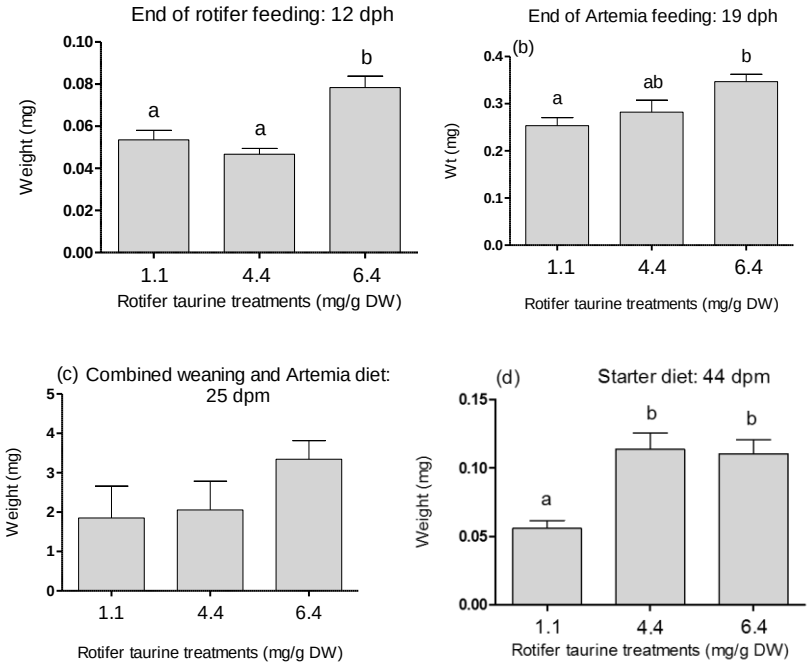


Figure 13.1.1 The effect of feeding rotifer taurine treatments to 3-12 dph grey mullet larvae on larval weight gain in 12, 25 and 44 dph grey mullet larvae and juveniles. One-way ANOVA of larval weight values of taurine treatments at each age was performed. Bar values, within an age, having different letters were significantly ($P<0.05$) different.

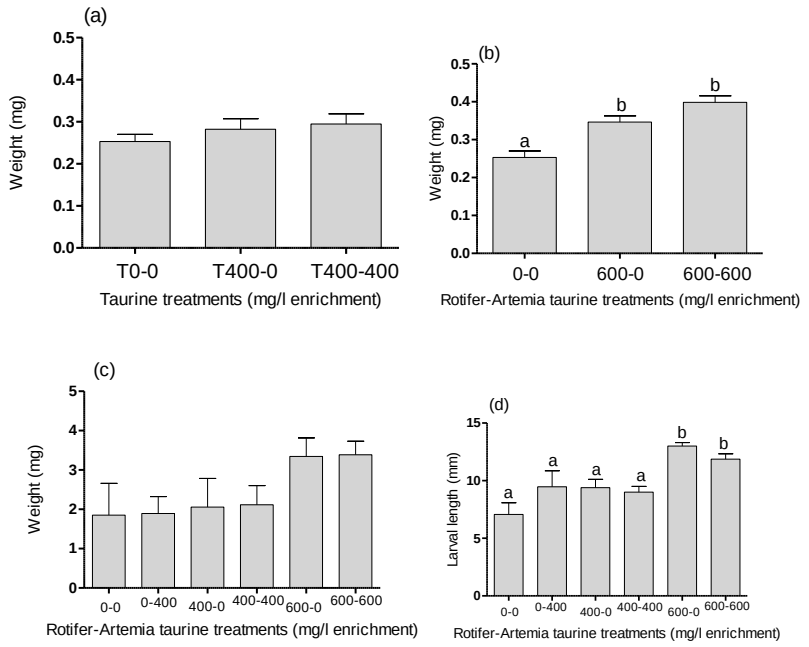


Figure 13.1.2 The effect of feeding 3-19 dph larvae with the rotifer and *Artemia* control treatment (T0-0) as well as the (a) T400-400 or (b) T600-600 treatments on weight gain in 19 dph larvae. In (c) and (d) the effect of feeding all 6 rotifer-*Artemia* treatments on 25 dph juveniles, which were also fed a combination of starter diet and dried Ulva from 19-24 dph, on larval weight and length is shown. One-way ANOVA on weight or length values of all treatments was carried out. Weight or length bar values having different letters were significantly ($P<0.05$) different.

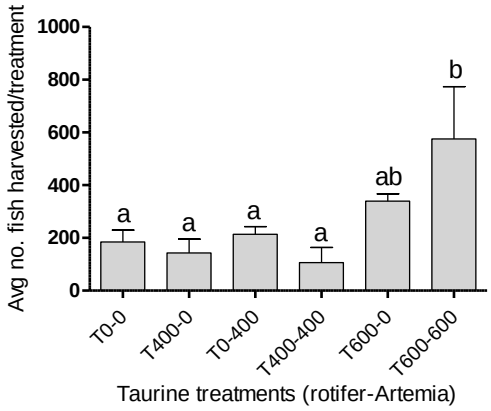


Figure 13.1.3 The effect of all rotifer-*Artemia* treatments on survival (average no. of fish harvested/treatment) in 44 dph. One-way ANOVA on survival values of all treatments was performed. Bar values having different letters were significantly (P<0.05).

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, we wanted to see if the taurine influence continued during juvenile growth. This was not a task in the DOW but the results have important implications for the growing of this species. To this end, about sixty 44 dph fish from each taurine treatment (1.1, 4.4 and 6.4 mg taurine g^{-1} DW rotifer) were stocked in three 20 l buckets, with mesh on bottom and sides, with 20 fish per bucket. All 9 buckets were placed in a 9 m^3 polypropylene tank in the outside nursery and the fish were all fed a taurine containing commercial starter feed for marine fish (Raanan Ltd., Israel) for 1 month where they reached about 500 mg. The fish from the taurine (4.4 and 6.4 mg g^{-1} DW) treatments were at the start of the experiment significantly larger (113.86±0.09 and 125.52±0.14, respectively) than the low taurine control (1.1 mg g^{-1} DW; 59.5±0.14). This meant that fish from each treatment were maintained with similar sized cohorts. The results showed that the smaller fish of the control exhibited a significantly (P<0.05) better percent weight gain than the taurine treatments (**Figure 13.1.4**). This attests to the (1) potential for compensatory growth in smaller individuals if they are maintained in a homogenous sized population and (2) the plasticity of this species as a growth disadvantage during earlier larval rearing can be reversed during the juvenile stage.

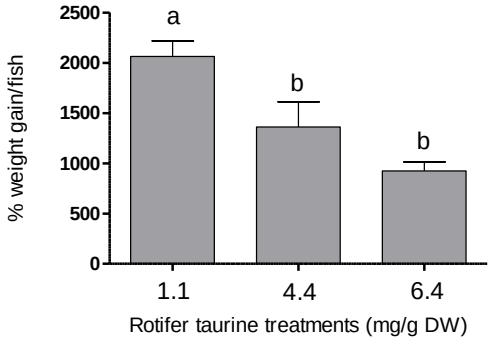


Figure 13.1.4 The percent weight gain of different size fish (~60-126 mg) previously fed different levels of dietary taurine (mg/g DW; 2-12 dph). One way ANOVA of percent weight gain (following arcsine transformation) values was performed. Bar values having different letters were significantly (P<0.05) different.

Sub-task 13.1.2

As the effect of supplemental ARA on grey mullet larval growth, survival, presence of urinary crystals, as well as synchrony in "silvering" during metamorphosis is dependent on determining the most effective DHA-taurine diet from (sub-task13.1), sub-task 13.1.2 has been postponed to the Fall 2017 season.

Task 13.2. Determining grey mullet nutritional needs for improved weaning to a dry diet (led by IOLR, Bill Koven).

Sub-task 13.2.1

This sub-task was to determine the gene expression of the rate-limiting enzyme for taurine synthesis, cysteine sulfinate decarboxylase (CSD) during the larval and juvenile stages of fish fed different levels of dietary taurine (Samples were taken from studies in Task 13.1). The RNA (from adult grey mullet liver) quantity and quality 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. Total RNA samples (up to 2 µg) were treated with 2 units of RNAse-free DNase (Promega, Madison, WI, USA). Following incubation (30 min, 37°C) the DNAse was inactivated (10 min, 65°C) and the reaction was stopped with 1 µl of "stop" solution followed by cDNA synthesis. A high capacity cDNA reverse transcription kit (Applied biosystems) was used for the synthesis of cDNA from RNA samples, according to manufacturer's protocol.

Although the gene for CSD is considered conserved over many fish species, the primers that were currently tried, based on conserved regions in the CSD of *Anguilla japonica*, *Seriola quiqueradiata*, *Cyprinus carpio* and *Danio rerio*, have not produced any PCR product after amplification. Studies are still on-going to determine if the problem is primer design, little or no expression of liver CSD gene in grey mullet or that a vector approach is necessary to increase amplification.

Sub-task 13.2.2

This sub-task was to determine the gene expression of the rate-limiting enzyme for bile salt synthesis, cholesterol 7α-hydroxylase (CYP7a1) at various developmental stages, larval and juvenile, in fish fed different levels of dietary taurine (Samples were taken from studies in Task 13.1). The initial step for carrying out this task is to sequence the gene for this protein has been completed after cDNA production from the liver from adult grey mullet (see 13.2.1).

The gene specific primers sets used for qRT PCR:

CYP7a1 F1 CATGACAGCTTCGACCC
CYP7a1 R1 CGGGCCTTACACTTATCTTCA

The expected amplicon length was 270 bp. After PCR amplification, cleaning of PCR product and sending gel band for gene sequencing at Hy Labs (Rehovot, Israel). Final sequences were:

GNNNCCCACNNACGGNAANCTCCCCNAACCTTTCTGAAAACCTTGCAGGGTGAAGCTCTCCCCT
CCCTGATAGAGACAAT
GGTGGGCCACCTTCAGGATGTCATGCTGAAATCTGACACACTCAGGCCCAGCAAAAACCACTG
GGAGGTGGATGGCATCT
TTGCTTTCTGCTACAAGGTGATGTTTGAGTCTGGTTACTTGACTCTGTTTGGTAAAGAGCTTGGT
GAAGATAAGTGTAAG
GCCCGA

Samples from the Task 13.1 studies will soon be analyzed for the expression of this gene.

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. This subtask is not due until the end of Y4. The DHA/EPA dietary requirement trial will begin in June 2016. Using the best performing DHA/EPA diet of this trial, another trial testing the ARA requirement will be implemented in the spring of 2017.

Sub-task 13.3.2

Effect of four levels of taurine supplementation to best performing DHA/EPA/ARA non-fish meal grow out diet from 13.3.1 on fish performance. Although the DHA/EPA and the ARA studies will be performed in 2016 and 2017, respectively, a taurine study was performed on juvenile grey mullet in 2015. In this study, forty 127 dph (~ 5.46 g) juvenile grey mullet were stocked in each of twenty 400 l tanks allowing for the testing of four taurine treatments (0, 0.5, 1.0 and 2.0 % DW diet) and the hatchery control (commercial diet Raanan Ltd., Israel: IOLR Ulva at a ratio of 1:1 w/w) in replicates of 4 tanks/treatment. The taurine treatments all were extruded (SPAROS, Portugal) and based on the IOLR formula for grey mullet. The fish in the various treatments were fed daily at 4% of tank biomass for 58 days or until 185 dph, where they approximately doubled their weight. Fish fed the T0.5 (0.5%) diet exhibited a significantly ($P<0.05$) higher percent weight gain than the control (T0), although further increases in dietary taurine (T1.0, T2.0) were not expressed as better growth (**Figure 13.3.1**). This suggests that the capability to synthesize taurine, despite the fact that juvenile grey mullet at this age are feeding mostly on taurine poor plant sources, is still insufficient so this nutrient must be provided in the diet. However, the hatchery control diet (HatCon) performed markedly ($P<0.05$) better than all other treatments (**Figure 13.3.1**). This means a complete biochemical analysis of the HatCon diet is necessary in order to determine which is the nutrient lacking or in insufficient supply in the IOLR grey mullet formula that might be inhibiting better performance.

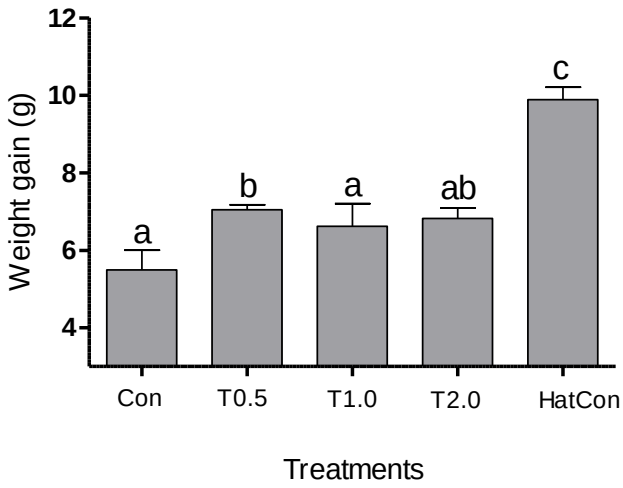


Figure 13.3.1 The effect of the control (0 % taurine), T0.5 (0.5% taurine DW), T1.0 (1.0% taurine DW), T2.0 (2% taurine DW) and hatchery control (HatCon; Starter diet: Ulva-1:1) diets on weight gain in 126-185 dph juvenile grey mullet. One-way ANOVA was performed on weight gain values. Weight value bars having different letters were significantly ($P<0.05$) different.

Sub-task 13.3.3

This sub-task will compare 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 is planned to be carried out in the fall of 2017 (will use 1 year old F2 juveniles)

Sub-task 13.3.4

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 grey mullet culture and fed to adult grey mullet until gonadal maturation (IOLR). This experiment will begin in 2017.

Sub-task 13.3.5

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 quality (CTAQUA). This task is not due until month 48 and there are no results to report at this time.

Task 13.4. Design adequate feeding regimes for brood stock to optimize reproduction success (led by UNIBA, Aldo Corriero).

Subtasks 13.4.1 and 13.4.2 are not due until month 47 and there are no results to report at this time.

Deviations from Annex I and their impact:

The effect of DHA/EPA ratio on larval and juvenile performance during rotifer and *Artemia* feeding was planned for the autumn of 2015 and was scheduled to conclude in June 2016. However, due to poor and very few grey mullet spawns, this sub-task is postponed to the autumn of 2016, as well as subtasks 13.3.2 and 13.3.3. However, about 400 F2 juvenile grey mullet were produced from 2015 season and these fish are presently being used to test the effect of dietary DHA/EPA ratio on older juveniles. Subtasks 13.2.1 and 13.2.2 were delayed due to the difficulties in finding student/technician to carry out the work on samples already collected from task 13.1. Having said this, these tasks are currently being carried out.

Group Work Packages

Larval husbandry

In meagre, we compared fish weaned from 20 dph (Control), with fish that were weaned from 10 dph. There was significantly ($P<0.05$) lower mortality and higher growth in the Control. Pancreatic enzyme activity in the Control and treatment fish were similar, and 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.

A great success was achieved in the greater amberjack larval rearing season of 2016 (Mo 31-32, outside the scope of this report). 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. In particular, the results from the trials with the modified "light environment" of tanks are better at one order of magnitude of previous reported showing the validity of the tested hypothesis. Also, we showed that rotifers enriched with marine lecithin supplemented with 20% Echium oil showed the best results. Gene expression of GHRH, GH, IGF-I and II and IGFBPs were not affected by semi-intensive or intensive larval rearing, while the optimum egg stocking density for larval performance was between 25 and 50 eggs l^{-1}. The study of the ontogeny of the digestive system showed that enzyme activity is independent of environmental rearing conditions. In comparing tank hydrodynamics in the semi-intensive tanks (2000 l) and mesocosm tanks (40,000 l) we found that the semi-intensive tanks had higher current velocity. The were no differences between the photoperiods of 24L:00D and 18L:6D in larval growth or mRNA expression levels of IGF, GH and GnRH. Also, tank color did not affect larval growth, although white tank larvae exhibited the highest survival rate and expression of IGF-1 and GH.

The study of pikeperch larvae recommended a light intensity of 50 lux, water renewal rate of 100%, afternoon tank cleaning and water entering the tank from the bottom for best larval performance. In these studies there was no marked effect on the histological organization and morphogenesis of the digestive tract and associated digestive glands.

In Atlantic halibut, comparison of recirculation (RAS) and flow through (FT) systems during yolk sac and first feeding, found very little difference in mortality, although RAS larvae were smaller. We are presently characterizing the bacterial microbiota associated with larval cultures using New Generation Sequencing. A production protocol for on-grown *Artemia* was further developed, but feeding on-grown *Artemia* had no marked effect on larval growth, survival, pigmentation and eye migration, although the study was shortened from 45 to 28 days post first feeding.

Experiments with wreckfish were limited by few and poor quality spawns in 2015, which were likely due to an unsuitable diets coupled with unusual environmental conditions. In the 10 spawns during this year, the fertilization varied between 62-97% and hatching between 4-56%, while no facility succeeded in growing larvae past 22 dph. Larvae exhibited a syndrome resembling swollen yolk sac, which has been related to poor broodstock nutrition. Growth performance and deformities were documented up to 22 dph larvae.

In grey mullet, 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 until 25 dph. This study concluded that rotifer consumption and survival of larvae and juveniles were dependent ($P<0.05$) on algal turbidity, but independent of algal type. Rotifer consumption in early development markedly influenced later juvenile survival. Higher survival resulted in large numbers of smaller fish, which contributed to a skewed size distribution in the population.

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 progress towards objectives and details for each task (13-30 Mo):

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).

Due to the high mortality obtained as a consequence of the high cannibalistic behaviour of the larvae the experiment was repeated in Y2 (2015), reducing the number of treatments and including different measures to reduce cannibalism and increase survival rate. Samples taken in this first period were also analysed in 2015. The task was completed in 2015 and 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*** delivered in May 2016.

Briefly, two trials were carried out, one in 2014 (described in the 1st Periodic Report) and another in 2015. In both trials different feeding regimes were assayed. In 2014, 4 treatments (see above) were used, whereas in 2015 only two regimes were tested using 5 replicated tanks per treatment:

- Group **A:** Weaning on dry feed started from 20 dph and completed on 30 dph, (control group)
- Group **B:** Started from 10 dph and completed on 23 dph

Considering the results obtained from the trial conducted in 2014, several changes in the rearing protocol were introduced in 2015 to avoid problems associated with larval cannibalism. Thus, light intensity was reduced from 500 lux to 150-200 lux from 13 dph, and the number of doses of *Artemia* metanauplii (given at 10, 13, 16 and 18h) and the amount of artificial diet were increased in order to ensure providing enough food to the larvae. Samples were periodically taken for biochemical, digestive and antioxidant enzyme activity analyses and growth in length and weight as well as skeletal deformations.

The most important results are as follows:

1. Growth was always higher in the control group weaned at 20 dph (**Figure 14.1.1**), although in the 2014 trial it was clearly affected by the incidence of cannibalism, resulting in the cannibalistic larvae exhibiting longer length and higher weight than their siblings. Thus groups A, C and D that showed the lowest survival (**Table 14.1.1**) and highest incidence of cannibalism (**Figure 14.1.2**) also demonstrated the highest growth.

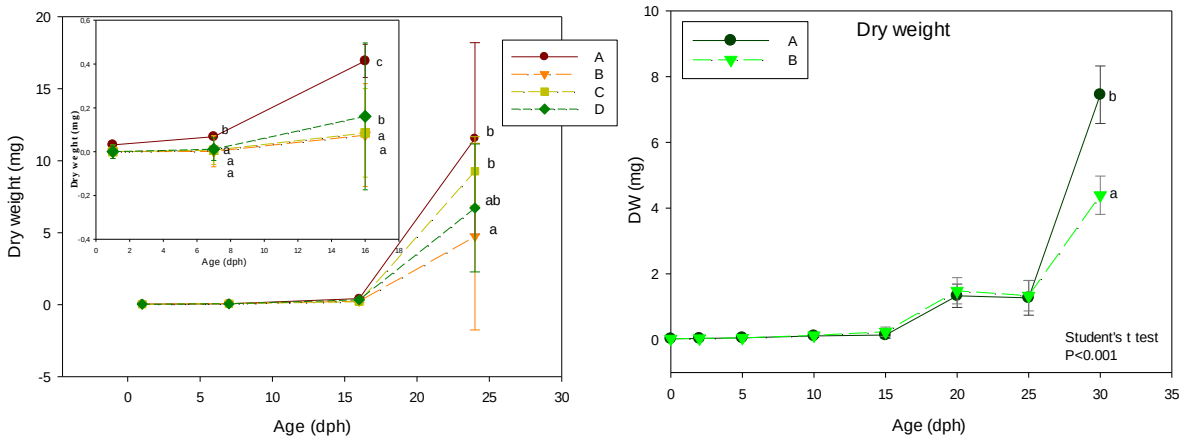


Figure 14.1.1. Growth in weight of the larvae used in 2014 (left) and 2015 (right) trials. Different letters show significant differences in growth (ANOVA, P>0.05 trial 2014 and Student's t test P<0.05 trial 2015)

Figure 14.1.2 Photographs showing the differences in the size of the larvae at the end of the experiment (24 dph) in the groups A, B and C due to the high incidence of cannibalism.

2. Survival was very low in 2014 trial whereas in 2015 the survival rate obtained was around 5%, which can be considered similar to commercial hatcheries (Table 14.1.1)

Table 14.1.1. Survival rate (% mean and SD) of the larvae at the end of 2014 (left) and 2015 (right) trials. One-way ANOVA of percent survival values (after transformation) was performed. Average treatment survival values having different letters were significantly (P<0.05) different.

T	%
A	$1,7 \pm 0,1^{b}$
B	$2,8 \pm 0,6^{a}$
C	$1,2 \pm 0,3^{b}$
D	$1,8 \pm 0,3^{b}$

ANOVA P=0,005

	Survival Rate (%)
A	$4,86 \pm 0,65^{a}$
B	$3,93 \pm 0,47^{b}$

Student's t test P=0,032

3. The pancreatic enzyme activity showed similar trends in both trials, where amylase level was not different between early-weaned and normally weaned larvae. The activities of lipase and trypsin (only in 2014) were higher in early-weaned larvae and alkaline phosphatase was lower in early-weaned larvae (**Figure 14.1.3**). However, the activity of pepsin, that indicates stomach development, was higher for control larvae and lower for early weaned (only analysed in 2015) as it is shown in **Figure 14.1.4**

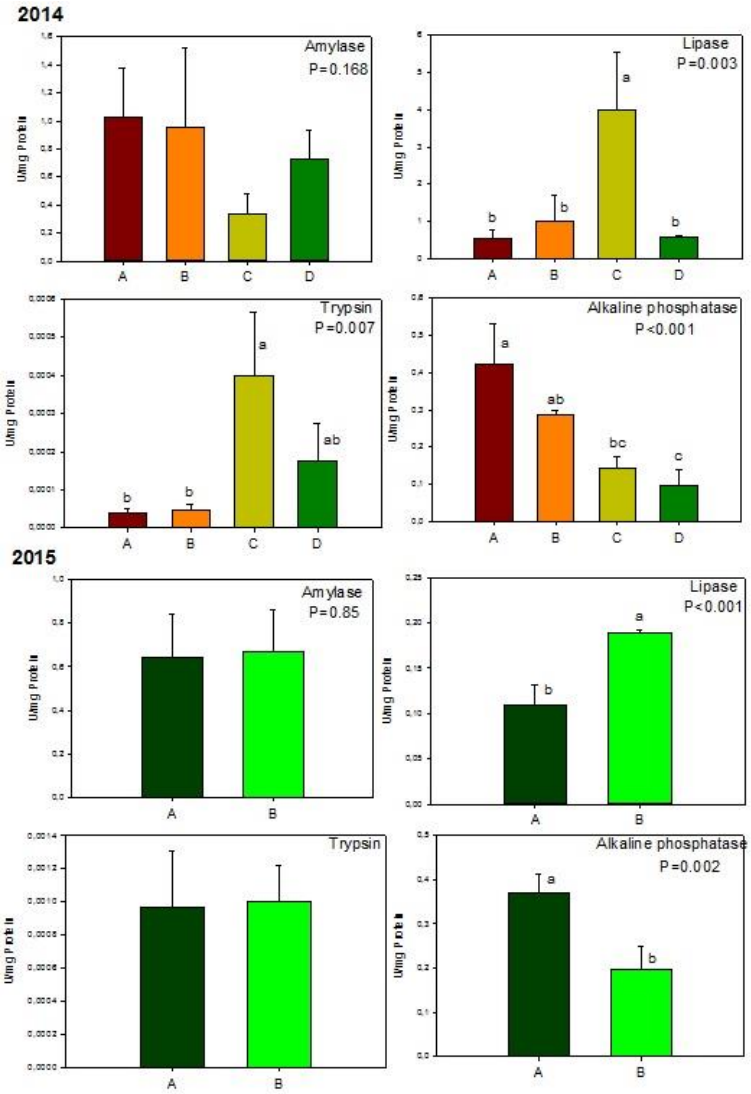


Figure 14.1.3 Results of digestive enzyme activity measured in the larvae from 2014 and 2015 trials at the end of the experiments. Different letters indicate significant differences (ANOVA, data from 2014 and Student's t test, data from 2015, P < 0.05).

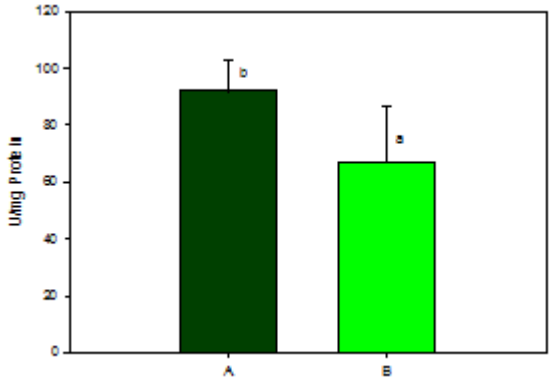


Fig. 14.1.4. Results of pepsin activity measured in the larvae from 2015 at the end of the experiment. Letters indicate significant differences (Student's t test, $P < 0.05$).

4. Early weaning of larvae did not have any effect on the incidence of total skeletal deformities in meagre. The frequency of deformed fish was not significantly ($P>0.05$) different between the two groups of larvae obtained from Trial 2 (Student's t test, $P = 0.621$), which ranged from 17.6% to 21.0% (**Figure 14.1.5**). Furthermore, severe deformities (lordosis, kyphosis, torsion, vertebral centrum) were similar for both treatments (4.25 ± 1.78 % for group A and 4.65 ± 2.22 % for group B). This was true as well in the case of minor deformities (haemal spines and neural spines and modified epural), which was 12.38 ± 7.26% for group A larvae and 16.8±2.48% for group B (**Figure 14.1.5)**.

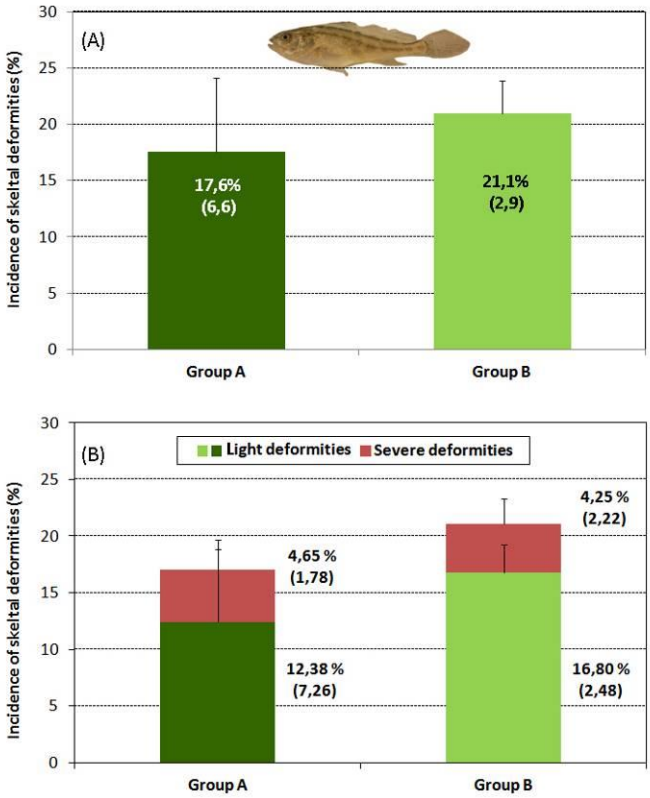


Figure 14.1.5. Incidence of skeletal deformities (A) and incidence of light and severe skeletal deformities (B) in meagre early juveniles (Trial 2015, Group A: control, Group B: early weaned). Data are expressed as mean ± SEM in brackets (n = 5).

Thus, based on the results obtained, meagre larvae can be weaned from live feed to an artificial diet as early as 10 dph, but other important aspects for production success including larval performance and survival should be considered. Particular care should be taken to avoid cannibalistic behavior in the rearing tanks, by reducing the light intensity, increasing larval feeding rate and daily rations, or grading the larvae periodically.

Deviations from Annex I and their impact:

The protocol for early weaning was scheduled for month 18 of the project, however taking into account the problems of cannibalism we decided to repeat the experiments and delay the submission of the Deliverable 14.1, which included the protocol until month 30, in order to be sure that the protocol designed was feasible. There was no impact on other tasks related to meagre rearing.

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), Ioannis Diakogeorgakis (P27)			

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 (led by IEO, Jerez Salvador). 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 (led by HCMR, Nikos Papandroulakis). 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.

Summary of progress towards objectives and details for each task (13-30):

The main objective of the present studies on larval greater amberjack was to improve the survival, growth and performance of greater amberjack larvae by improving the feeding regime, culture density and larval culture conditions. A great success was achieved in the greater amberjack larval rearing season of 2016 (Mo 31-32, outside the scope of this report). 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.

Task 15.1. Effect of feeding regime and probiotics. In this study different rotifers 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.

Task 15.2. Comparison of semi-intensive and intensive rearing. The comparison of semi-intensive and intensive rearing system in 2015 showed that the overall survival in the two systems was low. The gene expression of GHRH, GH, IGF-I and II and IGFBPs were not affected by the rearing method. However, there was a gradual increase in mRNA levels as development proceeded with statistically significant differences observed at 20 dph with peak levels at 25 dph and 30 dph of IGF-I and GHRH. In addition, IGF-II was higher ($P<0.05$) at 5 dph compared to the 2 dph and the 10 dph while GH exhibited higher ($P<0.05$) mRNA levels at 5 dph and 15 dph. The study determining the effect of stocking density of 25, 50 and 75 eggs l^{-1} on larval performance showed the optimum egg density for the larval rearing of greater amberjack is between 25 and 50 eggs l^{-1}. There was a marked appearance of different and severe anomalies in the larval stage that could lead to a lower survival. The study of the ontogeny of the greater amberjack larval digestive system showed that the average enzyme activity measured for a particular age range is independent of the larval rearing conditions. In general terms, the pancreatic enzymes; amylase and alkaline protease, were more active in the youngest larvae compared to the 30 dph larvae, whereas pepsin followed the opposite trend, displaying almost no activity at 12 dph. Intensive rearing seemed to favour amylase, alkaline protease and pepsin activities in the older larvae. Amylase was highly active in the eggs, decreasing ($P<0.05$) at 0-5 dph while increasing from 5 to 10 dph. From 10 to 30 dph, carbohydrates displayed a less relevant role in larval metabolism. Lipase and alkaline protease activities showed an increasing trend from 0-5 to 5-10 dph. However, although lipase decreased similarly to amylase activity after 10 dph, alkaline protease activity was still high at 10-15 dph, to further decrease in the oldest larvae (20-30 dph). Amylase activity was also higher at 12 dph for the intensive system larvae, whereas the opposite trend was observed for alkaline protease and lipase activities. According to the results, greater amberjack seem to use dietary proteins effectively from 20-30 dph.

Task 15.3 Effect of environmental parameters during rearing. The hydrodynamic field was estimated in tanks of 2,000 and 40,000 l. The applied water exchange rates (as % of total water volume) were of 10% and 4% per hour while the airflow was set at 350 and 1400 ml min^{-1} for the 2,000 and 40,000 l tank respectively. The conditions regarding water exchange and airflow were similar to the ones applied during larval rearing. The results showed differences between the conditions as the higher currents occurred in the 2000 l tanks followed by the 40,000 l tanks. The photoperiod study of (24L:00D vs 18L:6D) didn't appear to affect the mRNA expression levels of IGF-I. However, there was a gradual increase in mRNA levels as development proceeds with significantly ($P<0.05$) different observed at 20 dph and peak levels at 30 dph. The mRNA expression levels of IGF-II were not affected by the photoperiod and appeared high at 0 dph and at 5 dph and remained stable thereafter. In addition, 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 compared to the fish reared under the condition of 18D:6D at 25 dph and 30 dph. The study of the effect of tank color (white, green and black) showed no differences ($P>0.05$) 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. Larvae in the white tanks exhibiting the highest survival rate (1.3%) compared to the black tank (0.5%) and the green tank (0.02%). The gene expression analysis revealed significant ($P<0.05$) differences among the treatments. From 0 to 17 dph, the levels remained stable and low for all groups. However, at 25 dph and 30 dph the fish reared in the white and black tanks showed a significant ($P<0.05$) increase, whereas IGF-I levels in fish reared in the green tanks remained stable. 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.

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.

Task 15.1. Effect of feeding regime and probiotics (IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez).

To achieve the objectives proposed in this task, a first trial of rotifer enrichment was designed (P15. ULL/P8.IEO) and performed at P8. IEO in order to select products and enrichment time. In this assay,

rotifers were enriched with a polar lipid rich emulsion containing a marine phospholipid and arachidonic acid (20:4n-6) combined with 10 ppm of carotenoids (esterified astaxanthin). Echium oil was tested as probiotic / immunostimulant, given its role as modulator of the stress response in fish.

Experimental conditions

Different enrichment protocols were designed and prepared at ULL facilities (P15), based on preliminary results on larval nutrition (see Deliverable 9.1): T1= marine lecithin/20:4n-6/10ppm carotenoids (E1), T2= E1+20% Echium oil; T3= E1+10% Echium oil. Rotifer population status was compared with a commercial enrichment (C). The assays of rotifer culture were carried out at P8. IEO during 24 h, with the following conditions:

- Tank volume of 10 l, continuous light and aeration, maintained at room temperature (20ºC) at an initial density of 300 rot ml^{-1}.
- Sampling at 1, 3, 6, 10 and 24 hours to determine survival (%), ovigerous females (%), temperature (ºC), and oxygen (% saturation).

The results on rotifer culture parameters are shown in **Table 15.1.1**. The survival rates of treatments T2 and T3 were higher to that of the commercial C and T1 treatments after 10 hours. The oxygen saturation and ovigerous females remained unchanged among treatments. Overall, the experimental treatments with marine lecithin supplemented with 20 or 10% Echium oil showed the best results.

Table 15.1.1. Rotifer ml $^{-1}$, Survival (%), ovigerous female (%), temperature (ºC) and oxygen saturation (%) from the different experimental treatments (T1, T2 and T3) vs control treatment (C) used to enrich live prey (rotifers) for 24 hours. C= commercial enrichment product; T1= marine lecithin (LC60) /20:4n-6/10ppm carotenoids (E1), T2= E1+20% Echium oil; T3= E1+10% Echium oil. Values are means ± SD (n=3). Different letters within a column denote significant (P<0.05) differences between hours for a dietary treatment; different numbers within a column denote significant (P<0.05) differences among dietary treatments for an enrichment period (P<0.05).

T	h	Rotifer/ml		Survival (%)		Ovigers (%)		Temp. (ºC)		Oxigen (%)	
C	1	241 ± 24	a	80,3 ± 8,0	A	21,4 ± 4,6	A	22,9 ± 0,1	A	87,5 ± 6,0	
	3	236 ± 12	A	78,6 ± 4,1	A	18,8 ± 3,3	AB	22,5 ± 0,0	B	85,3 ± 5,5	
	6	205 ± 24	Ab 1	68,5 ± 8,1	AB 12	15,6 ± 6,2	AB	21,9 ± 0,1	C	86,4 ± 8,7	
	10	178 ± 22	AB 2	59,4 ± 7,4	AB 23	12,2 ± 5,4	AB	21,6 ± 0,0	D	86,5 ± 6,2	
	24	153 ± 35	b	51,1 ± 11,8	B	6,86 ± 2,1	B	20,2 ± 0,1	E	95,3 ± 1,0	
T1	1	266 ± 15	A	88,6 ± 5,0	A	22,6 ± 3,2	A	22,8 ± 0,1	A	85,9 ± 2,4	B
	3	219 ± 25	AB	73,2 ± 8,3	AB	20,5 ± 3,5	A	22,4 ± 0,1	B	80,9 ± 5,6	B
	6	164 ± 16	BC 2	54,8 ± 5,6	BC 2	9,46 ± 4,1	B	21,9 ± 0,1	C	83,8 ± 4,3	B
	10	141 ± 7	C 3	47,1 ± 2,5	C 3	8,60 ± 3,6	B	21,5 ± 0,1	D	81,6 ± 5,3	B
	24	124 ± 41	C	41,5 ± 13,9	C	4,16 ± 1,8	B	19,9 ± 0,1	E	96,0 ± 1,5	A
T2	1	265 ± 17	A	88,5 ± 5,9	A	25,0 ± 1,3	A	22,8 ± 0,1	A	86,2 ± 4,2	Bc
	3	259 ± 26	A	86,5 ± 8,8	A	20,2 ± 1,2	AB	22,5 ± 0,1	A	84,4 ± 5,4	C
	6	230 ± 14	AB 1	76,8 ± 4,8	AB 1	16,0 ± 4,9	BC	21,9 ± 0,1	B	88,9 ± 2,5	aB
	10	220 ± 12	AB 1	73,3 ± 4,0	AB 12	13,1 ± 2,9	BC	21,6 ± 0,1	C	88,6 ± 5,1	aB
	24	179 ± 47	B	59,8 ± 15,8	B	9,50 ± 2,6	C	20,0 ± 0,1	D	97,7 ± 0,7	A
T3	1	257 ± 28		85,6 ± 9,5		20,6 ± 5,6	A	22,8 ± 0,1	A	87,7 ± 4,8	B
	3	256 ± 29		85,4 ± 9,9		19,2 ± 0,9	A	22,5 ± 0,1	A	89,3 ± 3,5	AB
	6	252 ± 14	1	84,1 ± 4,9	1	17,1 ± 3,2	Ab	21,9 ± 0,1	B	93,5 ± 3,0	AB
	10	243 ± 31	1	81,2 ± 10,3	1	17,1 ± 2,6	AB	21,5 ± 0,2	B	92,9 ± 3,2	AB
	24	209 ± 31		69,7 ± 10,5		9,00 ± 3,9	B	20,0 ± 0,2	C	97,9 ± 2,3	A

Larval performance

From these preliminary experiments, it was suggested that rotifers enriched for a short period (3 h) with 6% of the marine lecithin/20:4n-6/10ppm carotenoids supplemented with 20 or 10% Echium oil, improve the species larval performance at early life stages.

The effect of these new combinations of enrichments formulated from results attained was assessed on greater amberjack larval performance. In addition to *Echium* oil as probiotic/immunostimulant, black cumin oil (*Nigella sativa*) was tested. This is because the use of black cumin seeds has been shown to enhance growth performance and immunity in fish (Awad *et al*., 2013; Aquaculture 388–391, 193–197). To this end the rotifer enrichment commercial protocol (T1) was compared with three experimental treatments (T2, T3 and T4) added at a 6% concentration for 3h to the rotifer enrichment tanks, under the same rearing conditions. T2 consisted of LC60/20:4n-6/10ppm carotenoids, the same emulsions described above, with T3 and T4 consisting of this lipid emulsion combined with 20% Echium oil and 20% black cumin oil, respectively.

Newly hatched larvae of greater amberjack at a total density of 100 larvae l^{-1} (mean total length 3.62±0.14 mm) were randomly distributed in 12 experimental tanks of 100 l capacity. Two prey concentrations were used (5 and 10 individuals ml^{-1}). Rotifers enriched with one of the four treatments mentioned above were added to the larval rearing tanks twice a day. All larval sampling performed at each age (1, 6 and 12 dph) was carried out randomly 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 was determined and daily prey intake was also estimated by the differences between added and remaining rotifers in larval rearing tanks.

The daily prey intake was similar for all treatments (7.2 ± 1.2 rotifers ml^{-1}) at 12 dph (**Figure 15.1.1**). Larval growth was significantly increased from 1 dph (3.502 ± 0.156 mm) to 7dph (3.751 ± 0.318 mm) and 12 dph (4.510 ± 0.424 mm), irrespective of enrichment treatment (**Figure 15.1.2)**. However, at 7 dph, larval growth was significantly lower in larvae fed with the commercial treatment (T1).

Larval survival was significantly ($P< 0.05$) affected by treatments assayed. The lowest survival was recorded in those larvae receiving the treatment T1 (**Figure 15.1.3**) and all larvae of this treatment died at 12 dph. The survival was also very low in the remaining treatments.

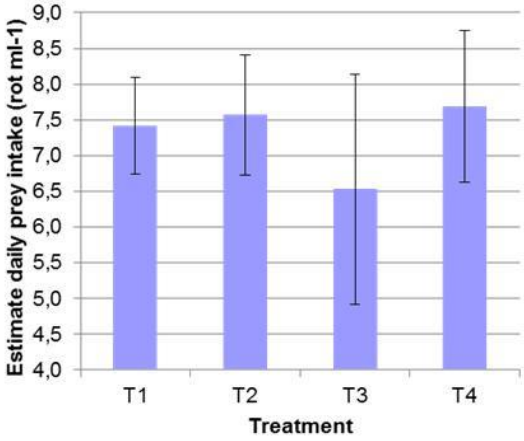


Figure 15.1.1. Estimate daily prey intake (rotifer ml^{-1}) of greater amberjack larvae, fed with rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids and 20% Echium oil) and T4 (LC60/20:4n-6/10ppm carotenoids+ 20% Black cumin oil) (ANOVA, P >0.05).

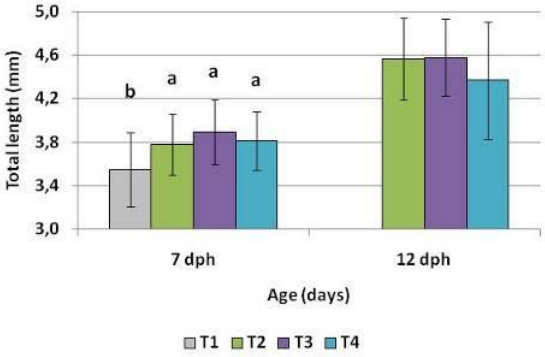


Figure 15.1.2. Total length (mm) of greater amberjack larvae, fed with rotifers from T1 (Commercial enrichment), T2 (LC60/20:4n-6/10 ppm carotenoids), T3 (LC60/20:4n-6/10 ppm carotenoids and 20% Echium oil) and T4 (LC60/20:4n-6/10 ppm carotenoids+ 20% Black cumin oil) at 7 and 12 dph. Values are mean ± SD, n=3. Different letters indicate significant difference between treatments at each age (ANOVA, P <0.05).

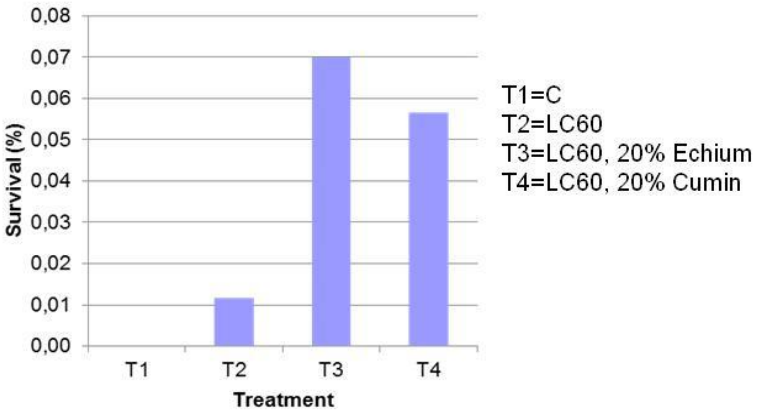


Figure 15.1.3. Final survival percentage (12 dph) of greater amberjack larvae, fed with rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/ 10ppm carotenoids), T3 (LC60/20:4n-6/ 10ppm carotenoids and 20% Echium oil) and T4 (LC60/20:4n-6/ 10ppm carotenoids+ 20% Black cumin oil).

The lower survival achieved did not allow enough larvae to carry out the activities planned. A second assay was carried out but the results were similar to the first one with very high mortalities from the very beginning of the assay. That means that an important part of the expected analyses were not performed and the attained results are not considered conclusive. A repetition of the present assay is scheduled for later in 2016 and, therefore, the submission of Deliverable 15.2 is delayed (as requested earlier this year and approved by the PC).

Task 15.2 Comparison of semi-intensive and intensive rearing (led by HCMR).

Sub-task 15.2.1 Comparison between intensive and semi-intensive larval rearing (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 is 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), and associated carrier proteins (IGFBPs) and receptors that represent the endocrine and autocrine regulator for skeletal muscle growth.

During the 1st Reporting Period, preliminary trials were performed in order to establish the larval rearing methodologies in the two rearing systems. During the present reporting period the actual trial was performed. 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 and N. Kopidakis.

Eggs from induced spawning (WP 3) of breeders kept in the P23. ARGO cage farm were used for the rearing. After collection, eggs were transported to the hatchery facilities of P1. HCMR in polystyrene boxes in ~ 12 hours, and then were incubated.

Semi-intensive Mesocosm Larval rearing

The **Mesocosm methodology** is an intermediate between the intensive and extensive methods of rearing, and can thus be considered as a semi-intensive technique of mass production. The most important characteristic of the infrastructure required by this method is the size of the larval tanks (the Mesocosm tank), which at P1. HCMR is 40 m^3 (**Figure 15.2.1.1**)

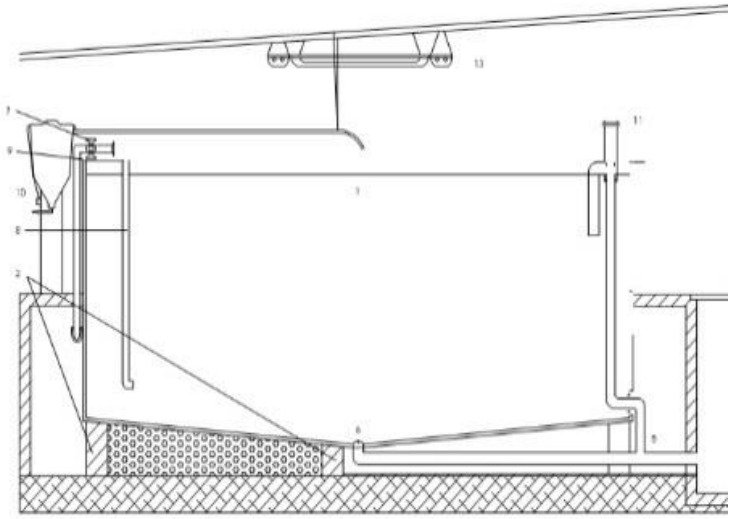


Figure 15.2.1.1. Graphical representation of a Mesocosm tank at P1. HCMR.

Two tanks were used inoculated with 110 and 15 $x10^3$ eggs each. Mesocosm tanks were filled with filtered natural seawater (salinity 40 psu) treated with UV, which was also the water for subsequent renewal. Temperature was at 24± 0.7ºC and the pH fluctuated from 7.99 to 8.18. Dissolved oxygen varied from 5.8 to 6.8 mg l^{-1} during the rearing. The rate of water renewal increased progressively during the rearing. Starting from 15% per day, reached 35% on 17 day post hatching (dph), 100% on 22 dph and 200% on 30 dph. Aeration was provided in the tanks by means of five pipes (without any wooden or stone diffuser) distributed in the perimeter and the center of the tank. A surface skimmer was operational during the appropriate period (5 to 13 dph) to keep the surface free from lipids, a prerequisite for good swim bladder inflation. The photophase was 24L:00D from mouth opening until 25 dph and then turned to 18L:06D for the remaining period. Light intensity varied according to the weather conditions between 500 lux on cloudy days to 1,000 lux on sunny days, while during the night when prolonged photophase was applied, light intensity was about 250 lux.

The phytoplankton organism used was *Chlorella sp*. that was added once daily at a concentration of 300 ± $100x10^3$ cells ml^{-1} after mouth opening until 20 dph. The rotifers *Brachionus* were mass-produced at P1. HCMR. Prior to distribution to the larvae, rotifers were enriched with commercial products (DHA Protein Selco, INVE Aquacultures S.A., Belgium) and were maintained during their use with *Chlorella*. The provided Instar II *Artemia* nauplii were also enriched with commercial products (Easy DHA Selco, INVE Aquacultures S.A., Belgium) according to the specifications of the provider.

Feeding was based on daily administration of enriched rotifers (3 to 20 dph) at a concentration of 2-3 ml^{-1}, Instar II *Artemia* nauplii, 0.1 to 0.5 ind ml^{-1}, (from 14 to 30 dph), and occasionally fish eggs (alive and frozen) since 16 dph. Yolk larvae (20-30,000 d^{-1}) were also delivered between 27 and 33 dph. The concentration of rotifers in the tank was maintained at 1.5 ind ml^{-1}, while that of *Artemia* at 0.1 ind ml^{-1} after measuring the concentration twice daily. Mesocosm tanks exhibited also some productivity of zooplankton (harpacticoida copepods) after 20 dph, which could potentially contribute to larval feeding. Starting on 22 dph, artificial diet was also delivered and was distributed with an automatic feeder.

Intensive rearing in closed water recirculation system

The second methodology applied was the **intensive rearing,** which is characterized by controlled conditions of water quality, light intensity, photophase and feeding. The variant of the methodology applied is the so-called "pseudo-green" water that is based on the frequent addition of phytoplankton and zooplankton in the larval rearing tanks. It integrates principles of both "clear water" and "green water" methods and minimizes biological problems and many of their technical, human and environmental constraints. The main difference from the classical "green water" technique is that phytoplankton is not produced nor bloom in the rearing tank, but its concentration remains constant by daily addition. The tanks used at P1. HCMR are 500-l cylindro-conical and are organized in pairs in a closed water system with a biological filter (**Figure 15.2.1.2**). The tanks are filled with borehole water of 35 psu. Temperature was kept at 22±0.5°C during the autotrophic stage and was gradually increased to 24±0.5°C after mouth opening. The pH fluctuated from 8.0 to 8.2 and the dissolved oxygen from 6.8 to 7.2 mg l^{-1}. Water circulation was achieved in two ways according to the stage of rearing. During embryogenesis, egg hatching and the autotrophic larval stage, water circulated in the tanks through a biological filter. Aeration was also provided in the tanks (150–250 ml min^{-1}). After first feeding, water circulation was autonomous for each tank by means of an airlift pump. The water in the biological filter was used for renewal in larval rearing tanks that was 3% daily until 15 dph followed by a gradual increase to 50% on 25dph. A skimmer was installed at the appropriate period (5 to 15 dph) to keep the surface free from lipids. The photophase was 24L:00D from mouth opening until 25 dph and then turned to 18L:06D for the remaining period. Light intensity varied between 200 - 800 lux during the day, and was about 200 lux during the night.

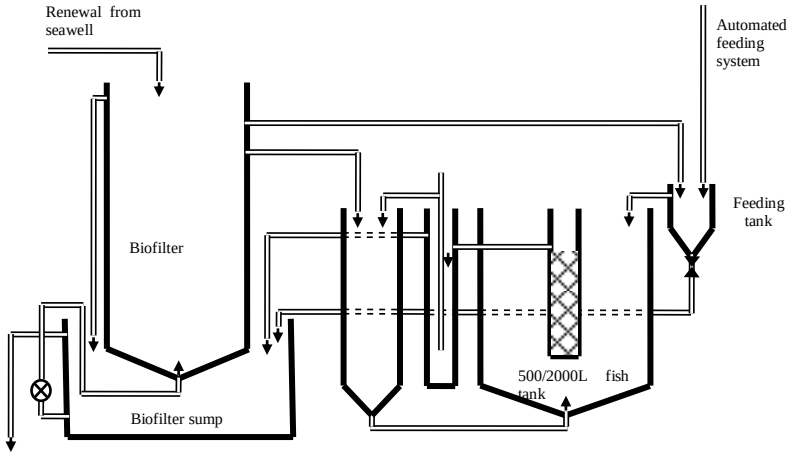


Figure 15.2.1.2. Larval rearing tanks in closed water system at P1. HCMR

Feeding was based on daily administration of enriched rotifers (since 3 until 21 dph), Instar II *Artemia* nauplii (from 12 dph onwards), and artificial diet (since 21 dph). The concentration of rotifers in the tank was maintained at 3.0 ind ml^{-1}, while of *Artemia* at 0.1 ind ml^{-1} after measurements of the concentration twice daily. Phytoplankton was added daily from 3 to 22 dph at 300 ± 100 x 10^3 cells ml^{-1}. The administration of the zooplankton was implemented with the use of an automated feeding system allowing the administration of food several times daily and also during the night.

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, samples were collected for

I. the ontogeny of visual system (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 (GHRH, GH, IGF-I and II, IGFBPs and receptors).

Primer design and qPCR experiments

Primer design

Primers for GH were based on the available sequence of greater amberjack in genomic databases (NCBI Gene Bank accession no. L43628) and primers for the gene of GHRH on the available sequence of *Sparus aurata* (no. DQ659328), IGF-I & IGF-II were designed based on available sequences of *Seriola quinqueradiata* (no. AB439208 and AB823704, respectively). The products of each primer pair were further checked with sequencing in order to confirm that they amplify the desired genes. Primers for IGF-BP1, IGF-BP2, IGF-BP3, IGF-BP5 were as described by Pedroso *et al.* 2009. Primers for b-actin were obtained from previous work of our group (Pavlidis *et al*., 2011), whereas for ribosomal 18S RNA (*18S*) were obtained by the work of Tom *et al*. 2004.

RNA purification and cDNA synthesis

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 GH, GHRH, IGF-I & IGF-II, IGF-BP1, IGF-BP2, IGF-BP3 and 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 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 2015 season, 5 pooled samples from both the mesocosm and intensive reared fish were taken at 0, 2, 5, 10, 15, 20, 25 and 30 days post hatch (dph) and were used for expression analysis of GH, GHRH, IGF-I & IGF-II, and IGF-BP1, IGF-BP2, IGF-BP3 and IGF-BP5.

Results

Rearing was performed in all cases as planned and all required samples were collected. Although no pathologies were presented, the overall survival in all cases was low. This was particularly obvious in the case of the Mesocosm in which survival was 0.12 and 0.02% in the two tanks used during the reporting period while it was 1.1 and 1.2% 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	0.0478 d^{-1}
500 l	0.0518 d^{-1}
Wet weight	
40,000 l	0.22 d^{-1}
500 l	0,25 d^{-1}

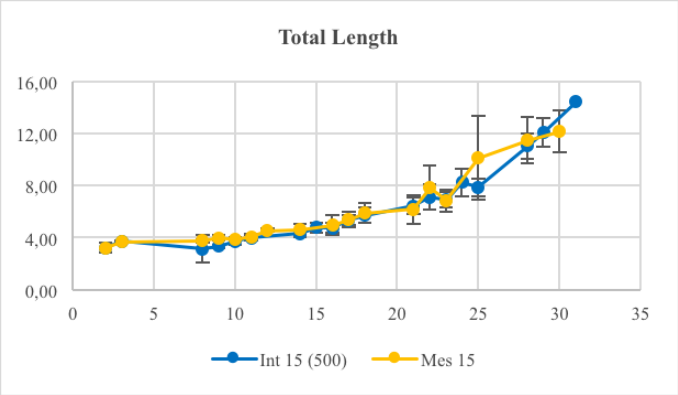


Figure 15.2.1.3: Evolution of the total length of greater amberjack larvae reared under intensive or semi-intensive conditions (Mean and SD).

The rearing method (mesocosm vs intensive) did not appear to affect the mRNA expression levels of IGF-I. However, there was a gradual increase in mRNA levels as development proceeds with significant differences observed ($P < 0.05$) at 20 dph with peak levels at 25 dph and 30 dph (**Figure 15.2.1.4**). The mRNA expression levels of IGF-II were not altered based on the rearing method but appeared significantly higher at 5 dph ($P < 0.05$) compared to the 2 dph and the 10 dph (**Figure 15.2.1.5**). The expression of GH was not affected by the rearing method used but higher ($P < 0.05$) mRNA levels were observed at 5 dph and 15 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 significantly increased ($P < 0.05$) at 25 dph and 30 dph (**Figure 15.2.1.7**).

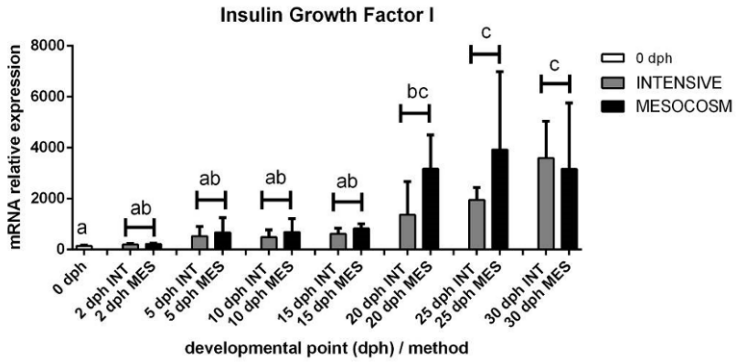


Figure 15.2.1.4. The mRNA relative expression levels of IGF-I between the different rearing methods during early ontogeny greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

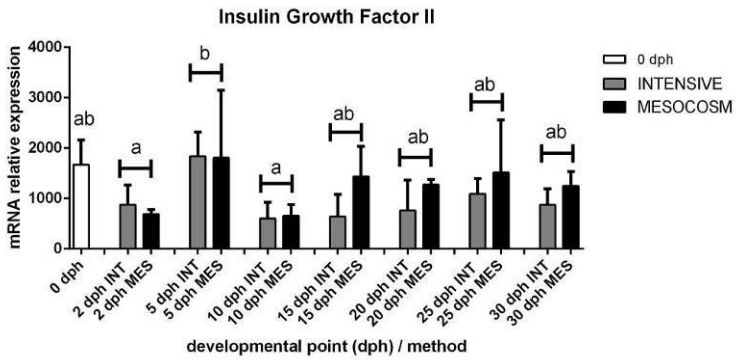


Figure 15.2.1.5. The mRNA relative expression levels of IGF-II between the different rearing methods during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

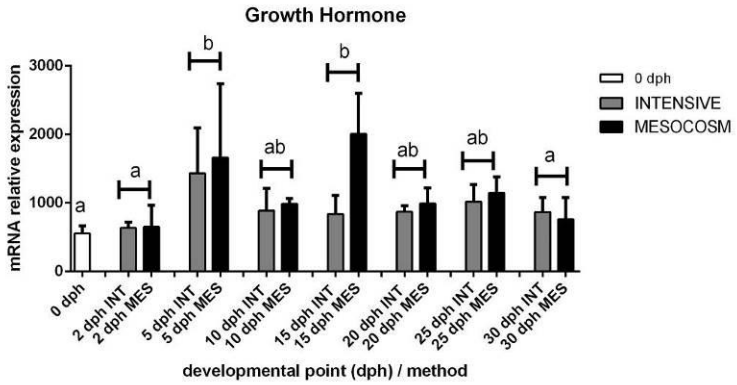


Figure 15.2.1.6. The mRNA relative expression levels of GH between the different rearing methods during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

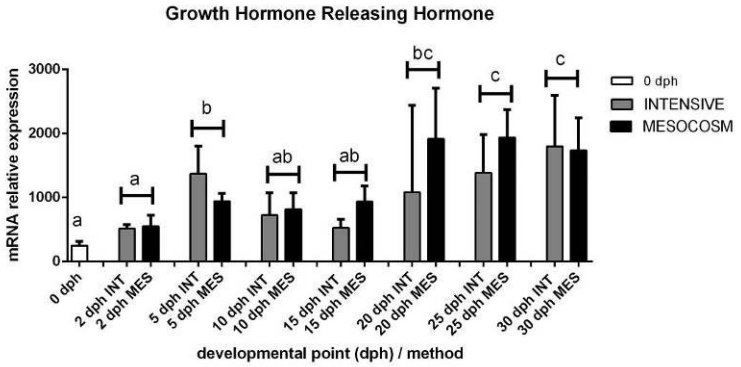
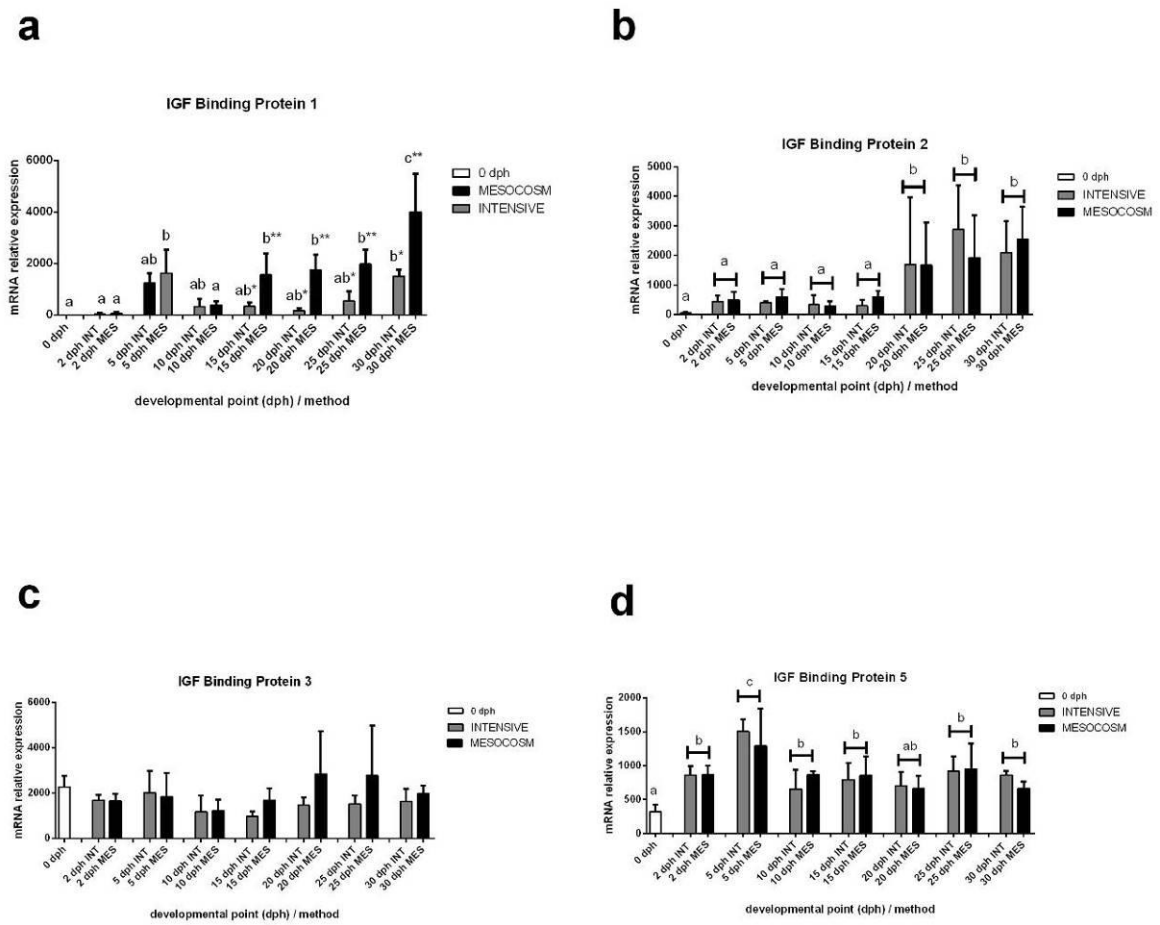


Figure 15.2.1.7. The mRNA relative expression levels of GHRH between the different rearing methods during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

The rearing method applied did not affect the mRNA expression of any of the IGF binding proteins studied apart from the expression of IGF-BP1 (**Figure15.2.1.8a**) where higher levels ($P < 0.001$) were observed in the mesocosm reared fish compared to the intensive reared fish from 15 dph until 30 dph. Additionally, as development progressed the mRNA levels appeared to gradually increase ($P < 0.001$) independent of the method applied (**Figure15.2.1.8a**).



(from previous page)

Figure 15.2.1.8. The mRNA relative expression levels of IGF-I binding proteins between the different rearing methods during early ontogeny of greater amberjack: (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 20 dph were there was a significant increase ($P < 0.05$) and remained high until 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 5 dph where peak values ($P < 0.05$) were observed (**Figure15.2.1.8d**).

Sub-task 15.2.2 effect of stocking density on larval performance (by FCPCT, Carmen Mª Hernández-Cruz).

The effect of stocking density on larval performance in terms of growth, survival, skeletal deformities and gene expression of stress and skeleton genes was studied. Three different larval rearing densities were evaluated: 25, 50 and 75 eggs l^{-1} in triplicate tanks for a period of 30 days. The full description of this work and results has been submitted in ***Deliverable 15.1. Effective greater amberjack larval stocking densities***.

The main objective of this study was to determine the effect of stocking density on larval performance. Due to the death of all the larvae of the first trial at around 15 days post hatching (dph) in 2014, the experiment was conducted again in 2015. Greater amberjack eggs from a natural spawning were stocked at densities of 25, 50 and 75 eggs l^{-1} in nine 2000-l tanks. Severe cannibalism and size dispersion were observed from 10-15 days post hatching (dph). Skeletal deformities evaluation and gene expression analysis were performed only during the second trial in 2015.

In the second trial, the treatment with 75 eggs l^{-1} showed significantly increased total length (TL, 17.43 ± 4.19 mm), whereas the treatment with 25 eggs l^{-1} showed significantly increased survival (11.25%± 4.92) compared to the other treatments (**Figure 15.2.1**). According to the results obtained in this study, the highest growth rates were obtained at a density of 50 eggs l^{-1} in the first trial and 75 eggs l^{-1} in the second trial, while the highest survival was 25 eggs l^{-1}, and 50 eggs l^{-1}. The results of evaluation of skeletal anomalies showed a marked appearance of different severe anomalies in the larval stage that could lead to a lower survival. With the results obtained, the methodology for larval rearing is established and the current protocol will be implemented during the project.

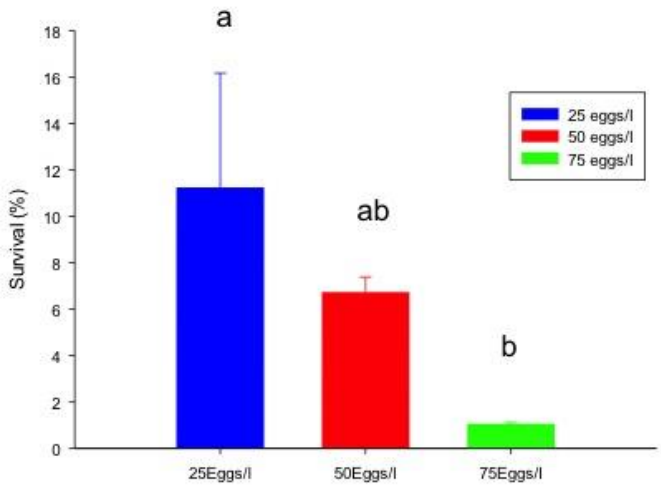


Figure 15.2.1 : Survival of greater amberjack greater amberjack (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1}. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Sub-task 15.2.3 Ontogeny of greater amberjack larval digestive system (by ULL, Covadonga Rodríguez).

A better knowledge of larval digestive ontogeny and its physiology by using different scientific approaches and techniques will contribute to the optimization of diets and rearing conditions. The analysis of the main digestive enzymes during larval development and under different rearing conditions will help understand the functions and limitations in the processing capacity of the digestive system of a new species such as greater amberjack. This will allow us to deliver nutrients to the rapidly growing larval tissues under changeable feeding and environmental conditions.

Determination of digestive enzyme activities

Samples of eggs and larvae were collected for digestive enzyme analysis from Subtasks 15.2.1 and 15.2.2 (P1. HCMR and P2. FCPCT). In action 15.2.1, comparisons of enzyme activities of larvae from the intensive and mesocosm tanks were carried out at 12 and 30 dph. In action 15.2.2, larvae were also available at 15, 25 and 30 dph but only from the treatment with the lowest egg initial density (25 eggs l^{-1}). Some additional larvae were analyzed from both Partners to establish greater amberjack average values of the main digestive enzymes at periods 0-5; 5-10; 10-15 and 20-30 dph, independently of their geographical location and rearing conditions, as a preliminary approximation to the digestive ontogeny of this species. The samples were completely homogenized (Ultra-Turrax T8, IKA©-Werke, Germany), in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4ºC, the supernatant removed for enzyme quantification and kept at -80ºC until further analysis. Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were based on methods performed and described by Dr. Gisbert (P3. IRTA) (see Gisbert *et al*. 2009). Enzyme activities are expressed as mean ± SD. The group data were statistically tested using one-way ANOVA followed by the Tukey test. The significant level for all the analysis was set at 5%.

Results

The average activities measured for a particular range of age was independent of the larval geographical origin and rearing and environmental conditions. Even though at 30 dph, lipase was more active and pepsin less marked in the Mediterranean larvae compared to the Atlantic ones. In general terms, the pancreatic enzymes; amylase and alkaline protease, were more active in the youngest larvae compared to the 30 dph larvae, whereas pepsin followed the opposite trend, displaying little activity at 12 dph (**Figure 15.2.3.1**). Similarly, amylase, alkaline protease and also lipase activities were higher at 15 dph than 30 dph. However, pepsin activity increased incrementally and significantly ($P<0.05$) from 15 to 30 dph (**Figure 15.2.3.2**). Intensive rearing seemed to favour amylase, alkaline protease and pepsin activities in the older larvae. Amylase activity was also higher at 12 dph for the RAS system larvae, whereas the opposite trend was observed for alkaline protease and lipase activities.

An approximation to the ontogeny of greater amberjack digestive system is shown through the evolution of average activities of the main digestive enzymes (**Figure 15.2.3.3**). Amylase was highly active in *Seriola* eggs, significantly decreasing at 0-5 dph while increasing at 5 to 10 dph **(Figure 15.2.3.3 a).** From 10 to 30 dph, carbohydrates displayed a decreasing role in larval metabolism. With the exception of the eggs, where both lipase and alkaline protease activities were moderate, an increasing trend of activity of these two groups of enzymes was also displayed from 0-5 to 5-10 dph **(Figure 15.2.3.3 b and c)**. However, although lipase decreased similarly to amylase activity after 10 dph, alkaline protease activity was still high at 10-15 dph and then decreased in the oldest larvae (20-30 dph) **(Figure 15.2.3 3 c)**. This decrease in this pancreatic protease coincided with a clear increment in the levels of pepsin activity **(Figure 15.2.3 3 d).**

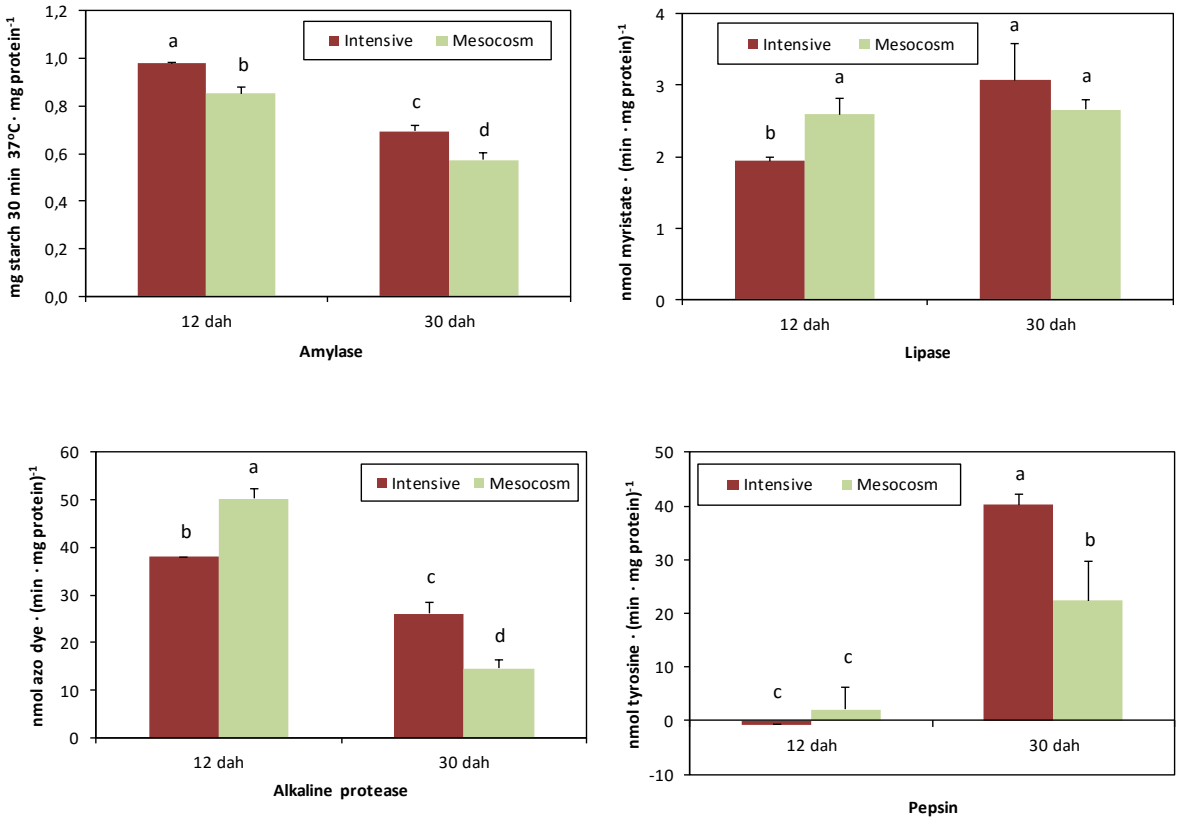


Figure 15.2.3.1. Digestive enzyme activities of Mediterranean greater amberjack larvae reared in intensive and mesocosm tanks. Different letters denote significant differences (ANOVA, P<0.05)

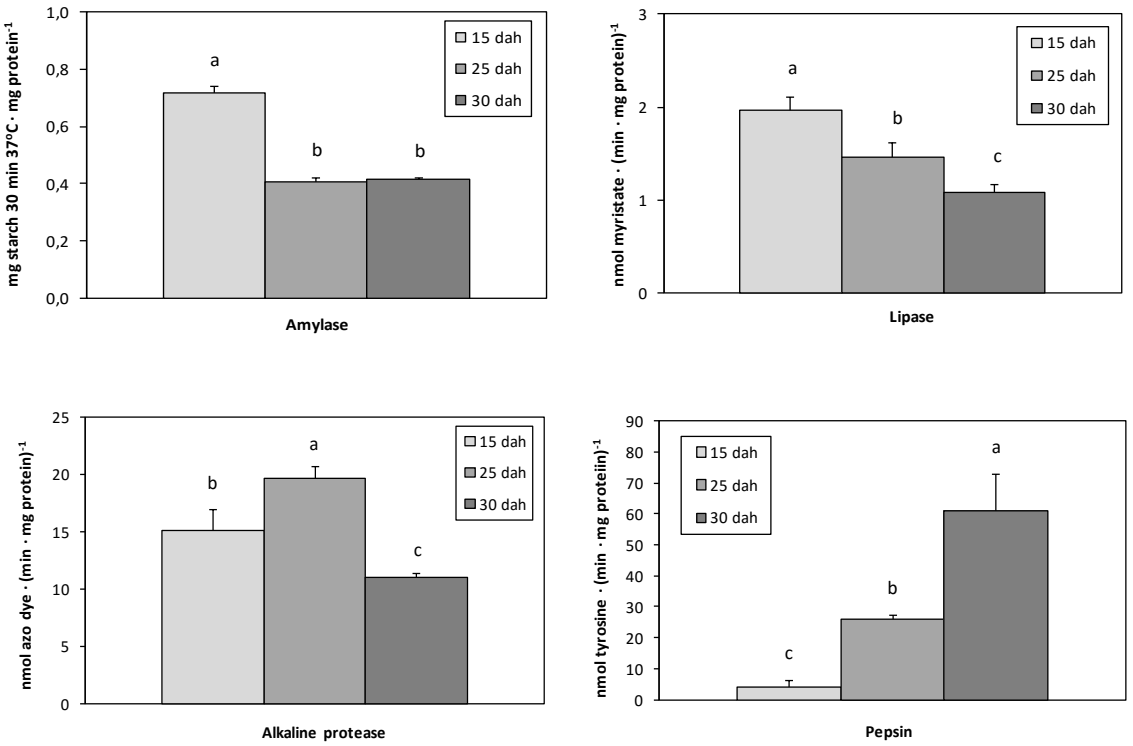


Figure 15.2.3.2. Digestive enzyme activities of Atlantic greater amberjack larvae reared in intensive tanks at an initial density of 25 eggs.l^{-1}. Different letters denote significant differences (ANOVA, P<0.05).

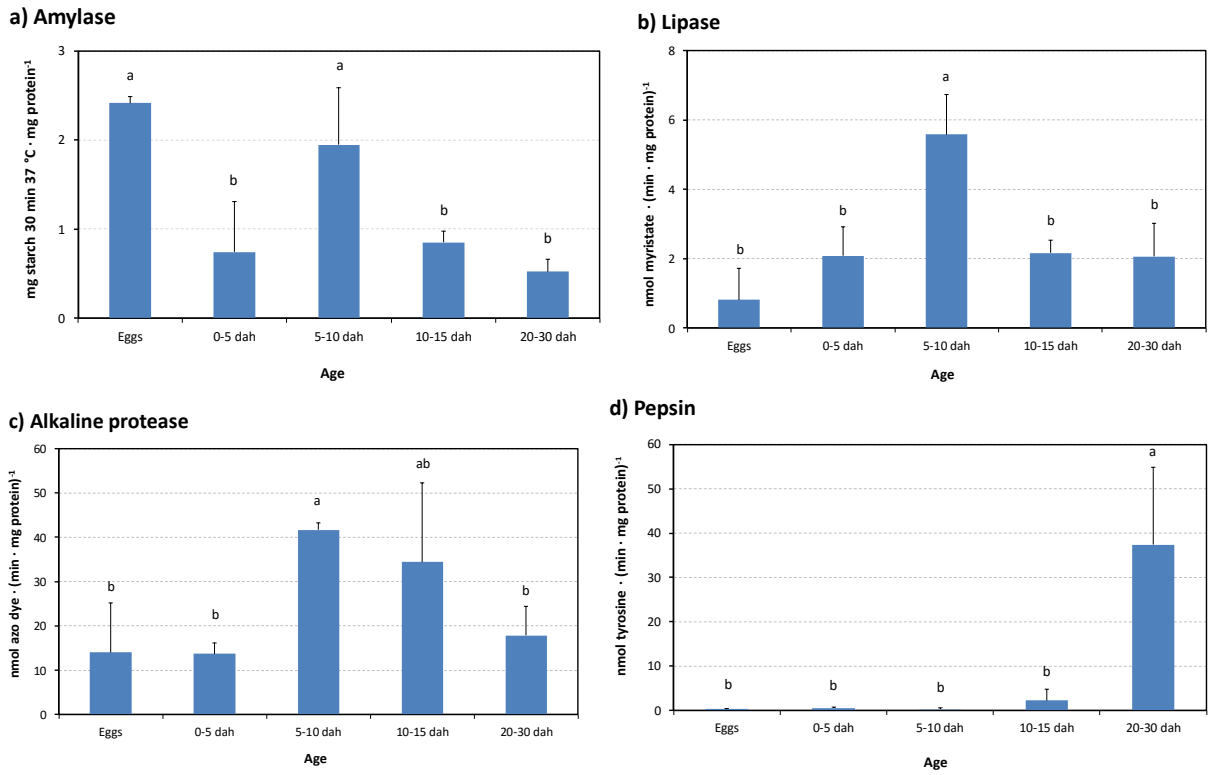


Figure 15.2.3.3 Average values of total amylase **(a)**, lipase **(b)**, alkaline protease **(c)** and pepsin **(d)**, measured in greater amberjack eggs and larvae sampled at different rearing protocols and geographical locations. Different letters denote significant differences (ANOVA, P<0.05).

According to our preliminary results greater amberjack seems to effectively use dietary proteins from 20-30 dph. Taking this into account, the weak ability of early-weaned larvae for proteolytic cleavage of proteins could be the reason for the poor larval performance achieved at early life stages of this species. The higher lipase but poorer pepsin activities, observed in the 30 dph mesocosm-reared-larvae could signal lower nutritional status compared to the Atlantic larvae, although further studies will be necessary to elucidate if initial larval quality differs among two stocks and if this affects further digestive performance.

Task 15.3 Effect of environmental parameters during rearing (led by FCPCT).

Sub-task 15.3.1 (by FCPCT, Carmen Maria Hernández Cruz, Antonio la Barbera, Mitrizakis Nikolaos, Nikos Papandroulakis)

The effect of tank hydrodynamics was studied. Two different tank types (40,000 l cylindrical and 2,000 l cylindro-conical) were tested in duplicates for a period of 30 days. The current profile was analysed with a Vectrino (high-resolution acoustic velocimeter). The effect of tank type on larval performance in terms of growth, survival, histology, biochemical composition, skeletal deformities and gene expression of stress and skeleton related genes were determined.

Current measurements

For the purpose of evaluating environmental conditions during larval culture rearing in different tank types, a technician from P1. HCMR traveled to P2. FCPCT in April 2015. In order to better evaluate the physical parameters in the tanks, measurements of water currents were performed independent of the rearing, as the

procedure could affect the survival and the performance of the reared groups. The Vectrino is a high-resolution acoustic velocimeter used to measure 3D water velocity in a wide variety of applications from the laboratory to the ocean. The basic measurement technology is coherent Doppler processing. The water velocity measurements have a range of ± 1 cm s^{-1} with an accuracy of ±0.5% of the measured value or ±1 mm s^{-1}. The sampling volume is at a distance of 5 cm from probe with a diameter of 6 mm and a height of 7 mm (**Figure 15.3.1.1**)

The hydrodynamic field was estimated in tanks of 2,000 and 40,000 l. The applied water exchange rates (as % of total water volume) were 10% and 4% per hour, while the airflow was set at 350 and 1400 ml min^{-1} for the 2,000 and 40,000 l tank, respectively. The conditions regarding water exchange and airflow were similar to the ones applied during larval rearing. Measurements of current field at specific depths or layers were performed. The 2,000 l tank was divided into 5 layers at 0.1 m, 0.65 m, 1.10 m, 1.25 m and 1.5 m depth. At the first 3 layers, 17 measurements were taken, while on the forth layer 5 measurements were taken and on the last layer, 1 was taken at normally distributed points. For the mesocosm 40,000 l tanks, 3 layers were defined at 30, 70 and 150 cm and 17 measurements were taken in each one. A specially prepared construction on the top of the tanks allowed the accurate positioning of the sensor in the tank in order to perform the measurements (**Fig. 15.3.1.2**).

Figure 15.3.1.1 Representation of current sampling volume.

Figure 15.3.1.2 The holding construction of the velocimeter on the top of the tank (a) and detail of the positioning of the sensor (b)

Larval rearing

The eggs that were used in these experiments came from spontaneous spawning at P2. FCPCT. Greater amberjack eggs were stocked at densities of 10 eggs l^{-1} in duplicate mesocosm tanks (**Mes 1** and **Mes 2**) of 40,000 l, 10 eggs l^{-1} in duplicate semi-intensive tanks (**Int 1** and **Int 2**) of 2,000 l and 20 eggs l^{-1} in duplicate semi-intensive tanks (**Int 3** and **Int 4**). Tanks were supplied with filtered (sand filter) and UV-treated seawater. All tanks were equipped with a surface skimmer for removing any lipid residue from the water surface while in the center there was an aeration tube, maintained at 125 ml min^{-1}. Larval rearing was performed according to the protocol of the P2. FCPCT, which required a natural photoperiod (14:10h, Light:Dark) and recommended salinity of 37 psu. Water renewal was increased progressively from 25% day^{-1} to 200% hour. The green water technique was used by adding live phytoplankton (*Nannochloropsis sp.*) to maintain a concentration of 250,000 cells ml^{-1} in the rearing tanks during feeding with rotifers

(*Brachionus sp*.), which were enriched with ORI-GREEN (Skretting TM) at 8:00 am and 15:00 pm. *Artemia* feeding started at 14 dph, and was enriched with ORI-GREEN (Skretting TM). The concentration of rotifers in the tank was maintained at 5 ind ml^{-1}, while that of *Artemia* was at 0.1 ind ml^{-1} after measuring the concentration twice daily. The weaning protocol also included manual feeding with microdiets from 20 dph to 30 dph (Gemma Micro, Skretting TM).

Total length and dry weight parameters were recorded during larval development from samples of 30 larvae per tank every 5 days. Sampling of larvae was made following the current regulations (Spanish Royal Decree 1201/2005) and accepted by the Spanish Ethic Welfare Committee (Comité Ético del Bienestar) of the University of Las Palmas de Gran Canaria (ULPGC) in 2011.

Results

Hydrodynamics in the tanks. There were differences between the conditions as higher currents occurred in the 2,000 L tanks followed by the 40,000 l tanks (**Table 15.3.1.1**). Current profiles in the different layers of the various tanks were determined. Arrows are in 3-d representation and the observed size does not represent the actual velocity value, **(Figure 15.3.1.3 (a, b).**

Table 15.3.1.1 Mean velocity at the different layers of the experimental tanks (mean and SD, n=1,500).

Tank volume (L)	Layer	Mean velocity value (cm s^{-1})
2,000	surface	$1{,}51x10^{-2}$ ±$1{,}55x10^{-2}$
	medium1	$0{,}92x10^{-2}$ ±$1{,}38x10^{-2}$
	medium2	$0{,}84x10^{-2}$ ±$3{,}32x10^{-3}$
	medium3	$0{,}23x10^{-2}$ ±$2{,}07x10^{-2}$
	bottom	$1{,}30x10^{-2}$ ±$3{,}65x10^{-2}$
40,000	surface	$9{,}10x10^{-3}$ ±$3{,}22x10^{-2}$
	medium	$8{,}70x10^{-3}$ ±$3{,}05x10^{-2}$
	bottom	$7{,}50x10^{-3}$ ±$8{,}69x10^{-2}$

(a) 2,000 l **(b) 40,000** l

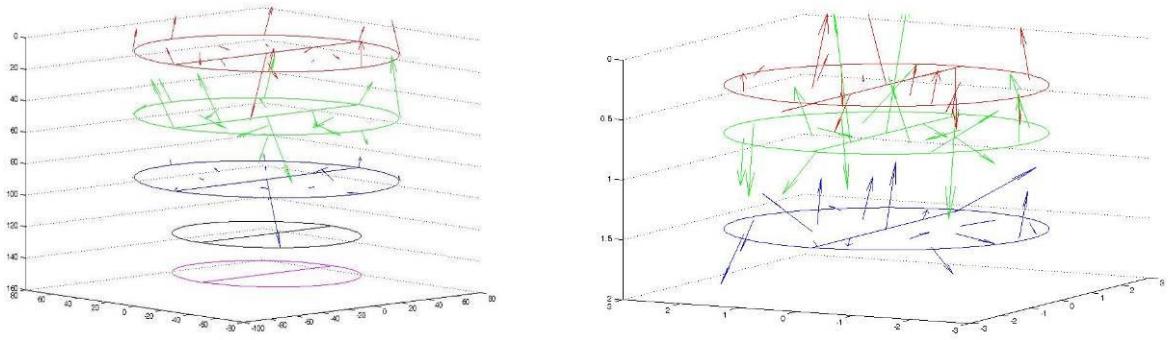


Figure 15.3.1.3 Current profiles in the different layers of the various tanks. Arrows are in 3-d representation and the observed size does not represent the actual velocity value.

Larval performance

No larvae survived in **Int 1** and **Int 2** tanks after 5 dph, or after 10 dph for the rest of the experimental tanks (**Mes 1** and **2**). Significant differences in total length were observed between the mesocosm and intensive system at 5 and 10 dph (**Figure 15.3.1.4**). This trial will be repeated in 2016 due to total larval mortality. In the present study water currents were in generally higher than in the 2000 l tanks.

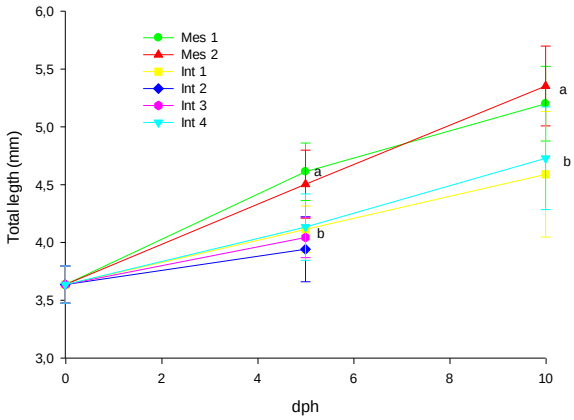


Figure 15.3.1.4 Progression of the total length of larvae reared in different tanks (Mean and SD).

Sub-task 15.3.2 Effect of light (intensity and duration) on larval rearing (by HCMR, Nikos Papandroulakis).

Two light intensity ranges (200-600 and 800-1200) and 2 photophases (18L: 06D and 24L: 00D) were tested. Tanks of 500 l were used in triplicate trials each having a duration of 30 days. The effect of light was evaluated in terms of larval growth, survival, quality and size dispersion. In addition, the somatotropic axis consisting of GHRH, GH, GHR, IGF-I and II and associated IGFBPs was investigated together with the receptors that represent the endocrine and autocrine regulators for skeletal muscle growth and are known to play key roles in the regulation of metabolism and physiological processes. During the 1st reporting period preliminary trials were performed in order to establish the larval rearing methodologies in the two rearing systems. During the present reporting period the actual trial was performed. The implementation of this work was achieved with the participation of the following group of P1. HCMR personnel: N. Papandroulakis, A. Tsalafouta, N. Mitrizakis, S. Stefanakis, P. Anastasiadis, M. Vassilakis, Y. Strakantounas E. Sfakaki, N. Kopidakis.

Eggs from induced spawning (WP 3) of breeders kept in the P23. ARGO cage farm were used for the rearing. After collection, eggs were transported to the hatchery facilities of P1. HCMR in polystyrene boxes in ~ 12 hours, and then were incubated.

A. Photoperiod

The duration of the photoperiod was tested during an experimental rearing in 2 triplicated systems of 2,000 l tanks. Although in the DOW it is stated that 500 l will be used, the trial was performed in 2,000 l instead, due to availability limitations and also as the general rearing conditions were kept similar to the ones proposed, *i.e.* closed water circulation and controlled environment. Two conditions were tested for 24 and 18 daylight hours d^{-1}. Tanks were covered with non-transparent plastic covers (**Figure 15.3.2 .1**) to prevent outside light from affecting the intensity and duration of lighting.

Figure 15.3.2.1 Tank configuration for controlling the photoperiod.

The methodology applied is the so-called "pseudo-green" water as explained also in task 15.2.1. The tanks used at P1. HCMR are 2,000-l cylindro-conical, organized in triplets in a closed water system with a biological filter (**Figure 15.3.2.2)**. The tanks are filled with borehole water at 35 psu. Temperature was kept at 22±0.5°C during the autortrophic stage and was gradually increased to 24±0.5°C after mouth opening. The pH fluctuated from 8.3 to 8.5 and the dissolved oxygen from 5.6 to 7.4 mg l^{-1}. Water circulation was achieved in two ways according to the stage of rearing. During embryogenesis, egg hatching and the autotrophic larval stage, water circulated in the tanks through a biological filter. Aeration was also provided in the tanks (150–250 ml min^{-1}). After first feeding, water circulation was autonomous for each tank by means of an airlift pump. The water in the biological filter was used for renewal in larval rearing tanks that was 3% daily until 15 dph then it was gradually increased to 50% on 25 dph. A skimmer was installed at the appropriate period (5 to 15 dph) to keep the surface free from lipids. Light intensity varied between 200 - 800 lux during the day, and was about 200 lux during the night.

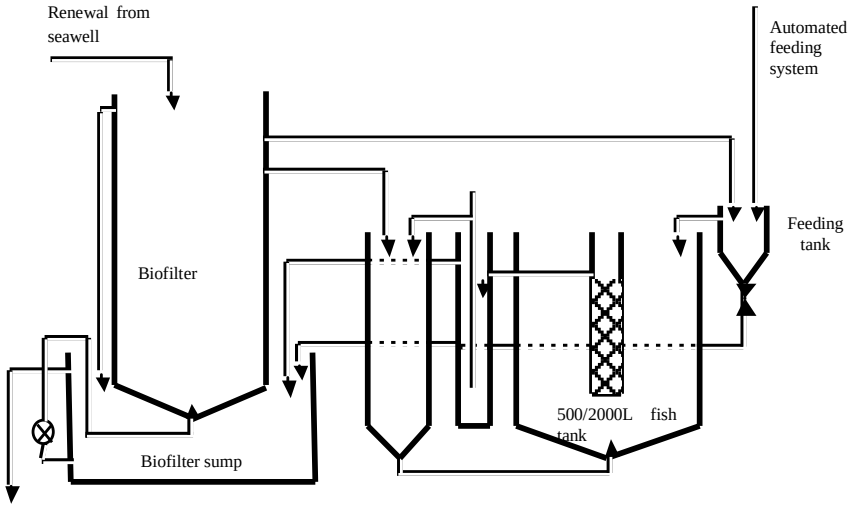


Figure 15.3.2.2. Larval rearing tanks in closed water system at P1. HCMR

Feeding was based on daily administration of enriched rotifers (from 3-21 dph), Instar II *Artemia* nauplii (from 12 dph onwards), and artificial diet (from 21 dph). The concentration of rotifers in the tank was maintained at 3.0 ind ml^{-1}, while of *Artemia* at 0.1 ind ml^{-1} after measurements of the concentration twice daily. Phytoplankton was added daily from 3 to 22 dph at 300 ± 100 x 10^3 cells ml^{-1}. The administration of the zooplankton was implemented with the use of an automated feeding system allowing the administration of food several times daily and also during the night. 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 (~27dph) populations were counted and transferred for pre-growing. Furthermore, samples were collected for investigating the larval somatotropic axis.. For primer design and qPCR experiments, the detailed procedures are similar t the ones presented earlier in subtask 15.2.1 description. For this particular trial, during the 2015 season 5 pooled samples were taken from fish reared under the 18L:06D and the 24L: 00D photoperiod scheme at 0, 2, 5, 10, 15, 20, 25 and 30.

Results

Rearing was performed as planned and all required samples were collected. Although no pathologies were presented, the overall survival in all cases was low and at the end of the trial only few individuals survived per tank. The results of the growth performance are shown in **Table 15.3.2.1**. and **Figure 15.3.2.3.**

Total Length	**2015**
18L:06D	0.0244 d^{-1}
24L:00D	0.0364 d^{-1}
Wet weight	
18L:06D	0.16 d^{-1}
24L:00D	0.21 d^{-1}

Table 15.3.2.1 Growth rate (exponential) of greater amberjack larvae in terms of total length and wet weight during the rearing.

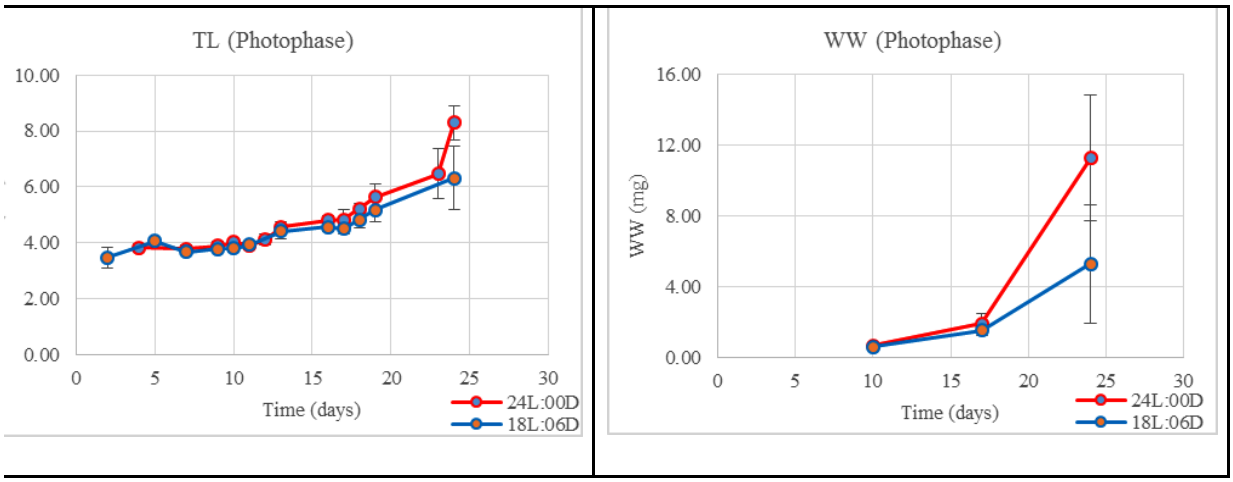


Figure 15.3.2.3: Progression of the total length and wet weight of g amberjack larvae reared in different photophases (Mean and SD).

The photoperiod (24L:00D vs 18L:6D) did not appear to affect the mRNA expression levels of IGF-I. However, there was a gradual increase in mRNA levels as development proceeded with significant differences observed ($P < 0.05$) at 20 dph and peak levels at 30 dph (**Figure 15.3.2.4**). The mRNA expression levels of IGF-II were not altered by the photoperiod and appeared high at 0 dph, dropped at 2 dph, peaked at 5 dph ($P < 0.05$) and remained stable thereafter **(Figure 15.3.2.5**). The expression of GH was not affected by the rearing method used and mRNA levels appeared to be generally stable throughout development, apart from 0 dph where lower levels were observed (**Figure 15.3.2.6**). The expression of GHRH was not affected by the rearing method used and remained generally at lower levels until 20 dph, where a significant increase was observed to reach peak values at 30 dph ($P < 0.05$; **Figure 15.3.2.7).**

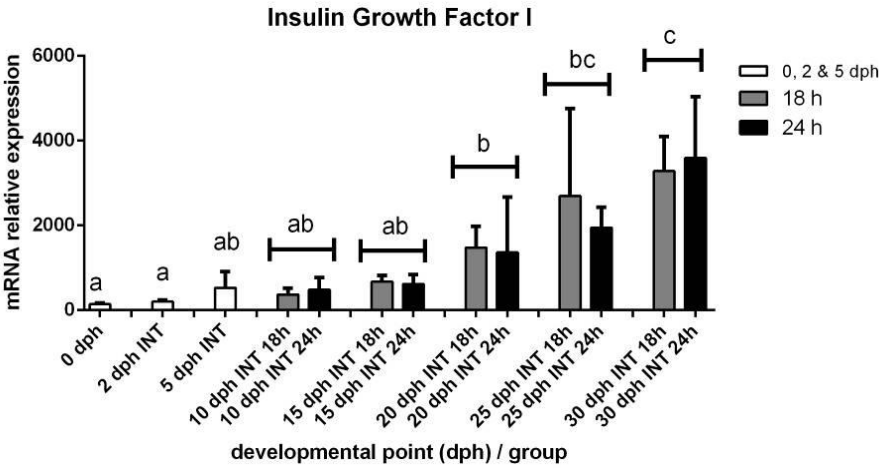


Figure 15.3.2.4. The mRNA relative expression levels of IGF-I between the different photoperiod schemes during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

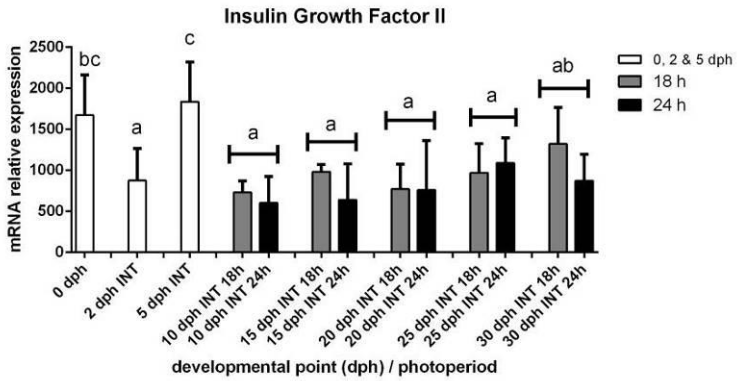


Figure 15.3.2.5. The mRNA relative expression levels of IGF-II between the different photoperiod schemes during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

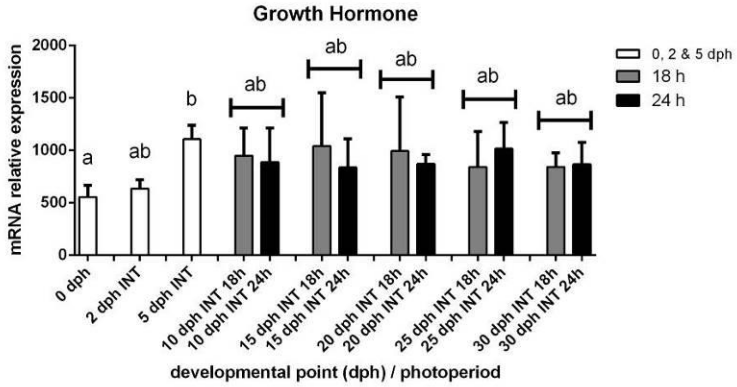


Figure 15.3.2.6. The mRNA relative expression levels of GH between the different photoperiod schemes during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

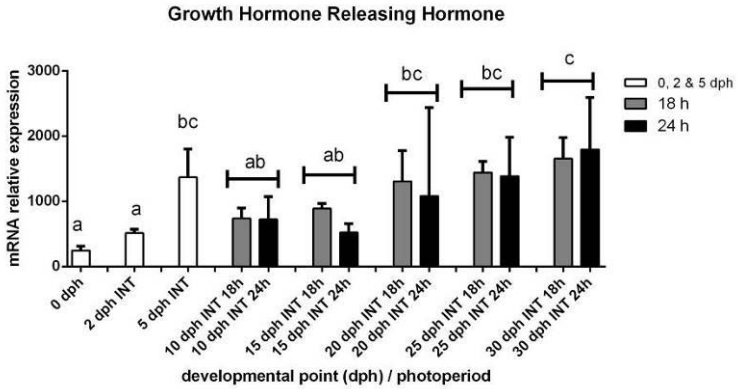


Figure 15.3.2.7. The mRNA relative expression levels of GHRH between the different photoperiod schemes during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

The photoperiod scheme applied did not affect the mRNA expression of any of the IGF binding proteins studied apart from the expression of IGF-BP1 where higher levels ($P < 0.05$) were observed in fish reared under the condition of 24L:00D compared to the fish reared under the condition of 18D:6D at 25 dph and 30 dph (**Figure 15.3.2.8a**). Additionally, throughout development the mRNA expression of IGF-BP1 was not consistent between the different groups but at 0 dph and 2 dph the minimum levels observed reached a peak at 5 dph ($P < 0.05$; **Figure 15.3.2.8a**) and remained stable thereafter with the exception of 30 dph where differences in fish from the two different photoperiod conditions (mentioned above) where observed. The mRNA expression levels of IGF-BP2 appeared low at the beginning of development until 20 dph were there was a statistically significant upregulation ($P < 0.05$), which continued to gradually increase until 30 dph (**Figure 15.3.2.8b**). No differences were observed in the case of IGF-BP3 (**Figure 15.3.2.8c**), however in the case of IGF-BP5 the mRNA expression levels showed a gradual increase from 0 dph until 5 dph ($P < 0.05$) but its levels appeared stable thereafter (**Figure 15.3.2.8d**).

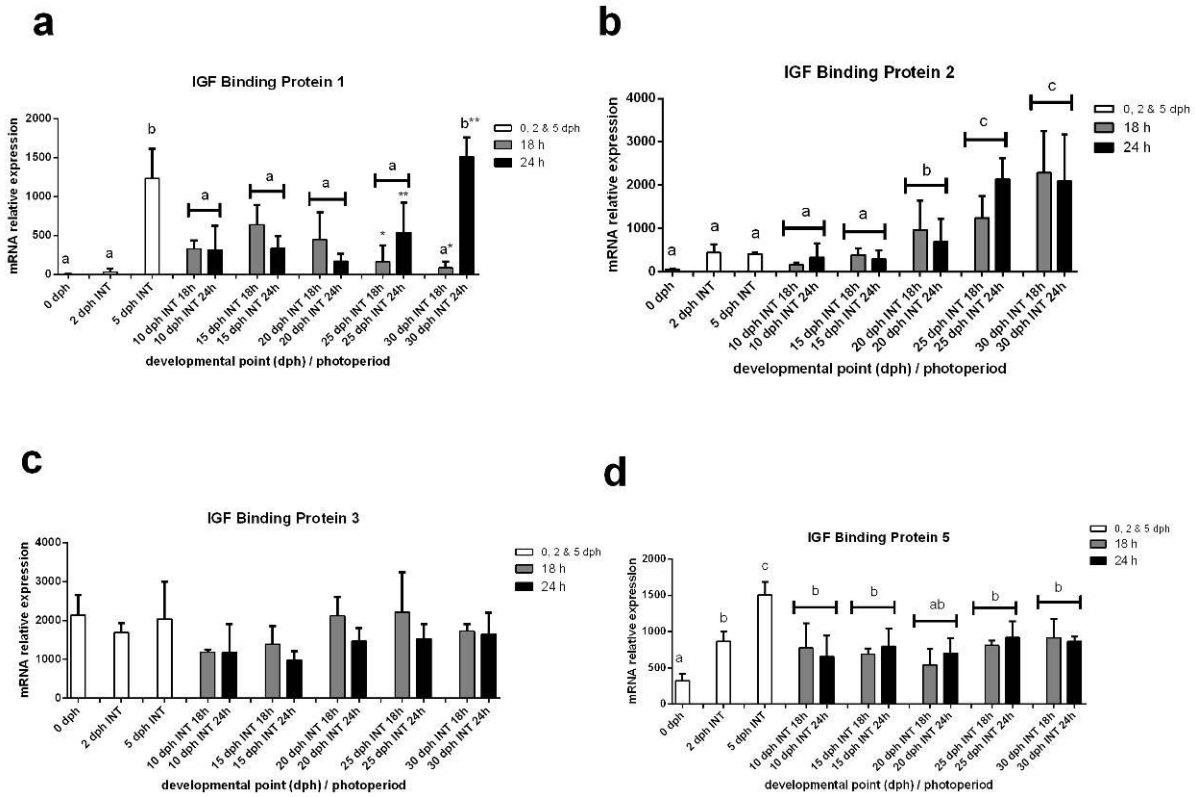


Figure 15.3.2.8. The mRNA relative expression levels of IGF-I binding proteins between the different photoperiod schemes during early ontogeny of greater amberjack: (a) IGF-BP1; (b) IGF-BP2; (c) IGF-BP3; (d) IGF-BP5. Values are means ± standard deviation (n = 5). Different letters indicate statistically significant differences between the sampling points during ontogeny whereas asterisks indicate differences between the different photoperiod schemes ($P< 0.05$).

B. Light intensity

The objective of the trial was to define the effect of light intensity on larval rearing. In the DOW it is stated that two light intensity ranges (200-600 and 800-1200) will be tested. Preliminary trials showed that low light intensities (below 500 lx) resulted in very poor performance of the larvae. Therefore, instead of testing the light intensity at the surface of the tanks, the background color of the tank was tested.

The trial was performed following the intensive methodology described earlier for task 15.2.1. Tanks with three different colors (black, green and white) were used for larval rearing, in duplicates. For this, white or green fabric that completely covered the inner walls was used, while black tanks served as controls. Furthermore, underwater lights were additionally used to improve light intensity in the water column without significantly changing the intensity on tank's surface. Underwater lighting was applied from 8:00 to 20:00 imitating the increased brightness during summer months. In **Figure 15.3.2.9** the tanks with different backgrounds are shown.

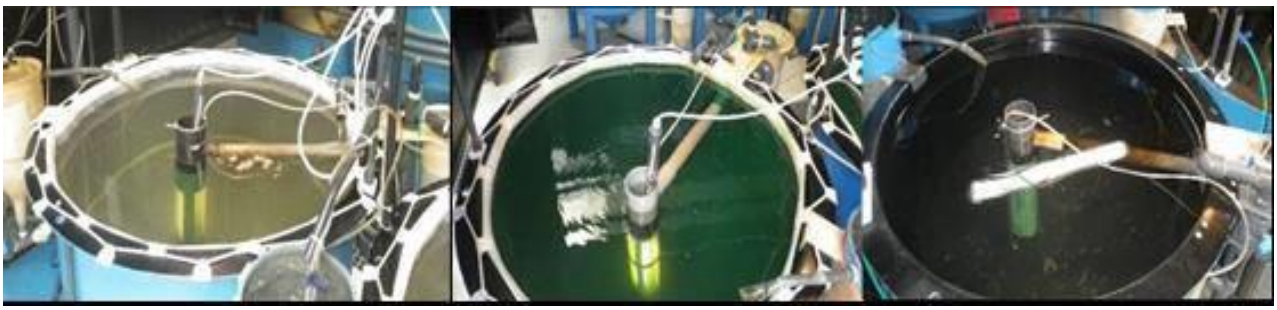


Figure 15.3.2.9. Tanks with different colour backgrounds.

Samples were collected regularly to measure the total length and body weight. At the end of the experiment, the survival of the larvae in each tank was estimated. Additionally, samples were collected at specific points of embryonic and larvae development (at 0, 2, 5, 10, 17, 20, 25 and 30 dph). These samples were for mRNA expression studies of genes related to the GH/ IGF, as described above

Results

No statistically significant differences were observed in the growth of the larvae in terms of total length and body weight between the different tank colours **(Figure 15.3.2.10)**. However, significant differences were observed at the end of the experimental period in the survival rates among the different groups with the larvae in the white tanks exhibiting the highest survival rate (1.3%) compared to the black (0.5%) and the green tanks (0.02%).

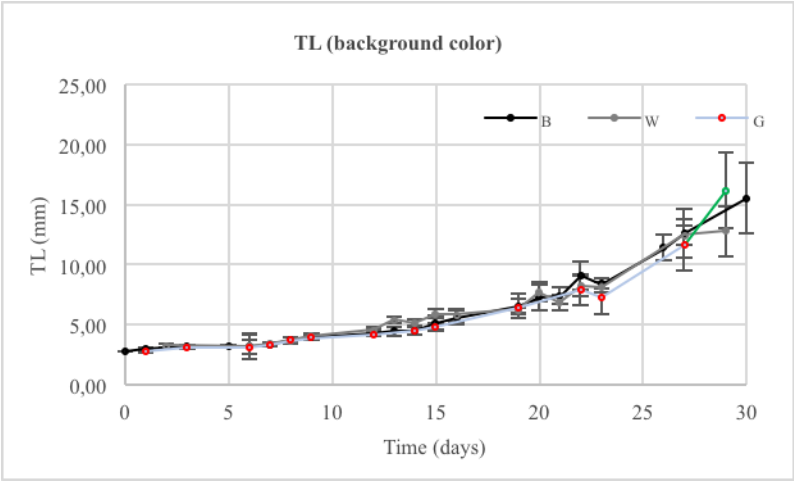


Figure 15.3.2.10: Progression of the total length of greater amberjack larvae reared in tanks with different background colour (Mean and SD).

Gene expression analysis revealed significant differences ($P<0.05$) among the groups as well as between the different developmental sampling points. The background colour appeared to affect the mRNA expression levels of IGF-I at 30 dph in all the three different background colours, with the fish reared in the white background showing higher levels of expression and the fish in the green background the lowest ($P <$

0.05; **Figure 15.3.2.11**). Additionally, during development the expression pattern observed for IGF-I was not consistent between the different groups. From 0 dph until 17 dph the levels remained stable and low from all groups but at 25 dph and 30 dph only in fish reared in the white and black background showed a significant increase ($P < 0.05$), whereas IGF-I levels in fish reared in the green background remained stable as before (**Figure 15.3.2.11**). The mRNA expression levels of IGF-II were not altered based on background colour but throughout development IGF-II expression was not consistent between the different groups as depicted in **Figure 15. 3.2.12**. The background colour appeared to have an effect at the mRNA expression levels of GH at 17 dph with fish reared in the white background exhibiting the highest levels of expression and fish reared in the green background the lowest ($P < 0.05$). However, throughout development GH expression was not consistent between the different groups as depicted in **Figure 15.3.2.13**. The expression of GHRH showed no statistically significant differences either depending on the background colour or as development progressed (**Figure 15. 3.2.14).**

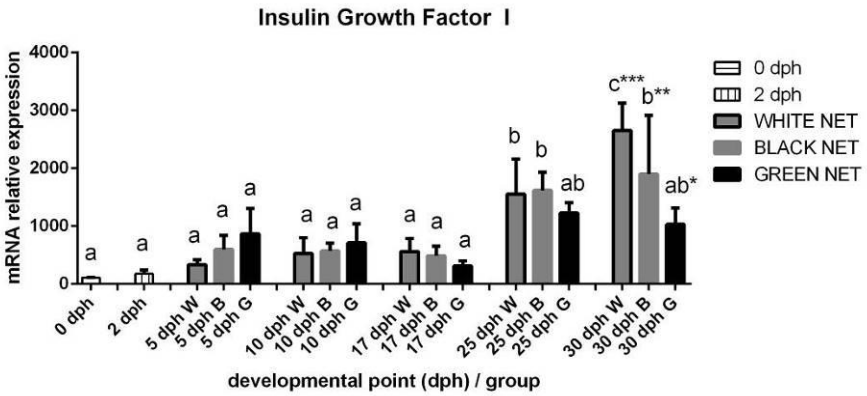


Figure 15.3.2.11. The mRNA relative expression levels of IGF-I between the different background colours during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Different letters indicate statistically significant differences between the sampling points during ontogeny whereas asterisks indicate differences between the different background colours ($P < 0.05$).

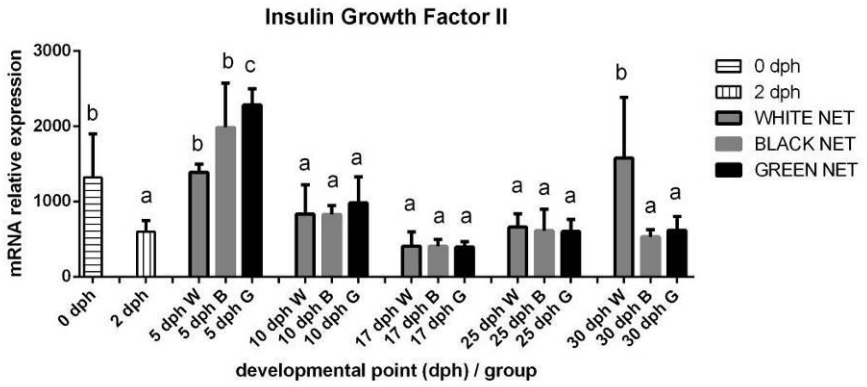


Figure 15.3.2.12. The mRNA relative expression levels of IGF-II between the different background colours during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Means with different letters differ significantly from one another ($P < 0.05$).

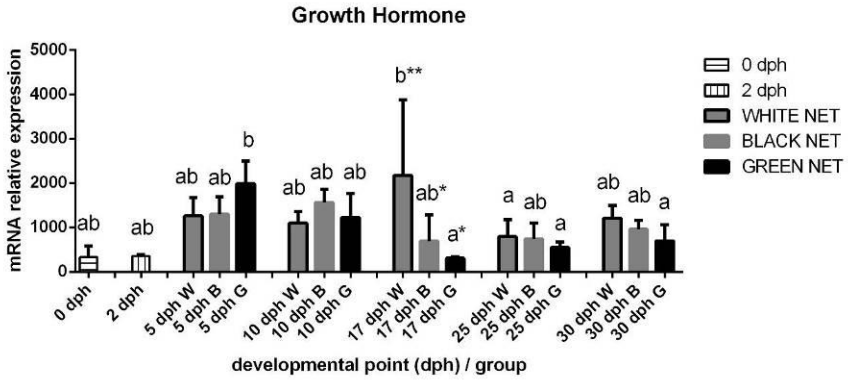


Figure 15.3.2.13. The mRNA relative expression levels of GH between the different background colours during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). Different letters indicate statistically significant differences between the sampling points during ontogeny whereas asterisks indicate differences between the different background colours ($P < 0.05$).

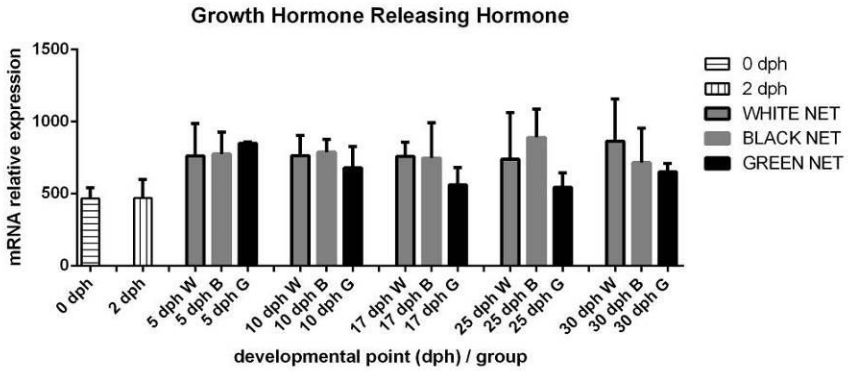


Figure 15.3.2.14. The mRNA relative expression levels of IGF-I between the different background colours during early ontogeny of greater amberjack. Values are means ± standard deviation (n = 5). No significant (P>0.05) differences were observed.

The different background colours used appeared to have an effect in the mRNA expression of IGF-BP1 in fish from all the different background colours at 5 dph, with fish reared in white background showing the lowest expression and fish reared in the green background showing the highest expression levels (**Figure 15.3.2.15a**). Additionally, throughout development the mRNA expression of IGF-BP1 appeared very low at 0 dph and 2 dph, while it demonstrated peak values at 5 dph for all groups, and then dropped and remained stable thereafter ($P < 0.05$; **Figure 15.3.2.15a**). The mRNA expression levels of IGF-BP2 appeared stable at the beginning of development until 20 dph where there was a significant up regulation ($P < 0.05$) for fish from all groups that was also observed at 30 dph. Additionally, at 30 dph the mRNA expression of IGF-BP2 appeared to be affected by the background colour in all groups ($P < 0.05$), with fish reared in the white background exhibiting the highest expression levels, fish reared in the black background exhibiting medium levels and fish in the green background the lowest (**Figure 15.3.2.15b**). In the case of IGF-BP3, expression levels appeared to gradually increase until 10 dph where peak values were observed ($P < 0.05$) that dropped back to the initial values as development proceeded (**Figure 15.3.2.15c**). At 30 dph an effect of the

background colour on the expression levels of IGF-BP3 was also observed, with an up regulation ($P < 0.05$) observed in fish reared in the white background compared to fish reared in the green background (**Figure 15.3.2.15c**). Finally, in the case of IGF-BP5 the mRNA expression levels remained generally stable from 0 dph until 25 dph where a significant ($P < 0.05$) up regulation was observed that continued until 30 dph. The background colour appeared to also have an effect on the expression of IGF-BP5 but only at 30 dph, where fish reared in the white background exhibiting higher mRNA expression levels ($P < 0.05$) compared to the other two groups (**Figure 15.3.2.15d**).

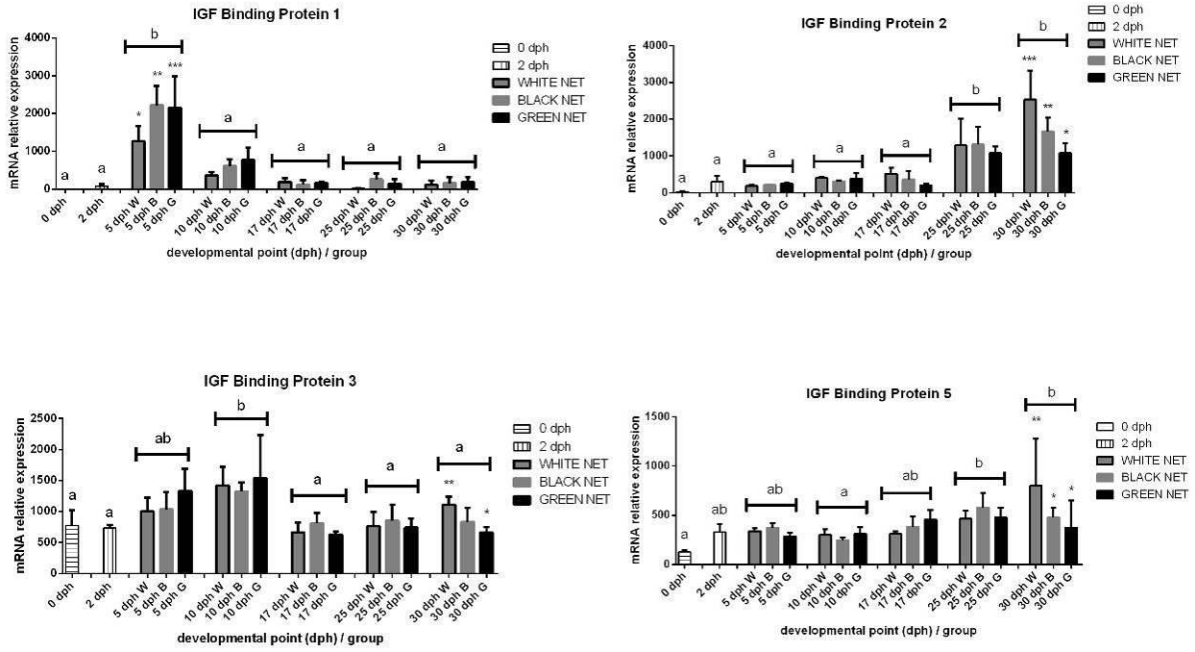


Figure 15.3.2.15. The mRNA relative expression levels of IGF-I binding proteins between the different background colours during early ontogeny of greater amberjack: (a) IGF-BP1; (b) IGF-BP2; (c) IGF-BP3; (d) IGF-BP5. Values are means ± standard deviation (n = 5). Different letters indicate statistically significant differences between the sampling points during ontogeny whereas asterisks indicate differences between the different background colours ($P < 0.05$).

This preliminary study provides for the first time information on the regulation of the various components of the IGF signaling pathway in greater amberjack and may serve for the better understanding of this complex system and its effects on fish performance at early ontogeny.

The 2016 Trials (Mo 31-32)

Although the experiments related to the definition of optimum light conditions were concluded in the 2015 trials, it was decided to repeat the trials in 2016 due to the low survival achieved and the poor, in general, performance of the reared individuals. In June 2016 eggs were obtained from the P1. HCMR experiments (Task 3.5 egg collection from sea cages) and P23. ARGO (Task 3.2 Induced spawning of Mediterranean greater amberjack) and were transferred to the hatchery of P1. HCMR. The photoperiod and the light intensity were tested in a similar experimental set-up as described earlier.

The results are still to be analysed, but some preliminary data are available related to the survival rate at the 25dph, presented in **Table 15.3.2.1**.

Table 15.3.2.1 Survival rate greater amberjack larvae at 25dph

Experimental Condition	24L:00D		18L:06D		White Background		Green Background	
initial number of eggs	44.800	42.000	42.000	43.400	48.048	49.452	51.870	51.870
final number of individuals	5.692	3.313	4.659	2.533	10.423	11.226	8.212	8.849
survival rate	13%	8%	11%	6%	**22%**	**23%**	**16%**	**17%**

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. In particular, the results from the trials with the modified "light environment" of tanks are better at one order of magnitude of previous reported showing the validity of the tested hypothesis.

The individuals resulted from the trials are currently weaned to artificial diets (**Figure 15.3.2.16**) and are expected to be used for the on-growing trials to be carried out at P1 HCMR and the SME Partners P.2723, as described in the DOW. The complete analysis of the results from the 2016 trials will be presented in the next report.

Figure 15.3.2.16. Greater amberjack juveniles during weaning.

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).

During this period, a preliminary assay of semi-intensive mesocosm larval rearing was performed in preparation for experiments in the following years. Eggs from induced F1 greater amberjack broodstock (GnRHa implant) kept in the P8. IEO-COC facilities were used for the larval culture. After collection, eggs were incubated in 90 l tanks. Newly hatched larvae were stocked in a 32 l indoor tank previously filled with filtered (10 µm) seawater at an initial density of 3 larvae l^{-1} **(Figure 15.4.1.1).**

Figure 15.4.1.1. Mesocosm tank at P8. IEO facilities**.**

The rate of water renewal was increased progressively during the rearing and the internal filter screen of 363 µm mesh size was changed to 500 µm mesh size at 25 dph. Aeration was provided in the tanks by means of five pipes distributed in the perimeter and the centre of the tank. A surface skimmer was operational during the rotifers feeding period to keep the surface free from lipids to improve the swim bladder inflation. Larval rearing was developed under natural conditions of temperature, salinity and photoperiod. Temperature was 22.8 ± 0.5 °C, and Oxygen concentration (%) fluctuated from 96.3 to 92.7 % **(Figure 15.4.1.2).** Natural light intensity in water surface ranged between 500 and 700 lux and no artificial light was used.

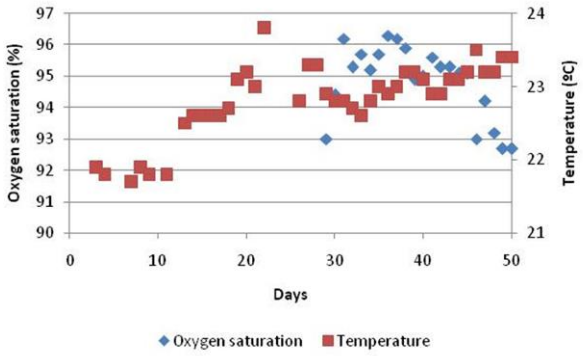


Figure 15.4.1.2. Temperature and oxygen concentration during Mesocosm rearing.

Phytoplankton (*Chlorella* sp) was added daily from start until 25 dph (~15000 cell ml^{-1}). Feeding was based on daily administration of rotifers (*Brachionus plicatilis*) distributed two times a day (8.00 and 16.00 h), and enriched with commercial products, from 3 to 25 dph. *Artemia* nauplii were administered (two times a day) from 12 to 30 dph, and artificial diet was initiated at 25 dph distributed each two hours from 8.00 to 20.00. The concentration of rotifers in the tank was maintained at 3 rotifers ml^{-1}, while *Artemia* concentration was 0.1 nauplii ml^{-1}. The total length of larvae at 50 days of rearing reached 26.5 ± 7.2 mm (**Figure 15.4.1.3**).

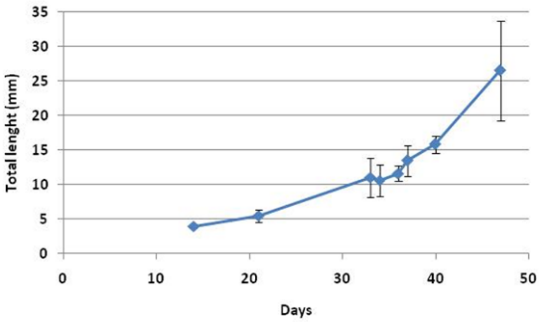


Figure 15.4.1.3. Progression of total length during Mesocosm rearing (± SEM).

The results obtained in Tasks 15.1, 15.2 and 15.3 will contribute to determine the effective stocking densities, prey density and environmental conditions. In addition Task 9.1 will contribute to obtain optimum levels and ratios of essential fatty acids and carotenoids in greater amberjack enrichment products. Based on this, an improved protocol will be tested in subsequent years.

Sub-task 15.4.2 Ossification pattern and incidence of skeletal deformities for amberjack larvae (by FCPCT, Carmen Maria Hernández Cruz)

Ossification pattern and incidence of skeletal deformities for amberjack larvae was evaluated under different levels of intensification. Samples of amberjack larvae from hatching to end of metamorphosis were collected at regular intervals, to evaluate ossification pattern. The ossification and deformity results are expressed as a function of size and not age as abnormal skeletal development may vary with the growth rate of individuals. Samples of larvae from hatching to end of metamorphosis were collected to evaluate ossification pattern. Staining protocols to evaluate these samples was determined in Sub-task 15.2.2. To study the bone ossification, all specimens were fixed in 10% buffered formalin, from hatching to 30 dph. Fixed larvae were cleared and stained with alizarin red and individually examined using stereomicroscopy (**Figure 15.4.2.1**). The study was underway when the report was prepared and no data has been analysed and evaluated available yet.

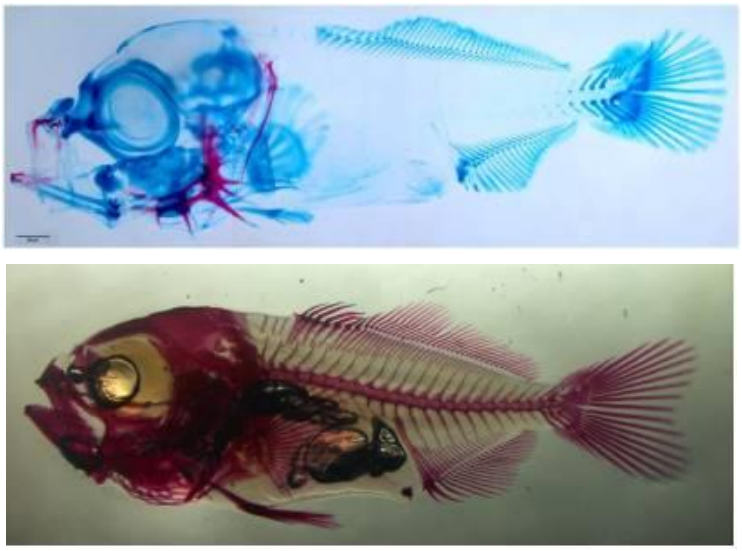


Figure 15.4.2.1. Larvae greater amberjack greater amberjack at 10 dph (upper) and 30 dph (lower).

Sub-task 15.4.3 Validation of the developed protocol initially at FCPTC and over two successive years in an SME hatchery (**by FORKYS)**

No work was done during this period

Deviations from Annex I and their impact:

In Task 15.1. a repetition is scheduled for later in 2016 and, therefore, the submission of Deliverable 15.2 is delayed and this was requested earlier this year and was approved by the PC. In Task 15.3. and subtask 15.3.1 a trial will be repeated in 2016 due to total larval mortality in the previous attempt. The submission of Deliverable 15.3 is also delayed as requested earlier this year and approved by the PC.

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. ASIALOR	
Lead Scientist preparing the Report (WP leader):	Pascal Fontaine			
Other Scientists participating:	Enric Gisbert (P3), Ivar Lund (P21), Tu-Linh Ly (P29)			

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 effectiveness of rearing larvae in a factorial design (4 factors tested in the 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 stable. However, due to an unstable RAS, all larvae died and this first experiment was postponed to January-March 2015.

Summary of progress towards objectives and details for each task (11-30 Mo):

1 – The first experiment (exp. 1), initially planned in 2014 was repeated in January-March 2015. Within this experiment, the effects of four environmental factors (light intensity, water renewal rate, water flow direction, tank cleaning time) on the effectiveness of rearing of pikeperch larvae were determined. Results have been analysed and included in Deliverable 6.1, which at the time this report was prepared was under final revision by the PC.

2 – A second experiment (exp. 2), aiming at the determination of the effect of four feeding-related factors on the effectiveness of pikeperch larviculture was performed between February and March 2016. Within the study four factors (feeding frequency, co-feeding or not, weaning timing, weaning duration) were tested. Results will be analysed by the end of 2016.

Task 16.1 Optimal combinations of factors to improve larval rearing (led by UL, Pascal Fontaine).

Introduction

Pikeperch has gained attention as a potential and promising new species in intensive fish farming and has great economic potential. Until now several bottlenecks prevented the success of the rearing of the larvae.

Three major bottlenecks have been identified: a high rate of mortality (mainly due to cannibalism), a high rate of deformities and a large growth heterogeneity. In order to determine optimal combination of factors allowing significant improvement of pikeperch larviculture, four experiments have been initially planned using original multifactorial approach. We used an indoor recirculated aquaculture system (RAS) which consisted of 10 tanks of 700 L each and a common water treatment system based on a mechanical filter, a biological filter and a UV sterilization.

The effects of environmental and nutritional factors have been studied in 2015 and 2016, respectively. For each experiment, 500,000 newly hatched larvae (<1 dph) were obtained from P. 29 ASIALOR and transferred to the experimental platform of P9. UL. Each time, larvae were distributed in 8 tanks (2^3) with water temperature set to 15-16°C.

In the first experiment, the effects of four environmental factors (light intensity, water renewal rate, current direction and time of cleaning) (**Table 1**) on growth, survival and development of pikeperch larvae were investigated. Next, a behavioural experiment was conducted in order to evaluate whether the individual behavioural or personality traits could be linked to cannibalism.

In the second experiment the effects of four feeding-related factors (frequency of food distribution, co-feeding, beginning of weaning, duration of weaning) (**Table 2**) were investigated.

Table 1. Applied modalities for each factor for the four environmental factors (experiment 1).

Factors	Modality 1	Modality 2
Light intensity (lx)	5	50
Water renewal rate (%)	50	100
Water current direction	At the water surface	At the bottom of the tank
Timing of tank cleaning	Morning	Afternoon

Table 2. Modalities applied in 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

Experiment 1: effects of environmental factors

The experiment lasted 35 days (from 30th January until 6th March 2015). Larvae were sampled every 7 days and at each sampling time (T0, on first day of feeding, T7, T14, T21, T28 and T35 on 35 day of rearing) the following analyses were performed:

(1) Morphological data included total length TL, body weight W, coefficient of variation of total length CV TL, coefficient of variation of weight CV W, skeletal and jaw deformities occurrence): 60 larvae per tank per sampling time;

(2) Histological data included intestinal, retinal and jaw development: 40 larvae per day per sampling time;
(3) Behavioral observations during 5 min day^{-1} of each tank in order to detect cannibalism.

Additionally, a complementary behavioural experiment was performed in March 2015, which aimed at evaluating a possible linkage between personality traits and cannibalism. For this experiment fish from the multifactorial experiment were used, and were tested between 50 and 64 dph (after the main rearing trial was finished). Overall, 42 pikeperch juveniles (5.78 ± 1.01 cm in TL) were used in two separate tests on the same day: cross-maze test and dyadic test.

Morphological analyses

In this experiment, it was demonstrated that weaned juveniles with 0.5-0.6 g mean body weight can be produced within 5 weeks. However, in such a case very low survival rate (0.3-2.6%) was recorded. Although survival rates were very low, results from this first experiment constitutes a first data base (growth, survival) of pikeperch larval rearing done under semi-commercial hatchery conditions, *i.e.* with large tank volume (700 l) and RAS conditions over a long period (5 weeks) and from newly hatched larvae. The fact that we used newly hatched larvae, and not, as in other studies, several days old larvae, might have contributed to increased mortality rates observed.

Considering the effect of the environmental factors tested, light intensity appeared to be the most important among the four factors evaluated. Compared to 5 lx, a light intensity of 50 lx resulted in larvae with higher TL and W. The negative impact of low light intensity was found to be dependent on the modality of the other factors. In particular, larval size was lower at 5 lux when the water renewal rate was also low (50%) or when the tanks were cleaned early morning. A higher light intensity allowed a better expression of the growth potential of pikeperch larvae, which could be linked with the carnivorous and predatory behaviour of this species at this developmental stage and thus highlights the importance of proper light conditions allowing effective predation in the water column. Consequently, under low light intensity, pikeperch larvae were more homogeneous in TL and W, as observed at T14. At T35, an inverse effect was observed where larvae demonstrated lower size heterogeneity under a higher light intensity. However, at that time, cannibalism also contributed to the elimination of the smaller fish from the population. Our results show also that it is better to clean the tank during the afternoon rather than in the morning hours. Considering all these results, we recommend a light intensity of 50 lux, water renewal rate of 100%, cleaning of the tank during the afternoon and an inlet of the water at the bottom level.

Histological analysis

The timing of organ development and its associated physiological functions are affected by the general life history and reproductive status of each species, as well as by a variety of abiotic and biotic factors. Among them, temperature, water quality, food availability and composition have been generally considered as some of the most important. In this sense, changes in the histological organization of the liver, the exocrine pancreas, the intestine, the visual organ and muscular fibers have been used on a regular basis as histological markers to analyze the nutritional condition of fish larvae and elucidate the effects of different dietary regimes or rearing conditions on larval physiology, nutrition and early development. These tissues and organs are especially sensitive to non-optimal feeding and rearing conditions during larval development, because they are under progressive and intensive morphogenesis, and consequently, they respond rapidly and sensitively to nutritional disorders and environmental stressors. Under present experimental conditions, any of the environmental factors tested by means of this multifactorial trial had any remarkable effect on the histological organization and morphogenesis of the different examined organs (digestive tract, accessory digestive glands). The absence of these differences were not only observed in samples from T35, but they were also noted at earlier ages of development (T7, T14, T21 and T28; data not shown). The lack of remarkable differences in terms of the histological organization of selected organs and tissues may be linked to the fact that organogenesis is not a synchronous process and the development of an organ takes place

within a range of sizes. Therefore, although significant differences were found in larval size at different sampling ages (*i.e.* T7, T35) depending on rearing and husbandry conditions, difference in body size among groups were not sufficiently larger to have resulted in a differential morphogenesis rate among them. Finally, it should be noted that regardless of the differences in growth, none of the tested rearing conditions affected negatively the formation and histological organization of the examined tissues in pikeperch during larval development, nor their nutritional condition assessed by the level of fat accumulation in target tissues like the intestine and liver.

Behavioural experiment

One of the most important results of the present study is that different behavioural traits were observed in very young pikeperch juveniles using maze and dyadic tests. This first experiment allowed us to know that it is possible to determine the personality of pikeperch juveniles and highlight a possible linkage between fish personality and cannibalism.

The full description of the work and results is provided in ***Deliverable 16.1 Determine effect of environmental factors on pikeperch larval rearing***.

Experiment 2: effects of feeding-related factors

The feeding-related factors were tested from 1st February until 24th March 2016. Larvae were sampled every 7 days: T0 (at first day of feeding), T7, T14, T21, T28, T35, T42 and T49 where several analytical methods were used.

1. Morphometric analysis (total length TL, body weight W, coefficient of variation of total length CV TL and coefficient of variation of weight CV W): 30 larvae per tank per sampling date;
2. Histological analysis: intestinal, retinal and jaw development of 15 larvae per day per sampling date;
3. Observations during 5 min day^{-1} of each tank in order to detect cannibalism.

Also, in March 2016 a complementary experiment to evaluate whether personality traits could be linked to cannibalism was performed. The results of this experiment will be analysed over the second semester of 2016, and will allow the identification of a significant effect of the tested variables on the rearing effectiveness. Two combinations of factors applied in tanks 3 and 9 seems to be particularly interesting because they were associated with the higher growth rates (**Figure 16.1.2.**), survival rates (9-11%) and swim bladder inflation rates (70-100%).

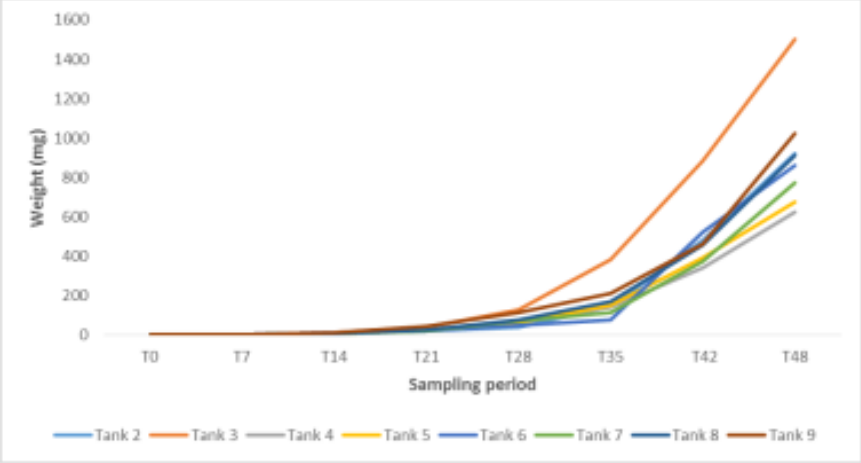


Figure 16.1.2. Growth curves of pikeperch larvae in the eight experimental tanks.

Deviations from Annex I and their impact:

The work for ***Deliverable 16.1 Determine effect of environmental factors on pikeperch larval rearing*** initially planned for the 1st Reporting Period, was delayed until month 30, because the experiment was repeated in 2015 due to high and abnormal mortality during the first trial done in 2014. As a result, the Deliverable was in its final revision by the PC at the time this report was prepared, and will be submitted promptly. The work for Deliverable D16.2 initially planned on month 24 was postponed to month 30 because the experiment was delayed in early 2016, as a consequence of the delay of the work for Deliverable 16.1. We do not expect any impact on the project from these delays.

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), 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 differerent *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 progress towards objectives and details for each task (13-30 Mo):

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).

First feeding stage

Introduction

The commercial production of halibut fry is currently carried out in flow through systems (FT), while there is a growing consensus that a RAS would offer more stable environmental and chemical water parameters that would lead to improved larval performance. At the P7. IMR facilities, it is standard practice to treat the larvae with antibiotics the first three days of the start feeding period, in case of dropping appetite during this period. To avoid use of antibiotics and also to decrease mortality, the use of a RAS has been tested.

It is not clear whether the intestinal microflora of Atlantic halibut larvae is determined by the feed or by water quality parameters. In order to elucidate this, samples were taken for examination of gut morphology (P17. NIFES) and bacterial flora (P7. IMR). Retention of nutrients (P17. NIFES), and digestive physiology (P5. ULL) is performed in Task 11.3 of WP11 Nutrition – Atlantic halibut. The results will give information on at what stage, and how the intestine is colonized, and will form the basis for the implementation of probiotics in the industrial protocol to be developed in Task 17.2.

Materials and methods:

Atlantic halibut larvae, at an age of 265 degree-days, were transferred from a yolk sac incubator to 6 first feeding tanks. Numbers of larvae were approximately 5,000 in each tank. Three of the tanks were connected to a RAS system (Tropical Marine Center, **Fig 17.1.1**). The three other tanks were supplied with a standard flow through water system with water coming from 160 m depth. The tanks had a volume of 1,400 liters and the water flow was 5 L $\cdot$ min^{-1}. Water temperature was 12 ± 0.3°C during the whole period.

Figure 17.1.1. RAS system and first feeding tanks.

The larvae were fed enriched *Artemia* nauplii three times a day and the amount of remaining *Artemia* in the tanks was estimated before each feeding to give an estimate of feeding activity in each tank. Samples of water and larvae were taken weekly for analysis of nitrogen compounds (NH_3 and NO) and larvae growth. In order to achieve feeding behavior and feed ingestion it is necessary to have turbid water. This is commonly achieved by the addition of microalgae to the water. At P7. IMR we have substituted the algae with dissolved clay, which was added to the first feeding tanks morning and evening.

Results

The experiment is still ongoing when this report was prepared and the final results will be reported in the next periodic report. So far, only small, if any, differences in larval mortality have been observed between the groups. However, the larvae in the RAS tanks are at present smaller than in the FT tanks. The clay particles are not fully removed by the RAS system, and only 10% of the amount used in the FT tanks is added to the RAS tanks to achieve the same turbidity.

Task 17.2 The effect of probiotics on larval microbiota and survival and development of an industrial protocol (led by IMR, Øivind Bergh).

Introduction

Due to rising market demands for high-value fish species and depletion of wild stocks, marine aquaculture is a growing industry. Like in any other food producing industry, dense monocultures are the predominant form of production due to economic considerations. As stocking densities are increased and culture is intensified, diseases can spread more easily and rapidly. Diseases caused by viruses, parasites, and pathogenic bacteria lead to welfare problems, reduced product quality, inefficient production, or complete loss of stocks. Infections with opportunistic pathogenic bacteria belonging to the *Vibrionaceae* family, which thrive well in the elevated nutrient concentrations in dense cultures, are accepted to be the most abundant and deleterious among the bacterial fish diseases. The most prominent fish pathogen among the *Vibrionaceae* is *Vibrio anguillarum*, which causes rapidly progressing infections in fish, crustaceans and bivalves that result in high mortalities and substantial economic losses. There are currently 23 known serotypes of *V. anguillarum*, however only serotypes O1, O2 and O3 seem to cause vibriosis in fish, and the majority of isolates from vibriosis outbreaks belongs to serotype O1.

The focus on challenge experiments is due to the impact of the probiotics on these pathogens. We have identified the key bacteria that should be controlled in a system for rearing of larvae. The studies on the microbiota (in progress) will reveal how these can be controlled. We are presently working with characterization of the bacterial microbiota associated with cultures of Atlantic halibut larvae (see also task 17.1). After trials with DGGE (denaturing gradient gel electrophoresis) we abandoned that method, and are now basing this work on New Generation Sequencing of bacteria in start-feeding cultures. Results from these trials will be reported later.

Materials and methods

A major challenge experiment with Atlantic halibut larvae was carried out in November-December 2014. The aim of the study was to test 35 different Vibrio spp. strains for virulence towards Atlantic halibut larvae. Some of the strains are known to be associated with bacteriophages, making phage therapy possible. Other possible strains are to be used as model strains in challenge experiments with probiotics following the model by D`Alvise et al 2012. Bacteria from frozen stock cultures (−80°C) were streaked on half-strength Marine Agar (½MA; 27.6 g Difco 212185 Marine Agar, 15 g Instant Ocean Sea Salts, 7.5 g Agar, 1 l deionized water). *V. anguillarum* was counted on Tryptone-Soy Agar (TSA; Oxoid CM0131) containing 6 mg/l chloramphenicol. The cells were harvested at 5,000 x *g*, washed twice, and used as inoculum for algae and rotifer experiments. Bacteria were diluted and washed in 80% autoclaved oxygenated seawater (ASW; 2% Sigma Sea Salts). Axenicity of algae and rotifer cultures was controlled by plating 100 µl on ½MA and incubating for 7 days at 25°C.

For the challenge trials, *V. anguillarum* HI610 was grown in tryptone-soy broth with additional 0.5% NaCl at 20°C with shaking at 60 rpm to an OD_{600} of about 0.5. The *P. gallaeciensis* strains were grown in MB without shaking at 20°C until stationary phase was reached. All strains were harvested by centrifugation (1,825 x *g*), washed twice, and resuspended in aerated autoclaved 80% seawater. The bacterial concentrations in these suspensions were determined using a counting chamber for *V. anguillarum*, and for the *P. gallaeciensis* strains by measuring OD_{600} after centrifugation and dissolving in 0.1M NaOH.

The protocol was adapted from Sandlund and Bergh 2008 and Sandlund et al. 2010. Atlantic halibut embryos were obtained from the commercial hatchery Norsk Kveite, in Askøy municipality, Western Norway. Transport of the embryos in polystyrene containers at around 6-7°C took 1.5 h by car. Upon arrival, the embryos were randomly picked and distributed to the wells of 6-well dishes (Nunc, Roskilde, Denmark) filled with 10 ml 80% autoclaved, aerated seawater, placing one embryo in each well. In each trial twelve dishes for each treatment (72 embryos) were prepared and inoculated immediately. All inocula were prepared in a volume of 100 µl, and initial bacterial concentrations were 1×10^6 cfu ml^{-1} and 1×10^4 cfu ml^{-1} for *V. anguillarum* HI610 in the two positive control groups (high and low concentration, respectively). The

plates were incubated in the dark at 7°C. The day when 50% of the larvae had hatched was defined as day 0, which was 3 days after the start of the experiment. Dead larvae were registered every second day for 30 d. A total of 35 different *Vibrio* strains were tested for pathogenicity towards the Atlantic halibut larvae. In a parallel project, the same 35 Vibrio strains were tested in controlled challenge experiments with turbot (*Scopthalmus maximus*) and cod (*Gadus morhua*) eggs and larvae in comparative experiments.

Results

The key results are presented in **Table 17.2.1**. Only five of the 35 strains gave high mortality in Atlantic halibut larvae, compared to ten in turbot and five in cod larvae. All pathogenic strains belonged to the species *V. anguillarum*. Interestingly, a large fraction of the strains did not give mortality significantly different from uninfected control larvae.

Table 17.2.1 Mortality in fish larvae after challenge with 35 different strains of *Vibrio sp*.

		Mortality in cod 10dph		Mortality in turbot 8dph		Mortality in halibut 34 dph	
Species	Strain	high dose	low dose	high dose	low dose	high dose	low dose
	neg. Control	2%	2%	35%	35%	24%	24%
V. anguillarum	DSM21597	80%	71%	100%	100%	74%	33%
	775	9%	6%	85%	42%	33%	24%
	NB10	24%	19%	79%	64%	49%	65%
	HI610	100%	97%	97%	69%	83%	66%
	90-11-287	33%	15%	90%	74%	64%	54%
	90-11-286	100%	96%	100%	100%	64%	53%
	87-9-116	23%	11%	81%	60%	58%	46%
	87-9-117	38%	16%	89%	81%	65%	58%
	91-7-154	81%	40%	99%	81%	60%	80%
	9014/8	35%	16%	90%	81%	56%	65%
	178/90	92%	58%	99%	93%	71%	88%
	601/91	80%	34%	100%	89%	56%	56%
	S2 2/9	51%	19%	99%	63%	46%	28%
	HI618	51%*	6%*	40%	42%	39%	18%
	4299	13%	7%	93%	69%	24%	38%
	VA1	21%	6%	88%	71%	58%	41%
	PF4	97%*	12%*	100%	100%	30%	37%
	PF430-3	97%*	88%*	100%	96%	40%	31%
	PF7	100%*	8%*	100%	94%	47%	32%
	VIB1	11%	19%	65%	68%	37%	47%
	VIB18	89%	23%	40%	49%	65%	71%
	VIB44	84%	19%	97%	50%	58%	55%
	VIB64	16%	13%	92%	74%	35%	47%
	VIB79	5%	12%	86%	39%	49%	48%
	VIB87	9%	6%	68%	40%	22%	19%
	VIB88	10%	7%	92%	69%	24%	20%
	VIB93	38%	12%	57%	53%	54%	32%
	VIB134	43%	16%	82%	68%	54%	49%
	VIB243	30%	9%	81%	68%	43%	37%
V. harveyi	DSM19623	3%	6%	53%	35%	10%	24%
	VH2	6%	4%	31%	31%	17%	11%
	VH5	11%	9%	54%	40%	24%	32%
	VIB391	15%	9%	33%	35%	33%	15%
V. alginolyticus	V2	6%	5%	39%	39%	24%	26%
	Vow	75%*	18%*	44%	39%	47%	20%
V. parahaem.	VH3	7%	8%	51%	36%	17%	29%
V. owensii	DY05	6%	12%	39%	43%	17%	35%
V. splendidus	VaAn	1%	13%	53%	44%	38%	52%

Task 17.3 Production of on-grown *Artemia* (led by IMR, Torstein Harboe).

Introduction

According to the current routine culture practise, Atlantic halibut larvae are fed *Artemia* nauplii through the whole first feeding period. An observed reduction in growth rate during the later phases of first feeding indicates that this feed is insufficient to maintain high growth. A larger prey size, with a higher nutrient content may be a more appropriate choice for those stages. Therefore, a production protocol based on Olsen et al., 1999, for on-grown *Artemia* was further developed, where water renewal and quality are crucial parameters. This 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* and have ben reported in ***Deliverable D11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia***.

Materials and methods

A total of five trial runs were conducted before the reported protocol was finalized. Adjustments were made as described in the following section. *Artemia* cysts (EG, INVE Aquaculture) were hatched in a separate tank, held for 24 hours from incubation, and then transferred to on-growing tanks. Conical fiberglass tanks, with a volume of 300 l, were used both for hatching and on-grown *Artemia*. All tanks were equipped with temperature (500W, and Carlo Gavazzi 600+ temperature regulator) and oxygen control systems (Ocea). The on-growing tanks had a flow-through system (**Figure 17.3.1**).

Figure 17.3.1. *Artemia* tank showing water supply and outlet sieve.

Seawater was pumped from 160 m of depth. For hatching of *Artemia* nauplii, the water was treated with chlorine and, thereafter, sodium thiosulphate for at least 18 hours. For the on-growing tanks, the water was filtered down to 5 µm before being supplied to the tanks. Flow rate was 15 $l \cdot h^{-1}$ from incubation and throughout the entire on-growing period. One hundred g of the disinfectant Sanocare ACE (INVE

Aquaculture, Belgium) were mixed with 1 L of freshwater using a blender (Hamilton Beach commercial) for 2 min and added to the tanks daily.

The enrichment medium ORI-GO (Skretting AS; Stavanger, Norway) was used for grow-out of *Artemia* nauplii. At first, the feed was administered to the tanks using a belt feeder, but due to variation in how the feed dispersed in the *Artemia* on-growing tanks, the feed was mixed with 1 l of freshwater using a blender (Hamilton Beach commercial) for 2 min and was then added to the tanks twice a day. *Artemia* were fed 20 g of ORI-GO in each meal. For the short-term enrichment of on-grown *Artemia*, LARVIVA MULTIGAIN (Biomar, Denmark) was used according to the manufacturer's standard procedure. Enrichment period was 2 h and density of *Artemia* was the same as in the on-growing tanks (100-110 individuals· mL^{-1}).

For measuring *Artemia* size and developmental stage, live *Artemia* were photographed using a dissecting microscope. For measuring number (density) and viability of *Artemia*, triplicate samples of 200 µl were taken daily and treated with buffodine. Treated samples were then counted using a dissecting microscope.

On day 3 the culture was transferred by a hose to an *Artemia* washer (**Fig. 17.3.2**) and concentrated from 280 l to approximately 70 l. The concentrate was then flushed (25 l· min^{-1}) under heavy aeration using 22°C seawater for 5 min, then washed with freshwater until the salinity reached less than 5 ppt and held there for 10 min. Thereafter, the salinity was taken back to >31 ppt by flushing with seawater. The *Artemia* were then transferred to a holding tank before being fed to the larvae.

Figure 17.3.2. *Artemia* washer.

After the five tests were carried out (from nauplii to on-grown *Artemia*), with necessary adjustments, the following protocol was established and used in four identical tanks during a 15-day period. Growth and production data of the *Artemia* have been reported in ***Deliverable 11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia***.

Protocol for on-grown *Artemia*

Day 0:
Add ~$25 \cdot 10^6$ dry *Artemia* cysts to hatching tank using standard procedures.

Day 1:
Filter the tank and wash the concentrate with seawater for 10 min. Transfer the nauplii to the on-growing tank at a concentration of 100 to 110 ind.· ml^{-1}. Turn on water flow and set at 15 l·h^{-1}. Set temperature to 21°C. Add Sanocare and then first meal of ORI-GO at 09.00 and second meal at 15.00.

Day 2:
Keep temperature at 21°C. Add Sanocare and first and second meal of ORI-GO as in day one. Raise air-stone 20 cm from the bottom and remove 5 L of sediment using a siphon.

Day 3:
Repeat procedure of day 2.

Day 4 (morning):
Transfer the on-grown *Artemia* by a hose to an *Artemia* washer and concentrate from 280 l to approximately 70 L. Flush concentrate (25 $L \cdot min^{-1}$) under heavy aeration with 22 °C seawater for 5 minutes. Then concentrate further to approximately 20 L and wash with freshwater (25 $L \cdot min^{-1}$) until the salinity is less than 5 ppt and hold for 10 min. Increase salinity back to >31 ppt by flushing with seawater. Transfer *Artemia* to a holding tank before being fed to the larvae.

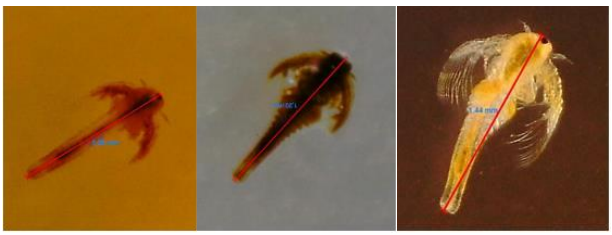


Figure 17.3.3. *Artemia* grown from nauplii for 2, 3 and 4 days (from left to right), having a length of 1.06, 1.2 and 1.4 mm, respectively.

Task 17.4 Comparison of feeding on-grown *Artemia* versus *Artemia* nauplii on larval performance (led by IMR, Torstein Harboe).

Introduction

A strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality **(Fig. 17.4.1)** 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 Atlantic halibut larvae fed on-grown *Artemia* develop into juveniles with better pigmentation and eye migration than Atlantic halibut fed *Artemia* nauplii. This was verified in a feeding experiment performed in 2005 (Hamre and Harboe, unpublished). The industry is considering implementing this knowledge in their production line, but will need further documentation.

Figure 17.4.1. Atlantic halibut juvenile quality (Photo: Øystein Sæle)

Materials and methods:

Atlantic halibut larvae, at an age of 265 day-degrees, were transferred from a yolk sac incubator to 6 first feeding tanks (**Fig. 17.4.2**). Numbers of larvae were approximately 5000 in each tank. The larvae were fed *Artemia* nauplii from 1 until 14 days post firstfeeding (dpff). Then one group of larvae (three tanks) was fed nauplii, and the other group on-grown *Artemia* (2+ out of 3 meals) in triplicate tanks until 28 dpff. The first feeding tanks have a volume of 1,400 liters and water flow of 5 l m^{-1}. Water temperature is 12 ± 0.3°C during the whole period.

The larvae were fed three times a day and remaining *Artemia* in the tanks were estimated before each feeding to give an estimate of feeding activity in each tank. In order to achieve feeding behavior and feed ingestion it was necessary to have turbid water. Normally this is made by adding microalgae to the water. At P7. IMR we have substituted the algae with dissolved clay, which was added to the first feeding tanks morning and evening.

Results and discussion:

There was no difference in larval performance. Survival, measured as number of Atlantic 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).

In the industry, the routine method is to feed *Artemia* nauplii and it is quite common to produce large fractions of Atlantic halibut larvae with abnormal pigmentation and lack of eye migration, although the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on-grown group had to be fed nauplii to get enough food. As the fish grew, more feed was needed and due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as was planned. These are all possible reasons that no differences between the groups were detected.

Samples from this experiment has been taken for nutrient analyses (NIFES) and analyses of digestive capacity (ULL) after end of feeding ongrown*Artemia*.

References cited

Attramadal, K.J.K., Tøndel, B., Salvesen, I., Øie, G., Vadstein, O., and Olsen, Y. (2011). Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. Aquacultural Engineering 49 (2012) 23– 34.

Bergh, Ø., Naas, K.E**.,** and Harboe, T. (1994)**.** Shift in the intestinal microflora of Atlantic halibut (*Hippoglossus hippoglossus*) larvae during first feeding. Can. J. Fish. Aquat. Sci. 51:1899-1903.

D'Alvise PW, Lillebø S, Prol-Garcia MJ, Wergeland HI, Nielsen KF, **Bergh Ø**, Gram LK. 2012. *Phaeobacter gallaeciensis* eliminates *Vibrio anguillarum*in cultures of microalgae and rotifers and prevents vibriosis in cod larvae. *PLOS One* 7(8): e43996. doi:10.1371/journal.pone.0043996

Olsen, A.I., Attramadal, Y., Jensen, A., and Olsen, Y. (1999). Influence of size and nutritional value of *Artemia franciscana* on growth and quality of halibut larvae *Hippoglossus hippoglossus* during the live feed period. Aquaculture 179, 475–487.

Sandlund N, **Bergh Ø** 2008. Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: Bath challenge trials using a multidish system. *Diseases of Aquatic Organisms*, 81:203-217

Deviations from Annex I and their impact:

In Task 17.4, a deviation was necessary as it was not possible to produce enough on-grown *Artemia* beyond 28 days post first-feeding. Therefore, the experiment was terminated at 28 dpff instead of 45 dpff. There are no other deviations during the reporting period.

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):	Tito Peleteiro			
Other Scientists participating:	Nikos Papandroulakis (P1), Fatima Linares, Jose Luis Rodriguez (P19), Antonio Vilar Peron (P32), Blanca Álvarez-Blázquez, Pedro Domingues, Montse Perez, Evaristo Perez, Rosa Cal (P8).			

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 progress towards objectives and details for each task (13-30 Mo):

Task 18.1 Development of feeding methodology (lead by HCMR, Nikos Papandroulakis)

The objective of this task was 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 included rearing in a 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 was to be evaluated in terms of ontogeny of larval digestive and visual system (influenced by feeding) through histological and image analysis

procedures. In addition, the ontogeny of the digestive enzymes for wreckfish larvae were to be studied under the different rearing regimes.

During the reported period only preliminary trials were performed due to the low availability of eggs, that did not allow the implementation of full scale trials. The implementation of this work was achieved with the participation of the following group of HCMR personnel: N. Papandroulakis, N. Mitrizakis, Y. Strakantounas E. Sfakaki.

Two batches of eggs were used at HCMR hatcheries.

A. From the Aquarium of P32. MC2, 2000 larvae were transported in May 2015, with 5 l seawater enclosed in polystyrene boxes (**Figure 18.1.1**). The duration of the transport was 24 hours. When the larvae arrived (19.5°C, 19.5 mg l^{-1} DO and pH 8) they were incubated in 500 l tanks.

B. From the broodstock of HCMR, in June 2016, that were induced to spawn, approximately 4,000 eggs were incubated in a 2000 l tank at 16.5°C, 7.2 mg l^{-1} DO and pH 8.2.

Figure 18.1.1. Transportation of wreckfish eggs from P32. MC2.

Trials were performed in tanks organised as closed water recirculating systems. Two types of tanks were used (500 l and 2,000 l) that had similar shape, but differ in their depth (1 and 2 m, respectively) (**Figure 18.1.2**).

Figure 18.1.2 Rearing tanks of 500 l (left) and 2,000 l (right).

After incubation and during the autotrophic stage, temperature was maintained at 16°C and was gradually increased afterwards to 17.5°C (**Figure 18.1.3**). First feeding was at 10 dph when larvae open their mouth and develop their eyes obtaining the characteristic black color.

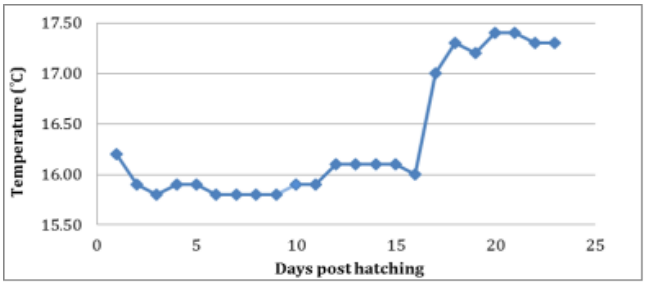


Figure 18.1.3. Temperature profile during the larval rearing.

Feeding was based on enriched rotifers, *Artemia* AF (from 13 dph) and *Artemia* EG (from 24 dph). The batch from the P32. MC2 did not survive beyond the autotrophic stage. The batch from HCMR survived until 24 days post hatching. The growth performance of the larvae is presented in **Figure 18.1.4**. During the rearing some malformed individuals were observed (**Figure 18.1.5**)

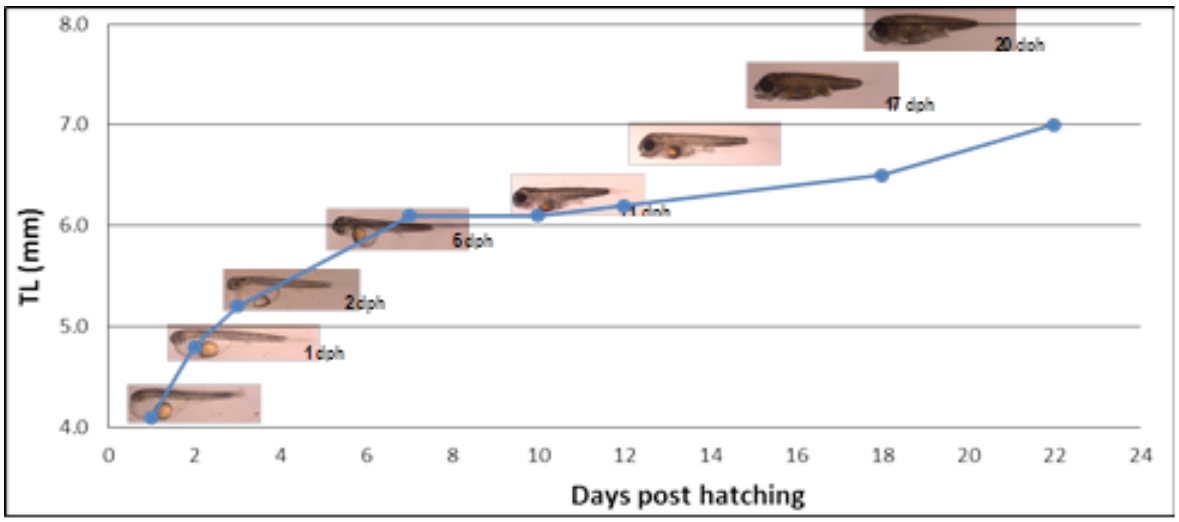


Figure 18.1.4. Growth performance of wreckfish larvae reared at P1. HCMR.

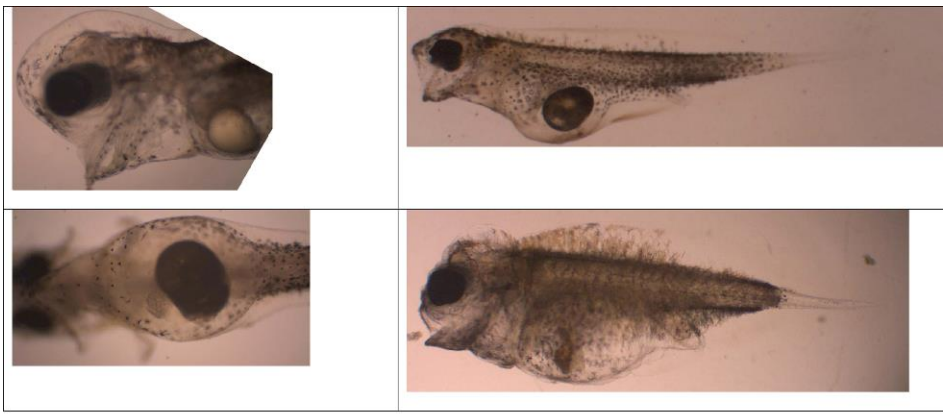


Figure 18.1.5 Malformed wreckfish larve observed during the rearing.

The problem was similar to a syndrome related to swollen yolk sac (SYSS) described in Murray cod, (freshwater fish in Australia) that was related to inadequate nutrition of the broodstock (Gunasekera et al., 1998). Furthermore, similar appearance has been also described in the Blue Sac Disease – (BSD) that is common in trout (Brzuzan et al., 2007). Several reasons have been suggested. The most credible is toxicity from nitrogen compounds such as ammonia or possibly oxidative stress. Nevertheless, the most likely cause for the wreckfish mortality was the Swollen Yolk Sac Syndrome although further studies are required.

Task 18.2 Defining optimum conditions for larval rearing (lead by IEO, Tito Peleteiro)

During 2015, 10 natural spawns were obtained at P32. MC2 and P8. IEO with fertilization between 62 and 97%, hatching between 4 and 56% and survival until 22 days post hatvching, as shown in **Table 18.2.1**.

Table 18.2.1. Parameters of the different spawns at P32. MC2 and P8. IEO and larval culture.

SPAWN TYPE	STOCK	DATE	FEC (%)	HATCH (%)	LARVAE (n²)	LARVAL DENSITY (n²	MEAN T²	FEED	SURVIVAL (dph)	WATER SYSTEM
1/ARTIF.	IEO	10-04-15	62		110	0,2	17,4	Enrich rot	14	CC UNTIL 10 DPH
2/NAT	CM2 (IEO)	05-06-15	84	30	1000	2,0	18,4	Enrich rot	10	CC UNTIL 10 DPH
3/NAT	CM2 (IEO)	27-05-16	86	22	100	0,2	19,1	Enrich rot	10	CC UNTIL 10 DPH
4/NAT	CM2	18-05-15	97	0,02	20	0,2	15,1	Enrich rot+copépods	10	WATER REN.*
5/NAT	CM2	22-05-16	81	4,3	2600	52,0	14,7	Enrich rot+copépods	18	*
5/NAT	CM2	27-05-15	86	22	10600	12,4	14,5	Enrich rot+copépods	18	*
6/NAT	CM2	01-06-15	95	56	180000	24,3	14,4	Enrich rot+copépods	22	*
7/NAT	CM2	05-06-15	84	30	18500	15,6	14,7	Enrich rot+copépods	17	*
8/NAT	CM2	08-06-15	75	3	500	10,0	15,3	Enrich rot+copépods	18	*

Larvae from the P32. MC2 natural spawns were cultured in 50 l tanks **(Figure 18.2.1)**, with rotifers enriched with phytoplankton and copepods in a flow-through system until 22 dph. Larvae at the P8. IEO were from an *in vitro* spawn, and cultured in 500 l tanks in closed system until 10 dph, using the "Green Water" system with rotifers enriched on phytoplankton until 14 dph. Larval density was 0.2 (P8. IEO) and 52 (P32. MC2) larvae l^{-1}, with natural photoperiod during endogenous feeding. After the opening of the mouth and consumption of all yolk sac, artificial light (410 Lux) was used for 12 h per day until the end of the culture period. Larval, yolk sac and lipid droplet length were measured (**Figure 18.2.2**) and developmental stages were documented **(Figure 18.2.3).** Malformed individuals were also observed during the rearing at the P8. IEO facilities **(Fig 18.2.4)**.

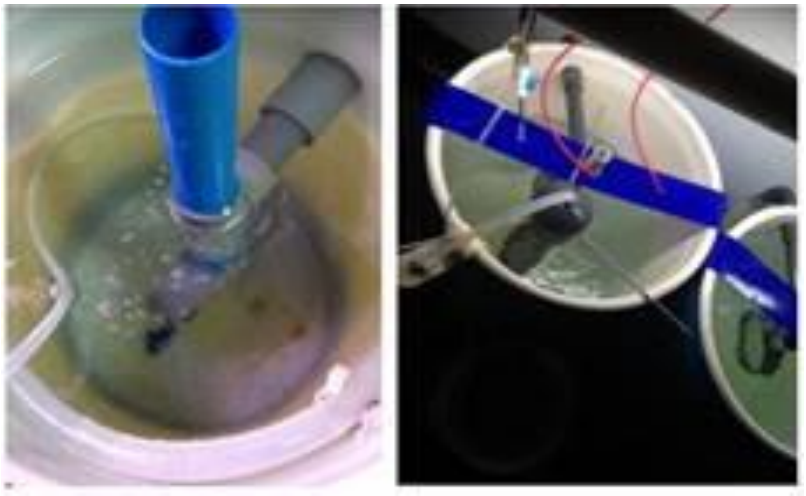


Figure 18.2.1. Culture tanks with recirculation at P32. MC2.

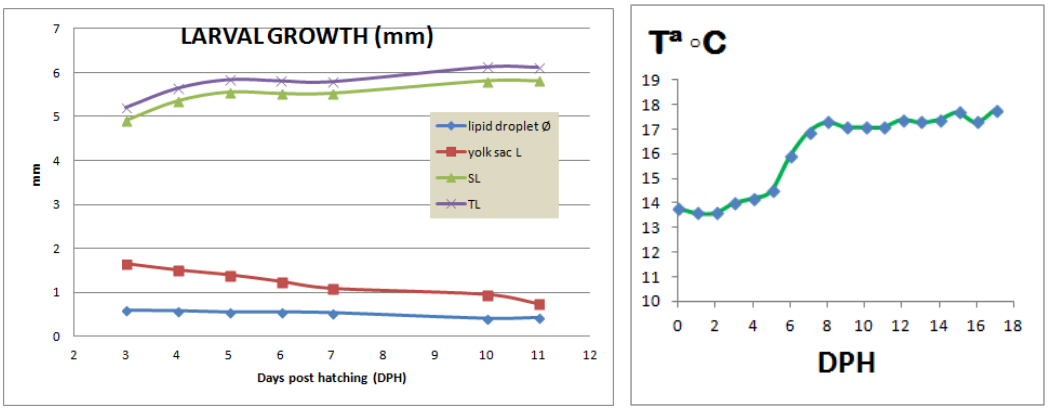


Figure 18.2.2. Water temperature (13.8-17.9ºC) and larval growth until 11 dph. Yolk sac (mm) and changes in the lipid droplet (mm)

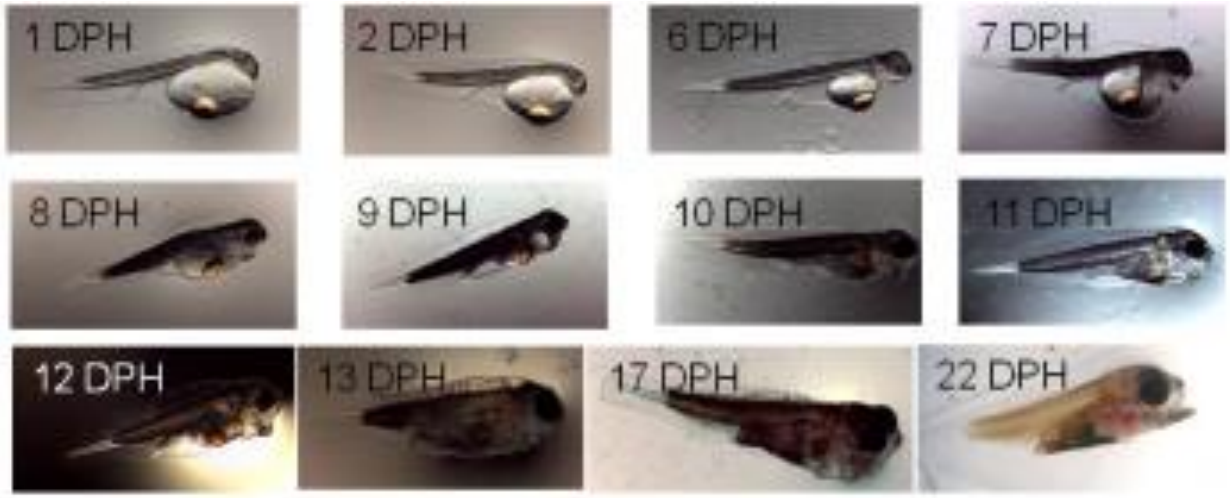


Figure. 18.2.3. Larval development of wreckfish until 22 days post-hatching (dph) at P32. MC2.

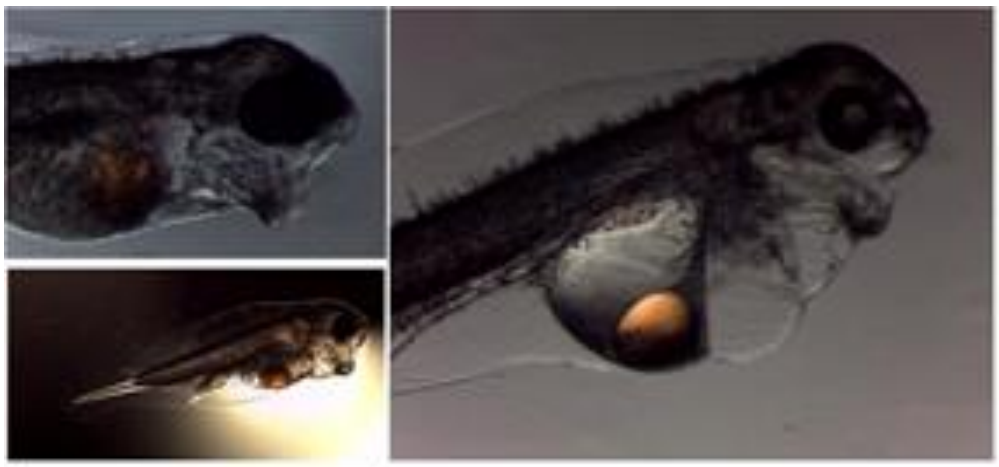


Figure 18.2.4. Malformed wreckfish observed during larval rearing in the IEO.

Year 2016

Sub-task 18.2.1 P8. IEO and P32. MC2 are currently testing the effect of two temperature ranges (14-17 and 19-22°C) in triplicate trials in 2,000 l tanks in a flow-through system and using the same photoperiod regime from the end of endogenous feeding to the weaning onto an inert diet. These studies will be evaluated in terms of growth, survival, larval quality and size. At the time this report was being prepared (June 2016, Mo 31) larval culture at the P8. IEO was attempted in duplicate, where 7,292 and 2,618 larvae were incubated in 150 l tanks and grown at 14-17ºC and 18-22ºC, respectively, in two 500 l tanks (**Figure 18.2.1.6**) Samples from both temperature regimes will be taken to determine dry weight, length, survival, and larval quality. In both cases, natural photoperiod will be maintained until the end of endogenous feeding. After that, a photoperiod of 12 h of light (410 lux at the surface) will be used.

Figure 18.2.1.1. Larval culture tanks at P8. IEO at 19-22ºC (A) and the cooling system at 14-17ºC (B).

References

Brzuzan, P., M Wozny, S Dobosz, H Kuzminski, M K Łuczynski and M Gora, 2007. Blue sac disease in larval whitefish, *Coregonus lavaretus* (L.): pathological changes in mRNA levels of CYP1A, ERa, and p53. Journal of Fish Diseases 2007, 30, 169–173

Gunasekera, R. M., G. J. Gooley, S. S. De Silva, 1998. Characterisation of 'swollen yolk-sac syndrome' in the Australian freshwater fish Murray cod,*Maccullochella peelii peelii*, and associated nutritional implications for large scale aquaculture. Aquaculture 169 _1998. 69–85

Deviations from Annex I and their impact:

The few spawns for poor quality obtained during 2015 was likely due to an unsuitable feeding protocol and the young age of the brood stocks (sexual maturity is achieved > 10 kg), as well as the variable and abnormal environmental conditions compared to previous years at the P32. MC2. Spawn quality at P32. MC2 has improved considerably while the brood stock at the P8. IEO has started to deliver good quality spawns, which suggests that 2016 will be a better year. Nevertheless, we will have to delay the submission of Deliverables D18.1, D18.2 and D18.3, which depend on the availability of an adequate number of eggs of good quality. In 2016 (Mo 31), better quantities and of better quality eggs have been obtained, and we expect to make significant progress in this WP as well.

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 progress towards objectives and details for each task (13-30 Mo):

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

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

The aim of the present study was to (1) compare the effect of adding microalgae type (*Nannochloropsis oculata* vs *Isochrysis galbana*) and concentration (cells ml^{-1}) to rearing tanks on larval rotifer ingestion rate, biochemical composition 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.

Grey mullet eggs (gastrula stage) were stocked in fifteen 1500 l tanks (100 eggs l^{-1}) in a flow through system where filtered (10 µm), UV-treated, 40‰ ambient sea water (25°C) entered the tank from the 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 in two algal species (*Nannochloropsis oculata* and *Isochrysis galbana*) on 2-25 dph mullet larvae. This meant that each of the 5 treatments were tested in three replicate tanks $treatment^{-1}$. 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 from tank flow rate and 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. The average turbidity values of the treatments in the tanks before rotifer feeding at each larval age (dph) showing the consistency of the treatments over the entire feeding trial are shown in **Figure 19.1.1**.

Table 19.1.1 Average turbidity levels of the no-algal control treatment, as well as the *Isochrysis* sp. And *Nannochloropsis* sp. concentrations added to experimental tanks to give two higher turbidity levels (A, B). One-way ANOVA of turbidity values of each treatment was carried out. Turbidity values having different letters were significantly ($P<0.05$) different.

Treatments	Turbidity (NTU)
control	0.26^{a}
Isochrysis A ($0.0144x10^{6}$cells\ml)	0.77^{b}
Nannochloropsis A ($0.2x10^{6}$ cells/ml)	0.75^{b}
Isochrysis B ($0.0228x10^{6}$cells\ml)	1.18^{c}
Nannochloropsis B ($0.4x10^{6}$cells\ml)	1.20^{c}

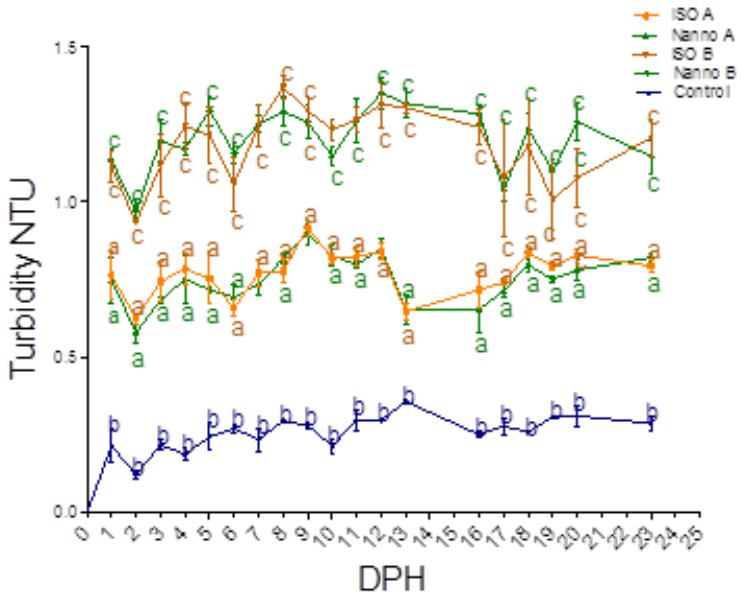


Figure 19.1.1 Average measured turbidity levels (NTU) of all experimental tanks following morning algal addition and before addition of rotifers. One-way ANOVA on turbidity treatment NTU values at each larval age (dph) was carried out. Average turbidity values within a larval age (dph) having different letters were significantly ($P<0.05$) different.

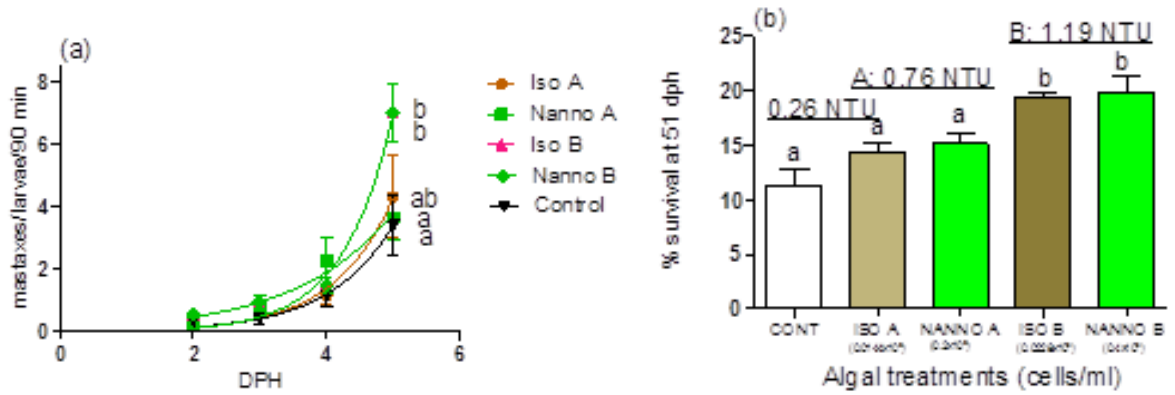


Figure 19.1.2 The effect of turbidity levels (Control, A and B) and algal type; *Nannochloropsis* (Nanno) and *Isochrysis* (Iso) on (a) average rotifer (mastax) consumption larva^{-1} found 90 min after feeding in 2-5 dph larvae and (b) average percent (%) larval survival at 51 dph. One-way ANOVA of mastax number in larvae at 5 dph and percent survival values of the fish in the algal treatments in 51 dph fish (rotifer treatments stopped at 23 dph) having different letters were significantly (P<0.05) different.

There was a turbidity treatment effect on rotifer (mastax consumption) that was significant (P<0.05) at 5 dph (**Figure 19.1.2a**) where 5 dph larvae from the Iso B and Nanno B treatments consumed markedly more rotifers than fish in the control (C) and Nanno A. On the other hand, Iso A was not significantly different (P>0.05) from the high turbidity fish. This feeding pattern appears to have influenced survival 46 days later (51 dph) and 28 days after the algal treatments were no longer administered to the tanks. Fish that were once exposed to the high turbidity treatments, regardless of algal type, demonstrated significantly better survival than fish exposed to the low and control turbidity treatments (**Figure 19.1.2b**).

On the other hand, there was no treatment effect on dry weight in 25 dph fish (**Figure 19.1.3**). There was a significant (P<0.05) algal type and turbidity effect on the larval essential fatty acids; docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) as well as the saturated (SAT), monounsaturated (MONO) and polyunsaturated (POLY) fatty acid composition in 15 dph larvae. The low turbidity *Nannochloropsis* treatment markedly (P<0.05) lowered DHA in these fish compared to all the other treatments while both EPA and ARA were at significantly decreased (P<0.05) levels under high *Isochrysis* and low*Nannochloropsis* turbidities. A similar but non-significant (P>0.05) ARA pattern was shown. Interestingly, the larval levels of ARA were equal or greater than EPA, which was independent of turbidity level and algal type. Fish exposed to low *Isochrysis* turbidity demonstrated higher (P<0.05) SAT and MONO fatty acid levels than fish exposed to the high *Isochrysis* turbidity. However, fish exposed to low *Isochrysis* turbidity exhibited higher PUFA than fish in the low *Nannochloropsis* treatment suggesting an algal type effect on this fatty acid class. Nevertheless, there were no significant (P>0.05) treatment differences in essential fatty acids or fatty acid classes in older 18 and 25 dph fish.

Digestive enzyme analyses are currently being processed at the time this report was being prepared and will be included in the relevant Deliverable, which will be submitted shortly.

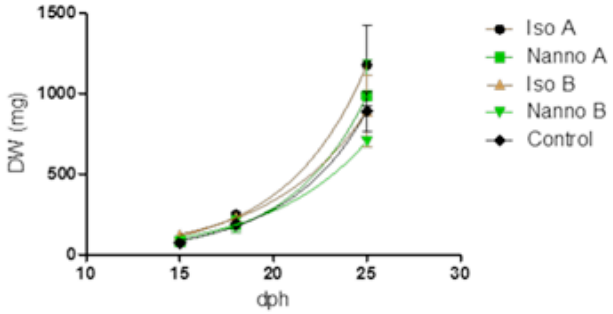


Figure 19.1.3. The effect of turbidity levels (Control, A and B) and algal type; *Nannochloropsis* (Nanno) and *Isochrysis* (Iso) on growth at 15, 18 and 25 dph. One-way ANOVA found no significant (P>0.05) treatment differences on growth on the days measured.

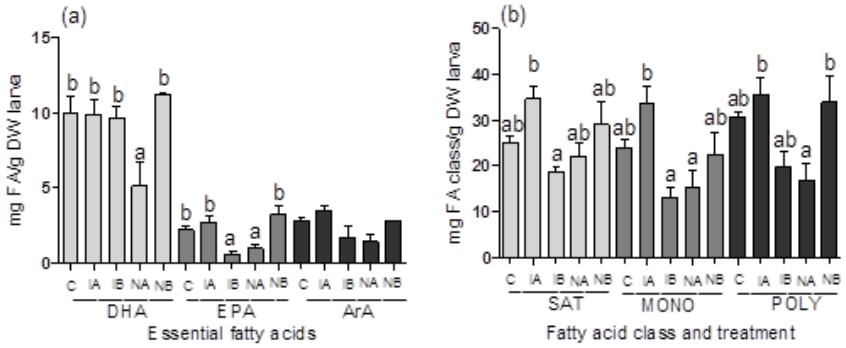


Fig. 19.1.4 The effect of the control (C), Isochrysis A (IA), Isochrysis B (IB), Nannochloropsis A (NA) and Nannochloropsis B (NB) treatments on 15 dph larval (a) essential fatty acids; DHA, EPA and ARA and (b) fatty acid classes; saturated (SAT), monounsaturated (MONO) and polyunsaturated (PUFA) fatty acids. One-way ANOVA of values of an essential fatty acid or within a fatty acid class having different letters indicated a significant (P<0.05) effect of turbidity treatments on this age fish.

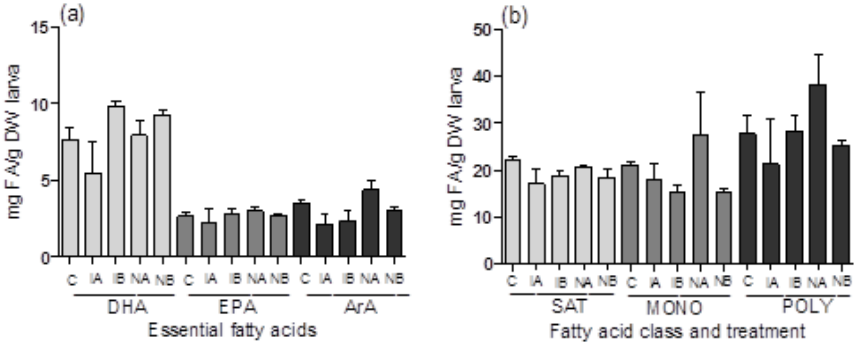


Figure 19.1.5 The effect of the control (C), Isochrysis A (IA), Isochrysis B (IB), Nannochloropsis A (NA) and Nannochloropsis B (NB) treatments on 18 dph larval (a) essential fatty acids; DHA, EPA and ARA and (b) fatty acid classes; saturated (SAT), monounsaturated (MONO) and polyunsaturated (PUFA) fatty acids. One-way ANOVA of values of an essential fatty acid or within a fatty acid class showed no significant (P>0.05) effect of the turbidity treatments on this age fish.

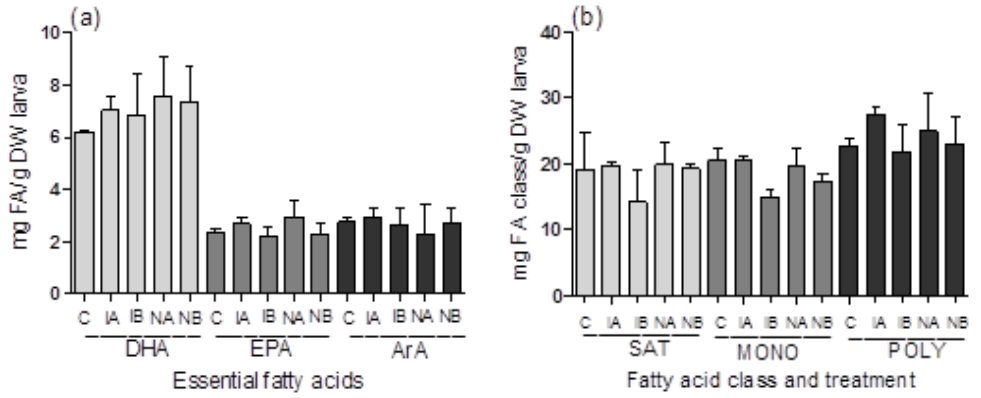


Figure 19.1.6 The effect of the control (C), *Isochrysis* A (IA), *Isochrysis* B (IB), *Nannochloropsis* A (NA) and *Nannochloropsis* B (NB) treatments on 25 dph larval (a) essential fatty acids; DHA, EPA and ARA and (b) fatty acid classes; saturated (SAT), monounsaturated (MONO) and polyunsaturated (PUFA) fatty acids. One-way ANOVA of values of an essential fatty acid or within a fatty acid class showed no significant ($P>0.05$) effect of the turbidity treatments on this age fish.

Conclusions

1. 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 greater numbers of smaller fish, which reduced average fish weight.
4. Larval essential fatty acid and fatty acid class composition at 15 dph, which marks the beginning of *Artemia* nauplii feeding, was influenced by turbidity level and algal type, but the implications are difficult to interpret and do not appear to be a major factor influencing growth and survival.

It is planned in the 2016 fall season to test the best performing algal-turbidity treatment against clay-produced turbidity to determine if algal benefit is solely due to its turbidity contribution.

Task 19.2 Comparing the selected microalgae type and protocol (Task 19.1) with lyophilized substitute (led by IRTA, Alicia Estevez/Enric Gisbert)

This task is not due until month 48 and therefore no work has been done in this task.

Task 19.3 Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production (led by IOLR, Bill Koven).

This task is not due until month 36. To carry out this task, it was originally planned to sub-contract the Israeli company Zoopt to produce large quantities of filtered and cleaned ciliates. However, since the time the project was approved, this company has closed its operations. We are now planning to use copepods instead of ciliates, which are now being produced using a local species, in the Larval Rearing and Physiology Department. of P4. IOLR in Eilat, Israel. (**Figure 19.3.1**). These trials will be undertaken in the autumn of 2016.

Figure 19.3.1 New copepod rearing system.

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).

Due to the poor spawning of grey mullet at the IOLR as well as other sites in Israel, this task will be postponed to the fall of 2017.

Task 19.5 Testing the improved grey mullet larval rearing protocol in a commercial hatchery (led by DOR, Hagay Sarusi)

This task is not due until month 54 consequently, and no work was undertaken so far.

Deviations from Annex I and their impact:

The delays in the planned experiments in 2015 were due to few and poor quality spawns during this season and these studies have been postponed to the 2016 season. We do not expect any negative impact on the outcomes of this WP.

Group Work Packages

Grow out husbandry

Size variability of **meagre** juveniles is related to the high cannibalism at the early stages. The different size classes performed similarly following grading, with no compensatory growth and thus slow growers present a commercial disadvantage. Different net depth (8 m vs 6 m) in cages resulted in no differences in growth, but mortality and feed conversion ratio (FCR) were lower in deep nets. Differences in physiological parameters, (glucose, lactate and lysozyme) being significantly elevated in fish in shallow cages, are possibly the underlying reasons. No differences in growth and mortality were observed between shaded and unshaded cages. Behaviour profiles of the fish described a significant difference between day and night (fish close to the bottom during the day and dispersed throughout the water column at night). Evidence of feeding during the night will be explored in developing feeding methodologies. We also tested the potential of different stimuli (mechanical, optical etc) to be used to entrain fish to feeding times with automatic feeders. Groups of two different size classes (50-100 and 700-900 g) at different tank sizes (500 and 5000 l respectively) were used for testing mechanical and optical feeding stimuli. The 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.

Juveniles of **greater amberjack** grew less when fed at 2.5% body weight d^{-1} or once daily compared to fish fed either 3.5% BW d^{-1} or at apparent satiation and 3 or 4 meals per day. For bigger individuals (200 g), between the feeding frequencies tested (1, 2, 3 and 7 meals d^{-1}), the best results in growth and feed conversion rates have been obtained with 7 meals daily. Environmental temperature affected significantly the performance of greater amberjack juveniles, as fish held at 26ºC grew better than those held at 22ºC while fish at 17ºC grew less. The morphological analysis showed that high temperature led (a) to elongated body shape, particularly the head, and (b) increased caudal propulsion efficiency. Regarding the stocking density, the conditions tested (final value of 3.66±0.46, 5.74±1.20 and 7.41±0.17 kg m^{-3}) influenced growth rates and feed intake. Fish at high density exhibited lower growth rate, condition index and feed intake.

Pikeperch juveniles prefer sinking feed than floating one, but a strong synergy was observed with the feed type and light spectrum, temperature, photoperiod and oxygen saturation levels. Final survival was affected by interactions between light intensity and temperature or stocking density. Neurotransmitters appear to be reliable stress indicators and were affected by the interactions between light intensity and temperature. A positive relationship between physiological status and immune competence was shown during the study. Combining the results on husbandry performances and on stress and immune status, apart from the sinking feed, the modalities considered as suitable for pikeperch differed mainly by their light characteristics. A validation of the selected modalities was performed with an *in vivo* experiment including a bacterial challenge using *Aeromonas salmonicida achromogen*. The results indicated that the three conditions had low impact on stress status. However, fish reared under two experimental conditions showed higher disease resistance after the induced infection.

In the grey mullet studies, we concluded that fishmeal substitution up to 75% by a mixture of different plant protein sources in compound weaning diets did not affect fish growth, survival, body composition, tissue organization, digestive capacity or the innate antioxidative stress response. The effect of stocking density on the grow-out of grey mullet, as a function of geographic location and environment in Israel, Greece and Spain is currently under evaluation.

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), 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 progress towards objectives and details for each task (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 progress towards objectives and details for each task (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 2.1) 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 were 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.

Task 20.1 Methodology to avoid size variability in meagre juveniles (led by IRTA, Alicia Estévez and Neil Duncan**)**

Size variability in juvenile pre-grow out makes regular grading essential to avoid cannibalism and grades of smaller fish may be related to poor performance when transferred to sea cages. Experiments were carried out with meagre juveniles of a mixture of 4-6 known families (from specific breeding groups), to simulate the commercial hatchery situation and in order to study differences in growth rate. Juvenile fish were stocked in triplicate tanks at the same initial density and fed the same commercial diet (P3. IRTA). At the end of the experiment, fish were sampled to be genetically characterised for parentage assignment (P1. HCMR, Task 2.4 from WP2 Reproduction and genetics - meagre) to establish if differences in growth rate was a consequence of genetic origin. Fish with low growth rates were used for compensatory growth studies to determine growth potential of small juveniles and estimate the economic cost of using these fish for production, compared to discarding and using only larger juveniles.

The objective of the studies was to develop a method to avoid size variability of meagre juveniles. The task is expected to define the influence of genetic origin on the size variability in juveniles and on the basis of this provide recommendations on how to avoid variability (*e.g.* genetic improvement and/or size grading including the possibility of recovering slow growing fish). Two trials were performed, one in 2014 already presented (1st Reporting Period) and another in 2015 that is included in this report. The complete results have been submitted in ***Deliverable 20.1. Methodology to avoid size variability in meagre juveniles***.

Results

In 2015 the experimental design was changed according to the suggestions given by the other participants in this task. In this trial four different spawns obtained from hormonal induction of paired fish (**Table 20.1.1**) were used for larval rearing. All the spawns were obtained on the same day (May 13th 2015) and after incubation the newly hatched larvae (May 15th 2015) were **mixed together** and distributed in four 1500-l tanks.

Table 20.1.1. Parents that contributed to each family and spawning date. The female and male number refers to the breeders unique ID and wild or cultured indicates the origin of the breeder.

Family	Spawning Date (Tank)	Female	Male	Hatched larvae (N)
1	13/05/2015 (V7)	5-wild	19-wild	122617
2	13/05/2015 (V6)	6-wild	23-cultured	141983
3	13/05/2015 (V8-1)	1-wild	20-wild	66500
4	13/05/2015 (V8-2)	8-wild	22-wild	8050

Larvae were reared under intensive conditions following the standard protocol of meagre culture. Fifty larvae per litre were used as initial density, a photoperiod of 16L:8D, 500 lux intensity, in the water surface, and fed

enriched rotifers from 2 to 14 days post hatch (dph), enriched *Artemia* metanauplii from 8 to 30 dph and weaned a commercial diet (Gemma Micro, Skretting, Norway) from 20 dph onwards. For enrichment, the commercial product Red Pepper was used following the enrichment procedure provided by Bernaqua.

Every week 20-30 larvae were sampled and anaesthetized with MS222 to estimate growth in weight and length. Standard length was determined by observation in a stereomicroscope Nikon SMZ800 (Nikon, Japan) equipped with a digital camera Olympus DP25 (Olympus, Germany) and an image analyzer (analysis, SIS GmbH, Germany). The same larvae were used to estimate wet and dry weight, placing the larvae on a pre-weighted coverslip and after drying at 60ºC for 24h in an oven, with a microbalance Mettler Toledo MX5 (Mettler Toledo, Spain).

Juveniles were fed a commercial diet for sea bass (Mar Perla, Skretting, Norway) *ad libitum* in 2014 and using a fixed feeding rate in 2015 (7.5% body weight for fish between 10-30 grams, 5% for fish from 30-60 grams). Growth of juveniles was also registered every 2-3 weeks during ongrowing and specific growth rate calculated following the formula: **SGR** = (lnWf - lnWi x100) / t, where Wf and Wi are the final and initial weights and t the time (days)

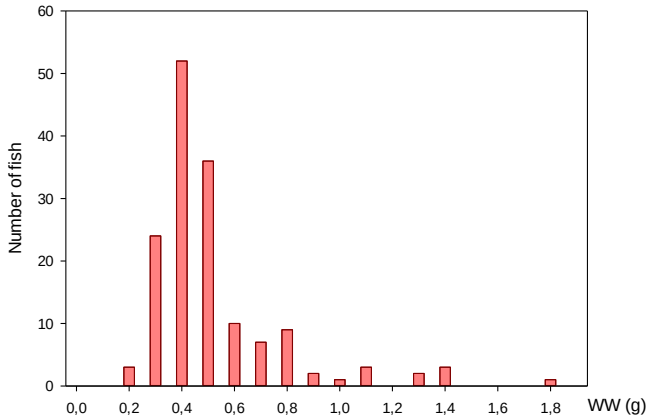


Figure 20.1.1. Size distribution of juveniles on July 2015

For the statistics (one way analysis of variance, ANOVA) and calculation of regression lines and growth curves the Sigma Plot 12.0 program (SyStat, USA) was used

All the larvae obtained from the 4 spawnings were reared mixed together in 4 tanks. On July 2nd 150 fish were individually weighed to check the size distribution before separating the fish in small, medium and large individuals (**Figure 20.1.1**). On July 17th all the fish were counted and graded into large, medium and small fish and distributed in two RAS modules according to **Table 20.1.2**.

Table 20.1.2. Distribution of fish in on-growing tanks on July 17th 2015

14/07/2015									
Mod 5						Mod 3			
Tank 1		Tank 2		Tank 3		Tank 1		Tank 2	
N	Average WW (g)	N	Average WW (g)	N	Average WW (g)	N	Average WW (g)	N	Average WW (g)
551	0,26	802	0,43	361	1,20	660	0,44	650	0,43
Biomass (g)	145,00		343,72		433,94		291,90		278,57
7,5% feeding rate (g)	10,88		25,78		32,55		21,89		20,89

The fish were kept in these tanks (an additional movement was carried out in August to redistribute M size fish and reduce the biomass in tank 2 Mod 5 and tanks 1 and 2 Mod 3) for 2 months and fed using automatic feeders at a feeding rate of 7.5% from July 17th until September 3rd when the fish were graded again in large (L 28-32 g), medium (M, 19-24 g) and small (S, 12-16 g) fish and fin clips taken for parental assignment. Fish were graded in L, M and S, and distributed in triplicate tanks each with 100 fish that were fed also using automatic feeders at a feeding rate of 7.5% for fish between 12 and 30 grams and 5% for fish weighing more than 30 g. Every 2-3 weeks until November 5th 2015, the fish were weighed to build the growth curve and calculate the standard growth rate as in 2014. Results are summarized in **Table 20.1.3** and **Fig. 20.1.2**.

Table 20.1.3. Summary of results obtained in the growth in weight of 2015 juveniles

	2015																	
	49 dph		83 dph			110 dph			112 dph			134 dph			155 dph			190 dph		
	02/07/2015		05/08/2015		SGR	01/09/2015		SGR	03/09/2015		SGR	25/09/2015		SGR	16/10/2015		SGR	05/11/2015		SGR
S	0,263	0,030	4,806	1,20	1,61	17,841	5,646	2,82	13,96	1,39	1,20	19,07	2,79	2,83	26,13	4,04	3,12	29,89	5,31	3,30
M	0,434	0,093	7,030	1,83	1,97	22,171	5,776	3,03	21,50	1,47	1,52	30,43	3,53	3,28	38,61	6,13	3,49	45,83	8,79	3,72
L	1,202	0,494	12,359	5,69	2,51	37,950	14,961	3,54	29,18	1,56	1,56	39,76	5,40	3,53	55,12	8,40	3,83	66,62	11,88	4,08

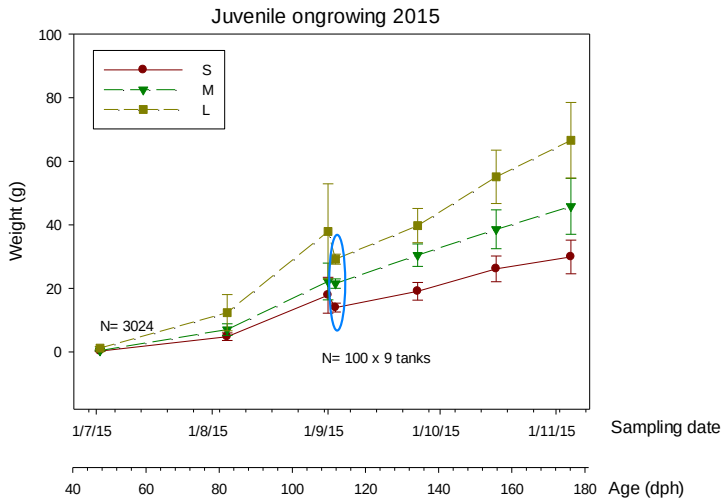


Figure 20.1.2. Growth in weight of juveniles obtained in 2014 after grading in small (S), medium (M) and large (L)

Table 20.1.4. Theoretical growth of S, M and L fish in 2014 and 2015

Growth of fish in 2014			
	S	**M**	**L**
100 d	7,82	10,84	15,67
200 d	66,67	85,94	104,18
360 d	160,83	206,10	261,79
540 d	266,76	341,28	439,11
Growth of fish in 2015			
	S	**M**	**L**
100 d	12,27	19,26	24,24
200 d	33,17	50,33	73,33
360 d	66,61	100,04	151,87
540 d	104,23	155,97	240,24

With the results obtained we have calculated the growth curves for 2014 and 2015 for S, M and L fish and calculated the differences in growth for the different groups of fish. The results are presented in table 20.1.4 and clearly show that S fish always grow more slowly than M and L fish and there is no compensatory growth when the fish are graded in different sizes. Thus, if S fish are kept in the fish farm they will have a

delay of around 6 months to attain the same size of L fish. Although the growth of the fish was different between the two years (2014 and 2015) the differences in growth of S versus L fish were almost the same

We have observed that in meagre there is no compensatory growth when the fish were graded in sizes during on-growing. Slow growing fish (S) always show a lower growth rate than medium (M) or fast (L) growing fish that have as a consequence a delay of around 6 months to reach commercial size with clear economic consequences for producers (**Table 20.1.5**, prices used were obtained from feed producer –Skretting- and central market of Madrid, Spain).

Table 20.1.5 Production cost of L- and S- growing fish.

PRODUCTION COST OF L- AND S- GROWING FISH (1000 juveniles)		
	L- fish	S- fish
Juveniles (0.6€/unit)	600	600
Food 10-30 gr (2.4€/Kg)	90	136,8
30-250 gr (2.04€/Kg)	1526	2557,7
250-500 gr	1943,1	3243,6
Total	**4159,1**	**6538,1**
Market price (9,3€/Kg)	4650	4650

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. The objective of this task is 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).

The specific objective of the trial is to test the performance of meagre in cages of different depth. Cages of 180 (6x6x5) and 290 (6x6x8) m^3 at the P1. HCMR pilot farm in duplicates indicated as Shallow and Deep were used. The fish origin was the hatchery of P1. HCMR. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery. Juveniles of 2 g were transferred at the cage facility and they were reared under similar conditions until the beginning g of the trial.

Two trials were performed. The 1st trial started in May 2014. Four groups were created, two of ~5,150 for the 180 m^3 cages and two of ~8,240 for the 290 m^3 cages. The wet weight at the beginning of the trial was 200 ± 20 g. The duration of the trial was planned to be 8 months and was completed at the end of 2014. The second trial started on March 2015. Four groups were created, two of ~2,000 individuals for the 180 m^3 cages and two with ~3,200 for the 290 m^3 ones. The initial weight at the beginning of the trial was 867 ± 18 g. The duration of the trial was planned to be 8 months and was completed at the beginning of 2016. The temperature profile during the two phases of the rearing is presented in **Figure 20.2.1**.

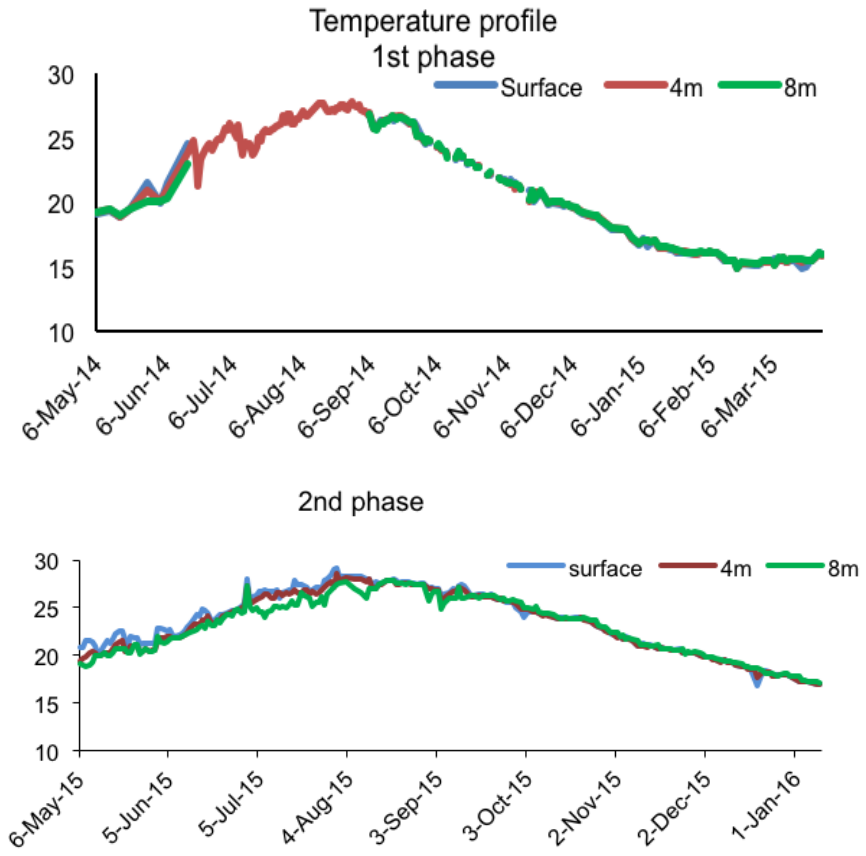


Figure 20.2.1 Temperature profile (surface, 4 m and 8 m depth) during the two experimental phases.

During the experimental period, growth performance was estimated with monthly samples in both trials. Every second month blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid, free fatty acids), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. 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.

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).

Hematocrite measurements were based on the use of special capillary tubes were blood samples were transferred and centrifuged for 10 min in a capillary centrifuge at 2000× *g*. Hemoglobin and lactate determinations were carried out using the corresponding commercial kits (SPINREACT). Glucose measurements were carried out using a commercial kit (Biosis). Serum samples were transported in dry ice and stored at -80°C until the immunoassay was performed. Lysozyme was measured using the turbidimetric method. Briefly, the kinetics of the membrane dissolution of *Micrococcus luteus* (0.2mg / ml) with 10 ul serum was followed at 450 nm for 20 minutes. The kinetics of antibacterial (anti-luminescent *E.coli*) serum activity was measured as previously described. Myeloperoxidase was measured as previously described ([2]). Most of the samples have been analyzed and only the immunological analysis of the 2nd experimental phase are still pending.

For the determination of cortisol, plasma samples were extracted with diethyl ether and water samples were extracted according to Ellis et al. (2004) using ethyl acetate. Briefly, 1 ml of diethyl ether was mixed with 100 μl of plasma and after allowing the phases to separate, the organic phase was transferred and evaporated under nitrogen gas. Residue was re-dissolved in 100 μl extraction buffer. **Cortisol** determinations were performed at the University of Crete (Lab of Fish Physiology). Plasma cortisol concentrations were measured using commercial cortisol enzyme immunoassay kit (Cayman).

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). Data are presented as means ± standard deviation (SD). Statistical comparisons of total length and body and also of the haematological, hormonal and biochemical parameters between the different groups and between the different sampling months were made using two-way ANOVA to assess differences among groups and Tukey's or Dunn's post-hoc tests to assess the level of significance. The significance level used was $P < 0.05$.

Results

Biological performance

Although analysis is not completed we present here some preliminary results. In **Figure 20.2.2** the growth performance is presented in the two experimental phases.

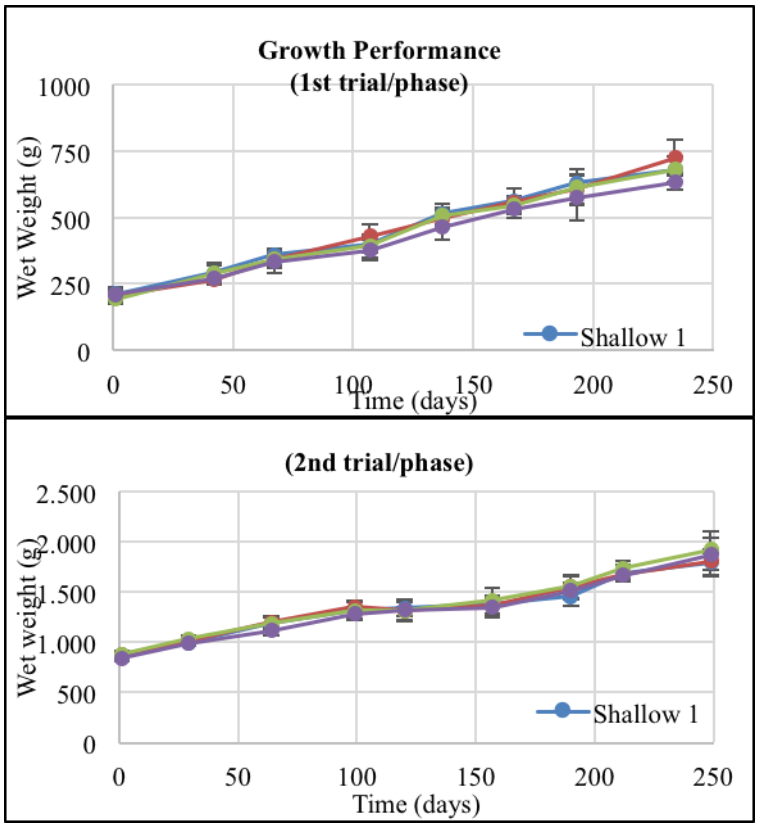


Figure 20.2.2 Growth performance, mean weight, of meagre. Error bars are the standard deviation (n=10)

During the 1st phase the growth rate was ~2 g d^{-1} while for the second phase it was increased to 3.5 g d^{-1}. In both phases no significant difference was observed between the tested conditions.

Table 20.2.1 Performance indicators during the two experimental phases.

		Shallow1	Shallow2	Deep1	Deep2
1st trial	**Mortality (%)**	23,5	24,2	12,1	13,9
	FCR	1,92	1,92	1,58	1,60
2nd trial	**Mortality (%)**	10.8	9.7	7.9	8.1
	FCR	1.67	1.70	1.50	1.47

Regarding other performance indicators, in **Table 20.2.1** the mortality (as %) and the food conversion ratio are presented. Significant differences are presented only during the first period with the groups reared in the deep nets to present almost half mortality rate and also ~25% lower FCR.

Hematological, Biochemical, Immunological and Hormonal parameters

1st Trial

Hematocrite levels appear to be affected by the sampling period ($P < 0.001$), but not by the depth of the net, with no statistically significant interaction between the two factors (**Figure 20.2.3**). There was a statistically significant difference in the hematocrite levels between November when peak levels were observed (NOV S = 40.6 ± 4.4 % ; NOV D = 37.4 ± 4.6 %; $P < 0.001$) and the rest of the months with the lowest levels observed during March (MAR S = 77.9 ± 2.1 % ; MAR D = 27.1 ± 2.8 %).

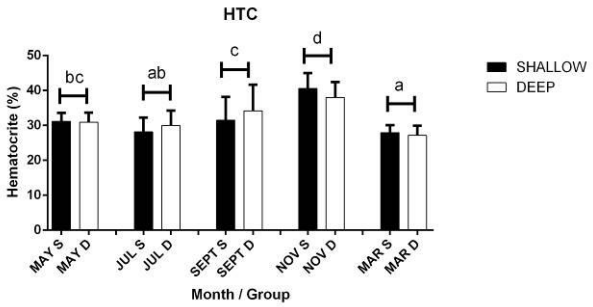


Figure 20.2.3. Hematocrite levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Hemoglobin

The net depth did not have a statistically significant effect on hemoglobin levels. However, there was a statistically significant difference on hemoglobin levels between the different months ($P < 0.001$). The pattern is characterized by low hemoglobin levels from May to September, peak values ($P < 0.05$) in November (NOV S = 9.5 ± 2.9 g dl^{-1} ; NOV D = 9.2 ± 2.1 g dl^{-1}), which dropped in March (MAR S = 7.8 ± 2.4 g dl^{-1} ; MAR D = 7.5 ± 1.5 g dl^{-1}) (**Figure 20.2.4**).

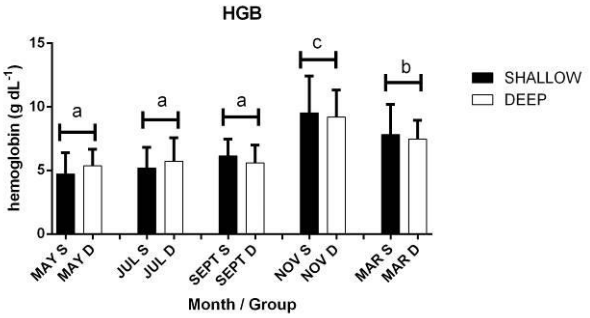


Figure 20.2.4 Hemoglobin levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Plasma osmotic pressure

The net depth didn't have a statistically significant effect on plasma osmotic pressure levels. However, there was a statistically significant gradual increase on osmotic pressure levels from May (MAY S = 313.0 ± 14.3 mOsm kg^{-1} ; MAY D = 320.3 ± 19.3 mOsm kg^{-1}) till November (NOV S = 385.7 ± 52.0 mOsm kg^{-1}; NOV D = 388.3 ± 33.5 mOsm kg^{-1}), and then on March plasma osmotic pressure levels started to decrease (MAR S = 355.6 ± 6.4 mOsm kg^{-1} ; MAR D = 357.1 ± 15.7 mOsm kg^{-1}) (**Figure 20.2.5**).

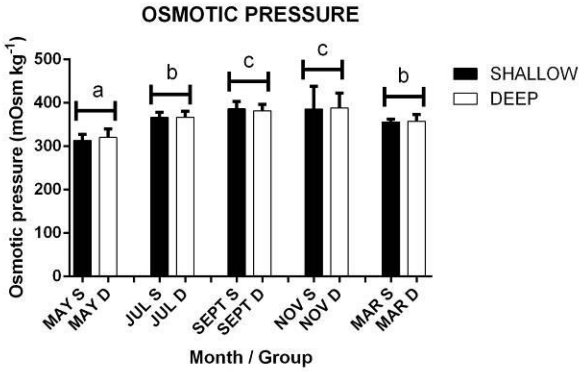


Figure 20.2.5 Plasma osmotic pressure during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Glucose

There is a statistically significant interaction between the net depth and the sampling month ($P < 0.001$). In the Shallow net there was a statistically significant difference between months May (MAY S = 9.7 ± 2.3 mmol dl^{-1}) and September (SEPT S = 7.3 ± 1.6 mmol dl^{-1}) when the highest values in glucose levels were observed and March (MAR S = 6.7 ± 1.7 mmol dl^{-1}) when the lowest levels were observed. In fish from the Deep net, highest glucose levels were observed in July (JULY D = 10.0 ± 2.3 mmol dl^{-1}) which gradually dropped to reach lowest levels in March (MAR D = 5.7 ± 1.6 mmol dl^{-1}), Figure 3.21. Additionally, the depth of the net had an effect on plasma glucose levels in all samplings apart from March (**Figure 20.2.6**).

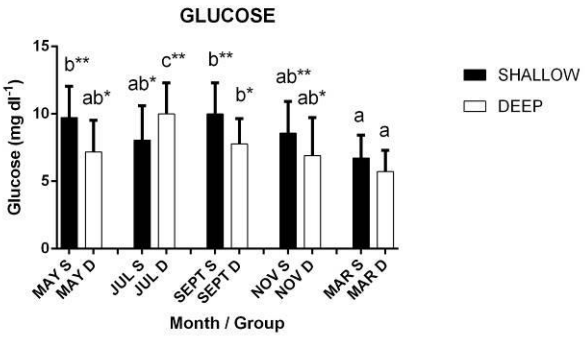


Figure 20.2.6. Plasma glucose levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different nets, $P < 0.05$.

Lactate

There is a statistically significant interaction between month and net ($P < 0.001$). In any case, highest lactate values were observed in July (JULY S = 4.7 ± 1.1 mmol dl^{-1}; JULY D = 3.7 ± 0.9 mmol dl^{-1}) and lowest in March (MAR S = 2.2 ± 0.4 mmol dl^{-1} ; MAR D = 2.1 ± 0.4 mmol dl^{-1}), regardless the depth of the net. The depth of the net affected the lactate levels of the plasma, as in fish from the shallow net lower levels were observed in May and November compared to the fish from the deep net but in months July and September fish from the shallow net exhibited higher lactate levels than fish from the deep net (**Figure 20.2.7**).

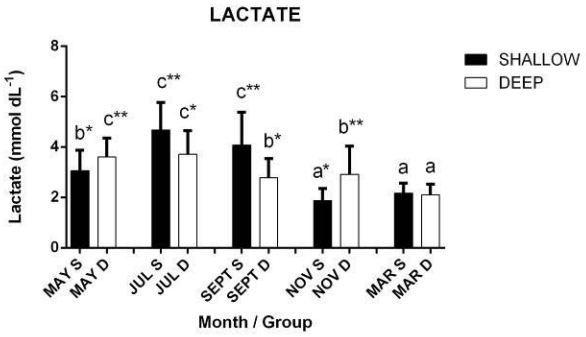


Figure 20.2.7. Plasma lactate levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different nets, $P < 0.05$.

Cholesterol

The depth of the net had no effect on the plasma cholesterol levels. However, there was an effect of the sampling month ($P < 0.001$) on the cholesterol levels with highest values observed in May (MAY S = 4.9 ± 1.2 mmol dl^{-1} ; MAY D = 4.7 ± 0.7 mmol dl^{-1}), dropped in July (JULY S = 4.1 ± 0.9 mmol dl^{-1} ; JULY D = 4.0 ± 0.3 mmol dl^{-1}), to gradually reach a minimum in November (NOV S = 1.0 ± 0.8 mmol dl^{-1} ; NOV D = 1.4 ± 0.6 mmol dl^{-1}) (**Figure 20.2.8**).

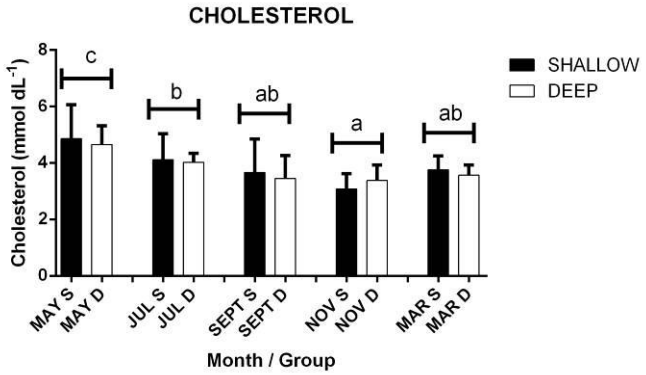


Figure 20.2.8. Plasma cholesterol levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Total Proteins

There was no effect of the net depth on the total protein levels in the plasma of the fish. However, the sampling month affected the levels of total proteins in the plasma of the fish ($P < 0.001$) regardless of the net depth, with the highest values observed in July (JULY S = 4.1 ± 0.9 gr dl^{-1} ; JULY D = 4.0 ± 0.3 gr dl^{-1}) and the lowest observed in September (SEPT S = 4.9 ± 1.2 gr dl^{-1} ; SEPT D = 4.7 ± 0.7 gr dl^{-1}) (**Figure 20.2.9**).

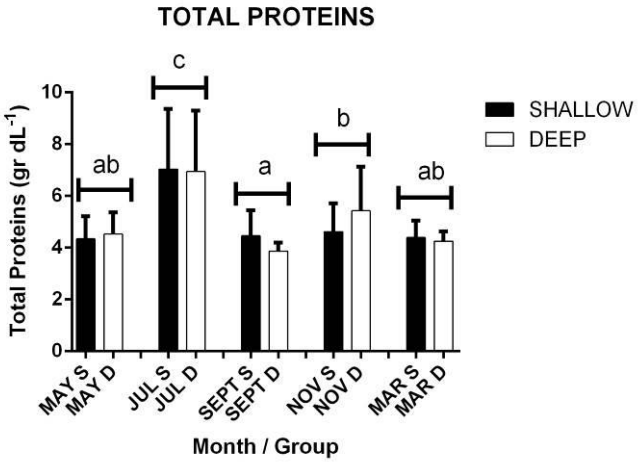


Figure 20.2.9. Total proteins levels in the plasma during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Lysozyme

There is a statistically significant interaction between the net depth and the sampling month. In fish reared in the deep net the activity of lysozyme is not affected by the sampling period. However, in fish reared in the

shallow net lysozyme activity levels are low in May (MAY S = 306.3 ± 134.2 k units l^{-1}) and rise during July (JULY S = 10.0 ± 2.3 k units l^{-1}) and September (SEPT S = 646.1 ± 296.0 k units l^{-1}) in a statistically significant manner ($P < 0.05$) to fall to the initial values during the following months (**Figure 3.25)**. Additionally, the depth of the net had an effect on lysozyme activity levels in July and September with fish reared in the shallow net exhibiting statistically significant higher activity levels ($P < 0.001$) than fish reared in the deep net (**Figure 20.2.10**).

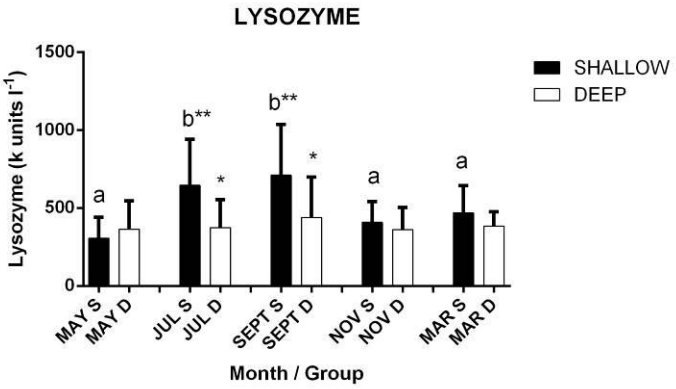


Figure 20.2.10. Lysozyme activity levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different nets, $P < 0.05$.

Free Fatty Acids

There was a statistically significant interaction between the net depth and the sampling month ($P < 0.001$). Statistically significant higher free fatty acids levels were observed for both net depths in July (JULY S = 4.1 ± 0.9 mM ; JULY D = 4.0 ± 0.3 mM) which gradually dropped to minimum levels in March SEPT S = 4.9 ± 1.2 mM ; SEPT D = 4.7 ± 0.7 mM) (**Figure 20.2.11**).

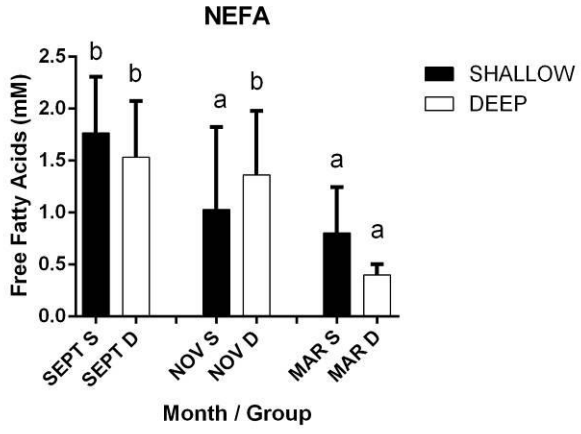


Figure 20.2.11 Free Fatty acids levels during the period from September 2014 to March 2015 (September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Cortisol

The depth of the net had no effect on cortisol levels and there was no statistical interaction between net depth and month sampling. In general, cortisol levels remained at lower values which did not differ statistically for the period between May to November [(MAY S = 0.18 ± 0.1 ng ml^{-1} ; MAY D = 0.32 ± 0.4 ng ml^{-1}) ; (JULY S = 0.58 ± 0.5 ng ml^{-1}; JULY D = 0.31 ± 0.3 ng ml^{-1}) ; (SEPT S = 0.34 ± 0.1 ng ml^{-1}; SEPT D = 0.42 ± 0.3 ng ml^{-1}) ; (NOV S = 0.22 ± 0.1 ng ml^{-1}; NOV D = 0.21 ± 0.2 ng ml^{-1})] but there was a statistically significant increase on cortisol levels in March (MAR S = 1.2 ± 0.6 ng ml^{-1}; MAR D = 1.4 ± 0.6 ng ml^{-1})(**Figure 20.2.12**).

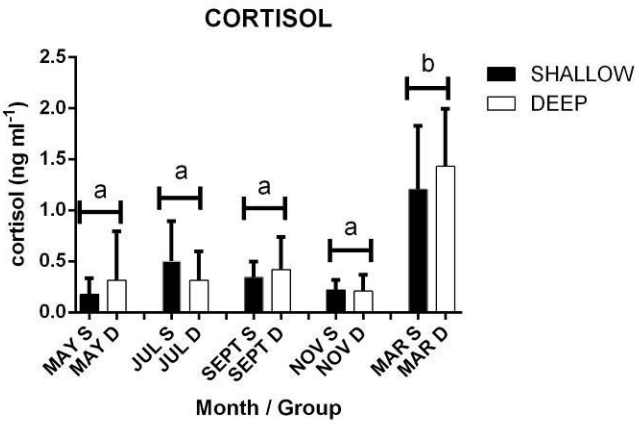


Figure 20.2.12. Plasma cortisol levels during the period from May 2014 to March 2015). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different nets, $P < 0.05$.

Triiodothyronine T3

Neither the depth of the net nor the sampling month had any statistically significant effect on T3 levels (**Figure 20.2.13**).

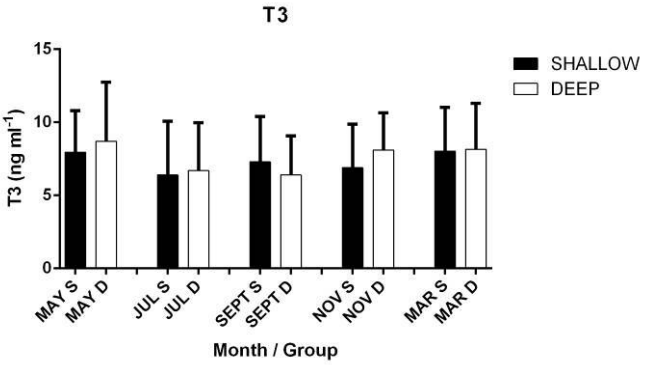


Figure 20.2.13 Triiodothyronine (T3) levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month).

Thyroxine T4

The net depth had no effect on T4 levels. However, the month of sampling had a statistically significant effect ($P < 0.001$) on T4 levels with minimum levels observed in July (JULY S = 5.31 ± 1.1 ng ml^{-1}; JULY D = 1.1 ± 0.3 ng ml^{-1}) which increased gradually to reach maximum values in November (NOV S = 11.4 ± 1.9 ng ml^{-1}; NOV D = 11.4 ± 2.6 ng ml^{-1}) and March (MAR S = 10.6 ± 2.1 ng ml^{-1}; MAR D = 10.7 ± 1.7 ng ml^{-1})(**Figure 20.2.14**).

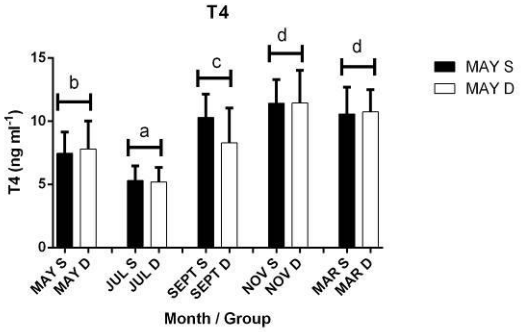


Figure 20.2.14 Thyroxine (T4) levels during the period from May 2014 to March 2015 (May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; March/ Shallow net: MAR S; March/ Deep net: MAR D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different nets, $P < 0.05$.

2nd trial

Hematocrite

The was a statistically significant difference in the hematocrite levels between November when peak levels were observed (NOV S = 39.93 ± 8.0 ; NOV D = 38.0 ± 5.5 ; $P < 0.001$) and the rest of the months when the hematocrite values fluctuated between similar levels. Moreover, the depth of the net appeared to affect the hematorite levels in July (JUL S = 30.45 ± 2.6 ; JUL D = 27.3 ± 6.6) and September (SEPT S = 27.65 ± 3.5 ; SEPT D = 30.45 ± 4.2) samplings ($P < 0.05$), **Figure 20.2.15**.

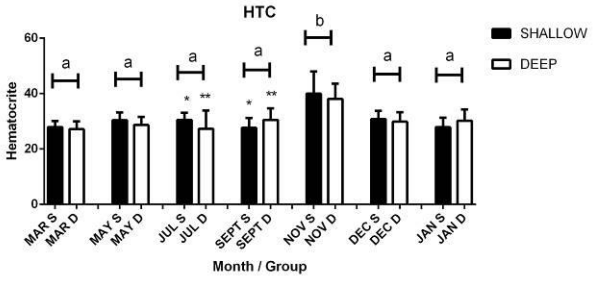


Fig. 20.2.15. Hematocrite levels during the period from March 2015 to January 2016 (March/ Shallow net: MAR S; March/ Deep net: MAR D; May/ Shallow net: MAY S; May/ Deep net: MAY D; July/ Shallow net: JULY S; July/ Deep net: JULY D; September/ Shallow net: SEPT S; September/ Deep net: SEPT D; November/ Shallow net: NOV S; November/ Deep net: NOV D; December/ Shallow net: DEC S; December/ Deep net: DEC D; January/ Shallow net: JAN S; January/ Deep net: JAN D). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months) while asterisks differences between the different nets, $P < 0.05$.

Hemoglobin

The net depth did not have a statistically significant effect on hemoglobin levels. However, there was a statistically significant difference on hemoglobin levels between the different months ($P < 0.001$). During March hemoglobin levels showed peak values (MAR S = 7.82 ± 2.4 g dl^{-1} ; MAR D = 7.46 ± 1.5 g dl^{-1}) which remained high during the following months of May (MAY S = 5.14 ± 1.8 g dl^{-1} ; MAY D = 6.29 ± 0.9 g dl^{-1}) and July (JULY S = 6.62 ± 1.5 g dl^{-1} ; JULY D = 6.26 ± 1.9 g dl^{-1}), to drop to lower levels during the following period of September to January [(SEPT S = 5.76 ± 0.6 g dl^{-1} ; SEPT D = 5.7 ± 0.7 g dl^{-1}) ; (NOV S = 4.55 ± 1.3 g dl^{-1} ; NOV D = 4.48 ± 1.3 g dl^{-1}) ; (DEC S = 4.49 ± 1.3 g dl^{-1} ; DEC D = 4.2 ± 1.2 g dl^{-1}) ; (JAN S = 4.75 ± 0.8 g dl^{-1} ; JAN D = 4.72 ± 0.8 g dl^{-1})], **Figure 20.2.16**.

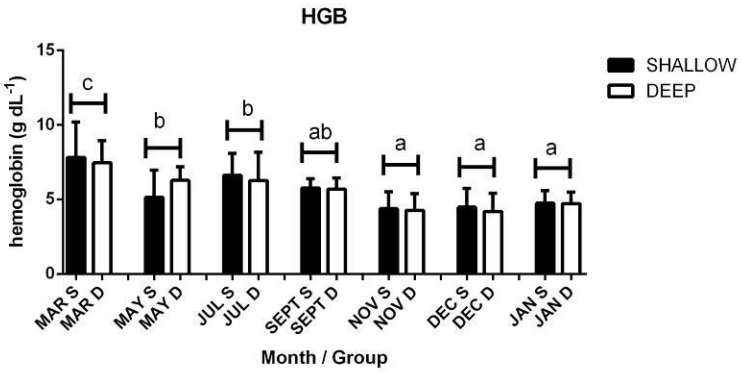


Fig. 20.2.16. Hemoglobin levels during the period from March 2015 to January 2016. Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Glucose

The net depth did not have a statistically significant effect on glucose levels. However, there was a statistically significant difference in glucose levels between the different months. Glucose levels remained low during March to September [(MAR S = 67.22 ± 16.9 mg dl^{-1} ; MAR D = 57.11 ± 15.6 mg dl^{-1}) ; (MAY S = 56.05 ± 36.5 mg dl^{-1} ; MAY D = 89.21 ± 38.5 mg dl^{-1}) ; (SEPT S = 81.99 ± 26.4 mg dl^{-1} ; SEPT D = 92.62 ± 34.9 mg dl^{-1})] with higher values for this period observed in July ((JULY S = 108.1 ± 35.3 mg dl^{-1} ; JULY D = 103.7 ± 35.8 mg dl^{-1})) and appeared statistically higher ($P < 0.001$) during the period from November to January [(NOV S = 149.13 ± 44.4 mg dl^{-1} ; NOV D = 172.43 ± 67.9 mg dl^{-1}) ; (DEC S = 158.21 ± 56.3 mg dl^{-1} ; DEC D = 159.71 ± 52.2 mg dl^{-1}) ; (JAN S = 123.67 ± 40.8 mg dl^{-1} ; JAN D = 137.28 ± 50.9 mg dl^{-1})], **Figure 20.2.17**.

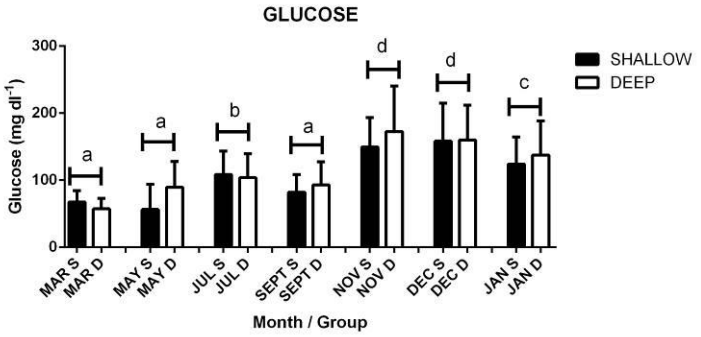


Fig. 20.2.17. Glucose levels during the period from March 2015 to January 2016 (as in **Fig. 20.2.16**). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Lactate

There is a statistically significant interaction between month and net ($P < 0.001$) and the effect of month on lactate levels depends on the depth of the net. The sampling month did not have any statistically significant effect on lactate levels in the case where the deep net was used. However, there was a statistically significant difference in lactate levels between the different months when the shallow net was used ($P < 0.001$), lactate levels statistically higher for months May (MAY S = 4.6 ± 1.6 mg dl^{-1} ; MAY D = 2.4 ± 0.6 mg dl^{-1}), July (JULY S = 3.58 ± 0.6 mg dl^{-1} ; JULY D = 3.05 ± 1.0 mg dl^{-1}) and January (JAN S = 4.73 ± 2.9 mg dl^{-1} ; JAN D = 2.48 ± 1.1 mg dl^{-1}) compared to March (MAR S = 2.17 ± 0.4 mg dl^{-1} ; MAR D = 2.1 ± 0.4 mg dl^{-1}), September (SEPT S = 1.93 ± 0.5 mg dl^{-1} ; SEPT D = 1.97 ± 0.5 mg dl^{-1}), November (NOV S = 2.47 ± 2.4 mg dl^{-1} ; NOV D = 2.47 ± 1.6 mg dl^{-1}) and December (DEC S = 2.2 ± 0.7 mg dl^{-1} ; DEC D = 2.17 ± 0.8 mg dl^{-1}), **Figure 20.2.18**.

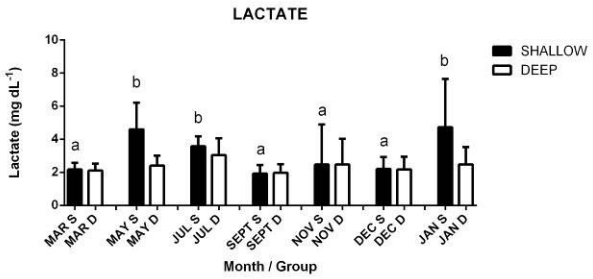


Fig. 20.2.18. Lactate levels during the period from March 2015 to January 2016 (as in **Fig. 20.2.16**). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Cortisol

The depth of the net had no effect on cortisol levels and there was no statistical interaction between net depth and month sampling. In general, cortisol levels remained at lower values which did not differ statistically for the period between March to November [(MAR S = 0.12 ± 0.1 ng ml^{-1} ; MAR D = 0.14 ± 0.1 ng ml^{-1}) ; (MAY S = 0.8 ± 0.04 ng ml^{-1}; MAY D = 0.9 ± 0.1 ng ml^{-1}) ; (JULY S = 0.27 ± 0.4 ng ml^{-1}; JULY D = 0.26 ± 0.4 ng ml^{-1}) ; (SEPT S = 0.13 ± 0.1 ng ml^{-1}; SEPT D = 0.15 ± 0.1 ng ml^{-1}) ; (NOV S = 0.25 ± 0.4 ng ml^{-1}; NOV D = 0.33 ± 0.3 ng ml^{-1})] but there was a statistically significant increase on cortisol levels in December (DEC S = 0.28 ± 0.8 ng ml^{-1}; DEC D = 0.53 ± 0.8 ng ml^{-1}) and January (JAN S = 0.68 ± 1.0 ng ml^{-1} ; JAN D = 0.17 ± 0.2 ng ml^{-1}), **Figure 20.2.19**.

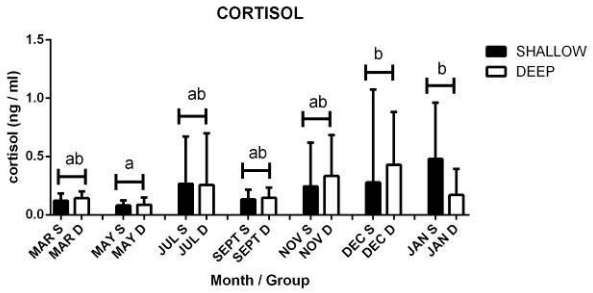


Fig. 20.2.19. Plasma cortisol levels during the period from March 2015 to January 2016 (as in **Fig. 20.2.16**). Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

Behavioral monitoring

Although the analysis of the data is not completed an interesting observation has been already made. The vertical distribution of meagre was mostly in the lower half of the cage for a period of approximately 12 hours while the rest of the period the meagre were distributed almost homogeneous in the whole available volume of the cage (**Figure 20.2.20**). This observation is independent of the cage depth and it is correlated with the light and dark period of the day. The pattern was repeated during the implementation period. To our knowledge this is the first time that such a behavior has been observed. The results will be analyzed after the termination of the trial in order to provide a better understanding on the meagre behavior in cages.

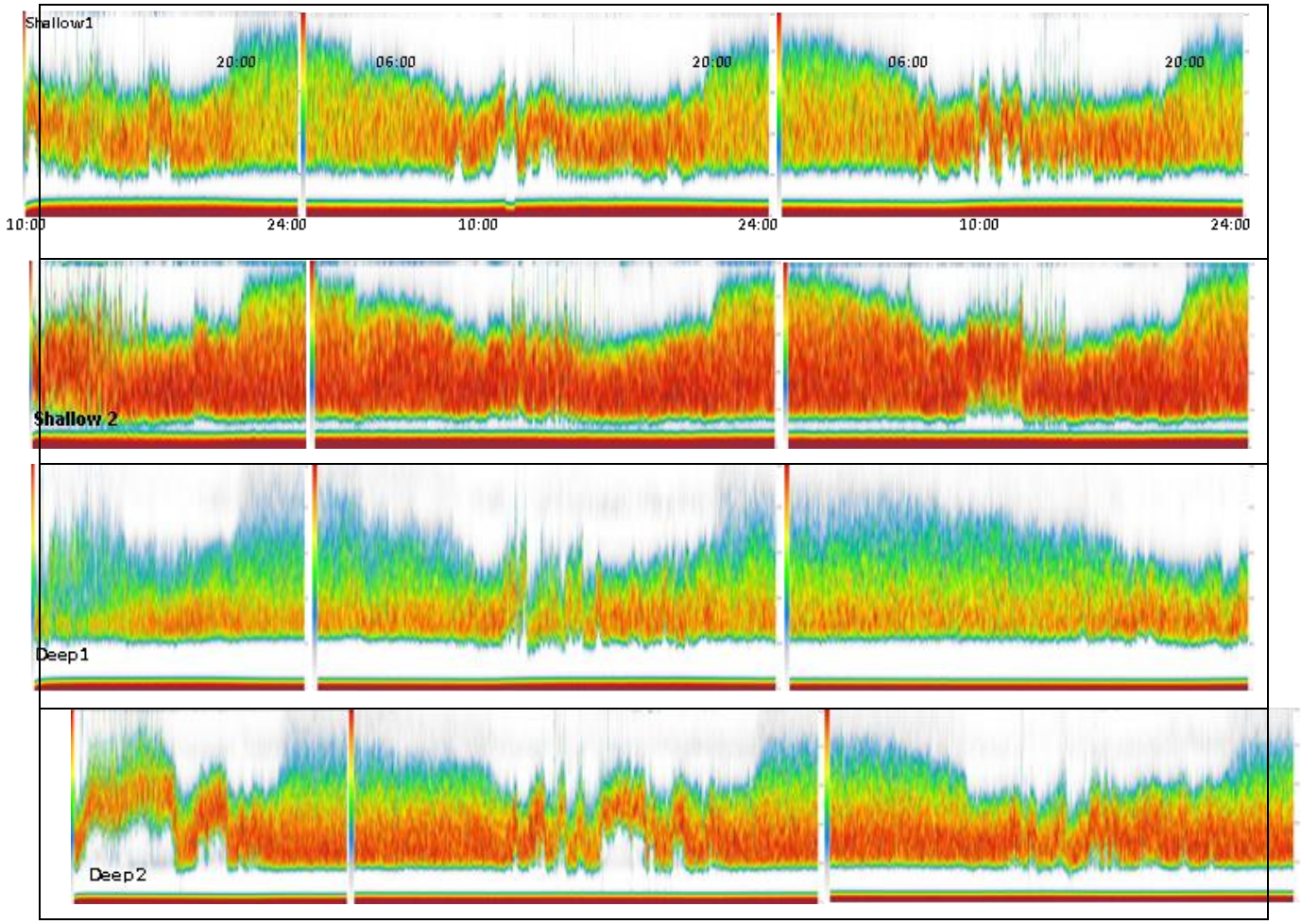


Figure 20.2.20 Vertical distribution of meagre in the experimental cages for a period of 3 days.

<u>Sub-task 20.2.2 Effect of light intensity in the cage (ARGO, Tasos Raftopoulos; HCMR, Nikos Papandroulakis)</u>

The objective is to test cage rearing with and without shading at the installations of P23. 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 are rectangular of 10x10x10 m. One of them is covered by net of 90-95% shading (**Figure 20.2.2.1**) while the second is covered only with a bird protecting net.

Figure 20.2.2.1 Experimental cages at P23. ARGO. Shaded (a) and not shaded (b).

Feed was provided daily by hand while weight samples were taken regularly to monitor the growth performance. For the behavior of the fish, the vertical distribution of the populations in cages has been monitored using an echo integrator (CageEye 1.3, Lindem Data Acquisition AS, Norway). The temperature profile of the area during the experimental period is presented in **Figure 20.2.2.2.**

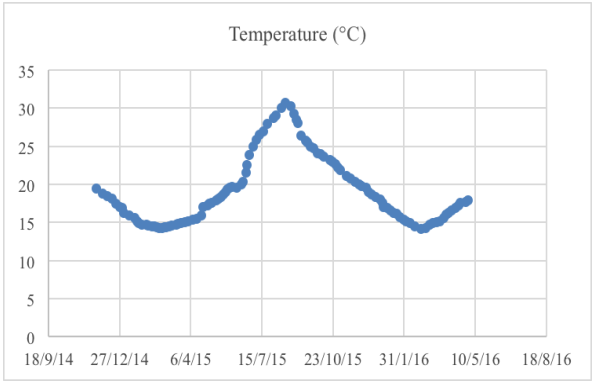


Figure 20.2.2.2 Temperature profile at 3 m depth at P3. ARGO.

Preliminary analysis of the results show that there was no significant difference between the populations neither in terms of survival (~90%), nor in terms of growth performance (**Fig 20.2.2.2**), although none of the groups performed well.

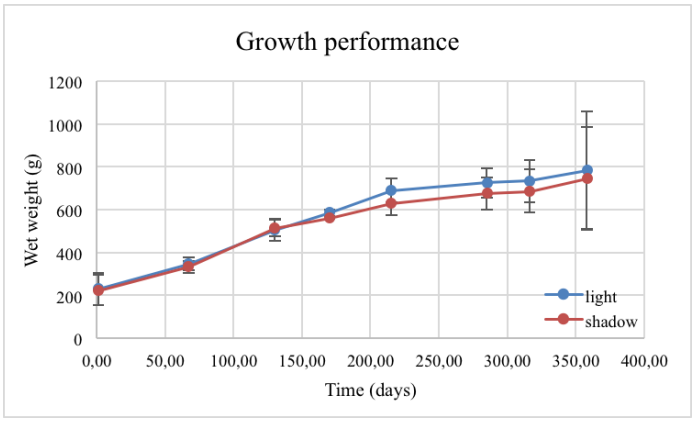


Figure 20.2.2.3 Growth performance of experimental groups. Error bars represent the SEM.

Regarding the behaviour of the groups, in terms of their vertical distribution in cages the pattern observed during day/night is repeated (**Figure 20.2.2.4**). Again observations are independent of the cage depth and are correlated with the light and dark period of the day. The second period will be initiated in June 2016, while the results will be also analysed further.

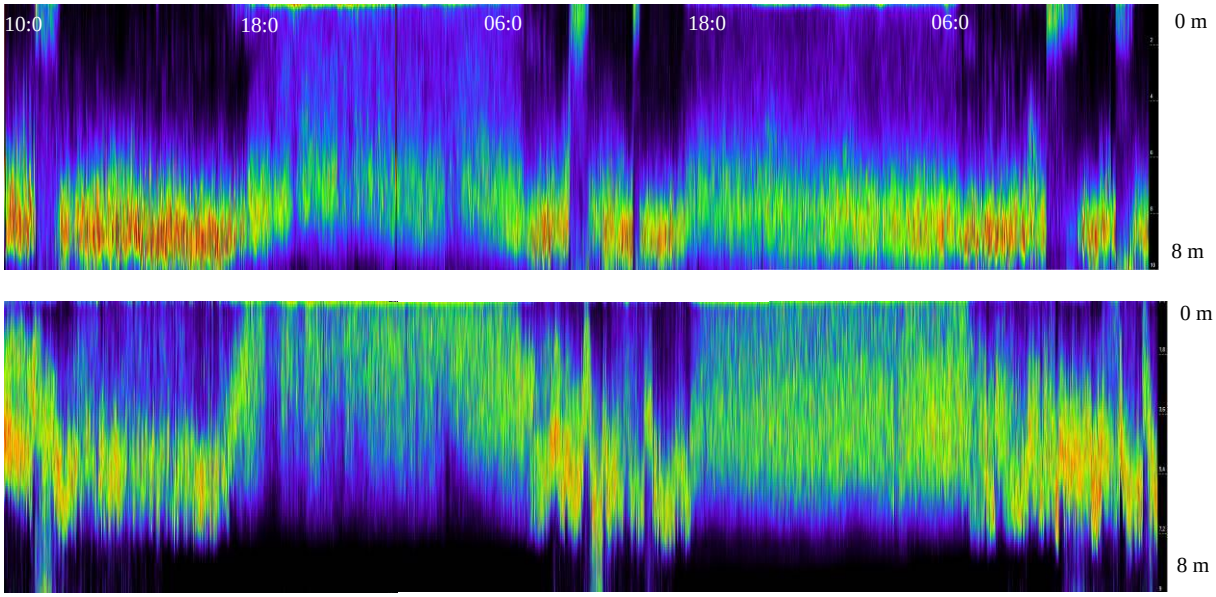


Figure 20.2.2.4 Echograms with the vertical distribution of the reared groups in (a) Not shaded and (b) shaded) cages

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).

Test of different feeding stimuli (mechanical, optical etc). Groups of two different size classes (50-100 and 700-900 g) at different tank sizes (500 and 5000 l respectively) were used for testing mechanical and optical feeding stimuli. Monitoring with video recordings was used and analysed allowing the definition of the optimal feeding stimuli.

First experiment with 50-100 g (in 500 l tanks)

The experiments, carried out during 2014-2015, focused on the effect of different stimuli on the feeding behavior of meagre. Two repeated experiments (Exp1, Exp2), 40 days duration each, with an intermediate pause of 1 month, were performed. The stimuli applied were light (L), air bubbles (A) and a combination of the two in different time-periods of the day. Each stimulus lasted 45 sec. Five seconds before the stimuli stopped an automatic electric feeder was activated, providing a constant amount of food pellets.

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 responded positively to the combined stimuli as well (group 3, **Fig. 20.3.1.1**). Furthermore, the population of Exp 2 in which the air stimulus was applied, responded this time positively to the stimulus, in contrast to the population of Exp 1.

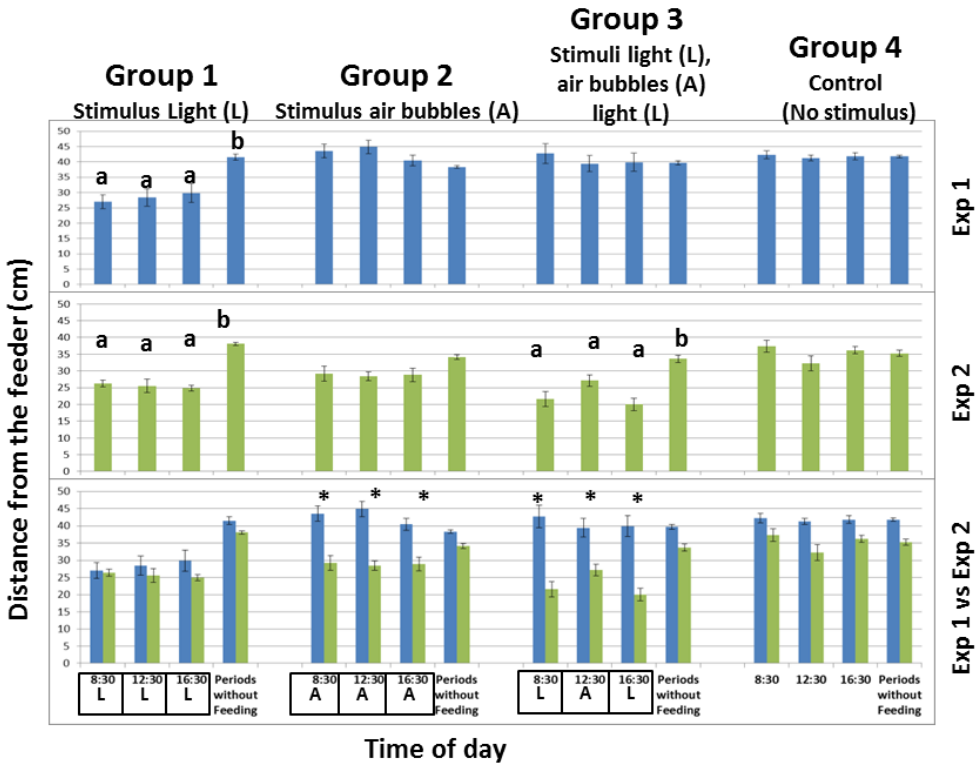


Figure 20.3.1.1. Evolution of the distance from the feeder during the different time periods and the different stimuli applications, in the experiments that were performed (values are mean ± SE). Latin characters (a, b) indicate differences between the different times of day and asterisks (*) indicate differences between experiments (ANOVA, Duncan test, P<0.05).

Second experiment in 700-900 g (in 5000 l tanks)

The experiments, carried out during 2015, focused on the effect of different stimuli on the feeding behavior of larger individuals of meagre (700-900 g) and were performed in outdoor 5000 l tanks in duplicate with a duration of 40 days. The stimuli applied were light (L), air bubbles (A) and a control condition without any stimulus. Each stimulus lasted 45 sec. Five seconds before the stimuli stopped an automatic electric feeder was activated, providing a constant amount of food pellets.

The preliminary results (data not shown) indicate that the stimulus of air bubbles had a direct positive effect on the population since it was shown that during its implementation fish gathered in the feeding area. In comparison the fish groups stimulated with light responded with 5-7 days delay. An important observation during this experiment is related to the effect of the weather condition and especially the sun light intensity on meagre feeding behavior. The constant daily movement of sun during the day resulted to areas with different light intensity in the tank, being bright or shaded. During the sunny days the fish in all replicates prefer to stay in the shadow.

The difference between light and air-bubble stimuli was more pronounced during the mid-day (12:30 am) feeding on sunny days. Although feed was distributed in a bright area, the air stimuli attracted the reared groups (which preferred to remain at the shaded area) and motivated them more intensive, than the light stimuli.

Based on this study, we can conclude that

- meagre is able to learn, to be trained and to remember specific stimuli that are associated with feeding time.

- Light is an acute stimulus to 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 is evident from the second day of its application.
- The environmental conditions and particularly light intensity affect the meagre feeding behavior.
- Both of stimuli (mechanical and optical) can be used in industrial scale as they be created, implemented and managed easily with existing technologies in the sea cages.

These behaviors are indicated for the first time in meagre.

Sub-task 20.3.2 Test of different feeding methods (HCMR, Yiannis Papadakis).

This task has not started yet and it will be planned after the implementation of subtasks 20.3.1.

Sub-task 20.3.3 Test in cages of 2 feed distribution methods (HCMR, Nikos Papandroulakis).

Although this task has not started yet and it will be planned after the implementation of Subtasks 20.3.1 and 20.3.2 some preliminary trials were performed at the cages of P1. HCMR based on the first results of Task 20.2. After observing the changes in the group behavior during the night a pre-trial was made to test whether night-feeding was an option for meagre. In **Figure 20.3.1** can be seen the feeding activity of the group during the dark period. Based on this finding a modification of the original plan was decided. Hence, 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.

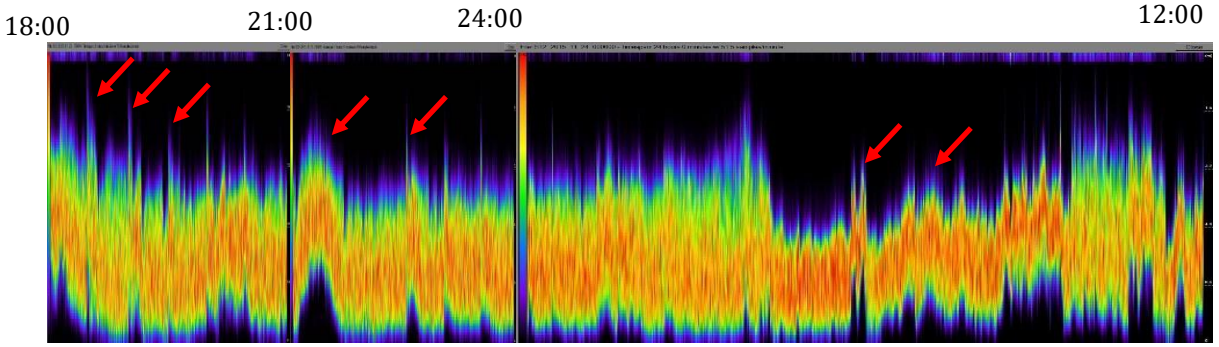


Figure 20.3.1 Echogram showing the vertical distribution of reared group. Red arrows indicate the group movements while feeding.

Sub-task 20.3.4 Comparison of hand and industrial demand type feeding in cages. (IRTA, Neil Duncan and Alicia Estevez)

Following the exit of P30. CULMAREX from the consortium (Amendment 2) the subtask was re-organized. In order to achieve the objectives set, i.e. to determine natural feeding rhythms and establish if these feeding rhythms improved growth performance (increase growth rate and decrease size dispersion) the trial will be performed in experimental scale at IRTA: Comparison in each season of the year of (a) demand feeding and (b) feeding with automatic feeders programmed to follow the feeding routines. Use three replicate control tanks (automated feeding) compared to three experimental demand feeding tanks. The parameters to be evaluated would be: feeding time, feed delivered, growth, size variation in the population, FCR, pattern of

fish activity, level of aggressive behaviours and fin condition. Experimental conditions will be natural photoperiod and simulated natural temperature controlled to be similar to sea cage growing areas for the specific season.

- Obtain 10 g juveniles in June-July,
- Initiate experiment after 2-4 week acclimation and training with demand feeders,
- Continue experiment for 1 year with 6-8 week periods of monitoring in each season of the year.

The amended task is on schedule, systems have been set up and delivery of juveniles was programmed for early June 2016.

No deviation following the amendment.

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.

Summary of progress towards objectives and details for each task (13-30 Mo):

Four experiments were performed during the second period for (1) the definition of feeding pattern for 5 g fish that it is currently implemented, (2) the definition of feeding pattern 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 5 g. The main results achieved so far can be summarized as follows.

Juveniles of greater amberjack grew less when fed at 2.5% body weight d^{-1} compared to fish fed 3.5% BW d^{-1} or at apparent satiation. Furthermore animals fed one meal daily showed lower growth compared to those fed 3 or 4 meals per day. Similarly, FCR was higher for fish fed 2.5% body weight d^{-1} or once daily compared to the other conditions tested. For bigger individuals (200 g), between the feeding frequencies tested (1, 2, 3 and 7 meals d^{-1}), the better results in growth and feed conversion rates have been obtained with 7 meals daily. The absence of changes among the hematological and biochemical parameters suggests that greater amberjack juveniles were able to adapt to the different feeding frequencies under the particular culture conditions. However, results from immunological parameters reveal differences in the immune status among fish subjected to different feeding frequencies that could influence the health status of fish.

Environmental temperature significantly affects the performance of greater 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. In terms of fish length, there were no significant differences between fish held at 22ºC and 26ºC, but both groups were significantly larger than those held at 17ºC. The morphological analysis performed during the trial resulted in significant differences between the experimental conditions. 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. Also, the mean values of caudal propulsion efficiency differed among the groups, noting higher propulsion of fish as temperature increases. The specimens reared at 26ºC showed significant swimming differences compared to the individuals reared at 17ºC and 22ºC whilst, there was no difference between the later.

Regarding the stocking density, for greater amberjack juveniles, the conditions tested was for values 3.66±0.46, 5.74±1.20 and 7.41±0.17 kg m^{-3} for Low (LD), Medium (MD) and High (HD) densities, respectively. 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.

These results are contributing to the overall objectives of the work package together with the expected outcomes from the trials in industrial scale (Task 21.1) in order to define the optimum conditions for the rearing of greater amberjack in cages.

Task 21.1 Development of rearing method in cages (led by FCPCT, Lidia Robaina)

Action 21.1.1 Effect of rearing volume (depth) on performance.

This Action has not started yet. It is expected to start after the reproductive period of 2016 (Mo 31-32) and the production of an adequate number of juveniles, which has been achieved at the time this report was being prepared (~150,000 juveniles produced).

Action 21.1.2 Effect of cage type on performance.

A comparison of surface and submerged cages will be performed in trials with commercial cages (P28. CANEXMAR), for 2 successive rearing periods of 12 months each. The final stocking density will be kept at 15 kg m^3. Growth performance and health status will be estimated every second month (P2. FCPCT). Partner 28. CANEXMAR has finally solved the bureaucratic problems to keep the greater amberjack in their facilities. A total of 3,000 juvenile amberjack of 100 g body weight were produced with the standardized methodology for greater amberjack in 2015 and were placed in an experimental cage of 5x5 m (**Figure 21.1.2.1),** waiting for the actual trial which is expected to start by the end of Aug 2016.

Figure 21.1.2.1 Transport of the amberjack juveniles to the experimental cage in P28. CANEXMAR.

Task 21.2 Development of feeding methods (led by IEO, Salvador Jerez).

Test of different feeding methods including estimation of daily rhythm and frequency (continuous vs fixed ratios) will be tested with individuals at different developmental stages of juveniles (5 g and 200 g individuals).

Action 21.2.1 Definition of feeding pattern for 5 g fish reared in 500 l-tanks for 4 months (led by FCPCT, Lidia Robaina).

An experiment is currently being conducted to define feeding pattern of greater amberjack juveniles. The duration of the trial will be 4 months. Currently the trial is in its 2nd month. Six hundred greater amberjack (20 g initial body weight) at P2. FCPCT were randomly distributed within 24 x 500-l fiberglass tanks (25 fish per tank). Water temperature was 21.10±1.44˚C. Fifteen additional fish (3 pools of 5 fish) were sacrificed and frozen at -80ºC for whole body biochemical composition analysis.

Figure. 21.2.1.1. Experimental tanks at P1. FCPCT.

Fish are fed with a commercial diet (R-5 EUROPA 22, Skretting, Burgos, Spain) and 8 different feeding strategies (triplicate tanks for each strategy) were defined as shown in **Table 21.2.1.1**.

Table 21.2.1.1 Feeding strategies applied.

	Treatment nº	Ratio	Nº of meals per day
Fed by hand	1	Apparent satiation	3
	2	Apparent satiation	1
Automatic feeder	3	3.5% B.W.	3
	4	3.5 % B.W.	4
	5	3.5 % B.W.	1
	6	2.5 % B.W.	3
	7	2.5 % B.W.	4
	8	2.5% B.W.	1

Feeding time was decided to be at 8:30, 11:30, 14:00, 16:00 for each experimental group (1, 3 or 4 meals per day). Those treatments with one meal per day were fed at 8:30 A.M. Those treatments fed with automatic feeders, these were programmed to distribute the amount of feed corresponding to the fish biomass previously calculated. Each month, all fish of each tank were anesthetized with clove oil and individual body weight and length were obtained. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated following monthly sampling using as follows: SGR = (Ln(final weight)- Ln(initial weight))*100/feeding time (days) and FCR= (total feed fed/total weight gained).

Preliminary Results

The experiment is currently in progress (May 2016, Mo 30). Fish fed at 2.5% body weight per day showed lower ($P<0.05$) growth at the second month of the trial when compared with fish fed 3.5% BW or fed at apparent satiation. Besides, those animals fed only one meal daily showed lower ($P<0.05$) growth when compared with those fed at 3 or 4 meals per day (**Table 21.2.1.2**). There were no significant differences on SGR after 2 months of different feeding patterns, but FCR was higher ($p<0.05$) in fish fed 2.5%, whereas fish fed only 1 meal per day showed also significant ($p<0.05$) higher FCR when compared with the other experimental groups (**Table 21.2.1.3**)

Table 21.2.1.2. Fish growth (g body weight) after 2 months of different feeding patterns (Mean ± SD). Different letter within a column denote significant ($p<0.05$) differences.

		M0	M1	M2
Apparent satiation	1 meal	21.78 ± 1.73	57.38 ± 11.10a	90.81 ± 19.13b
	3 meal	21.96 ± 1.79	60.51 ± 11.38a	100.69 ± 21.90a
3.5% B.W.	1	22.47 ± 1.65	57.80 ± 10.28a	100.73 ± 20.84a
	3	22.16 ± 1.64	58.57 ± 11.31a	102.90 ± 22.31a
	4	22.22 ± 1.65	57.50 ± 2.31a	103.58 ± 27.90a
2.5% B.W.	1	22.14 ± 2.36	52.14 ±8.92b	92.64 ± 22.50b
	3	21.93 ± 1.79	48.54 ± 6.08b	88.60 ± 16.18b
	4	22.32 ± 2.00	51.23 ± 7.30b	95.32 ± 19.04ab

Table 21.2.1.3. Fish growth (SGR) and FCR after 2 months of different feeding patterns.

		M0	M1	M2
Apparent satiation	1 meal	21.78 ± 1.73	57.38 ± 11.10a	90.81 ± 19.13b
	3 meal	21.96 ± 1.79	60.51 ± 11.38a	100.69 ± 21.90a
3.5% B.W.	1	22.47 ± 1.65	57.80 ± 10.28a	100.73 ± 20.84a
	3	22.16 ± 1.64	58.57 ± 11.31a	102.90 ± 22.31a
	4	22.22 ± 1.65	57.50 ± 2.31a	103.58 ± 27.90a
2.5% B.W.	1	22.14 ± 2.36	52.14 ±8.92b	92.64 ± 22.50b
	3	21.93 ± 1.79	48.54 ± 6.08b	88.60 ± 16.18b
	4	22.32 ± 2.00	51.23 ± 7.30b	95.32 ± 19.04ab

Action 21.2.2 Definition of feeding pattern for 200 g reared in 500 l-tanks for 4 months (led by IEO, Salvador Jerez, Virginia Martín, Marta Arizcun, Elena Chaves, Veracruz Rubio).

Monitoring included growth performance, feed efficiency, k index, juvenile quality (morphological aspects) and haematological, histological, biochemical and immunological analysis. The appropriate feeding strategy is important in grow-out operation. Among the different feed management practices proved to maximize the benefit of feeding, feeding frequency has an important role in regulating the feed intake, growth and waste outputs of fish. Optimizing feeding frequency may improve growth, feed intake, feed conversion, welfare and survival, minimizing feed wastage, leading to improvement in culture environment and or reduction in size heterogeneity. All these problems result in decreased production efficiency which ultimately increases cost of production. However, optimum feeding frequency varies depending on the fish species, size and rearing system and a lack of information exists in this regard for greater amberjack.

To achieve this objective, different feeding frequencies have been tested in greater amberjack juveniles (~200 g) cultured in P8. IEO facilities during 2015. The juveniles were tagged with a passive integrated transponder (PIT) and randomly divided into 12 homogeneous groups. Each three groups were fed during four months at a feeding frequency of either 1, 2, 3 and 7 meals per day, resulting in 4 treatments by triplicate. Fish were sampled monthly and the daily feed intake was recorded. At the sampling time, blood and mucus samples were collected to evaluate the influence of feeding strategies tested on immunological, hematological and biochemical parameters indicative of the health status, nutritional condition and welfare of fish. At the beginning and end of the study samples of fish tissue were obtained to oxidative stress and condition studies.

Results

The results showed that at day 60, the Specific Growth Rate (SGR) tended to increase with the increasing of the feeding frequency, but significant differences were only found between fish groups fed 7 meals per day that showed a higher value than fish groups fed once per day. However, between 60 and 120 day, this tendency changed, and the SGR was similar in all feeding strategies tested. In the overall period (0-120 days) the fish fed 1 meal per day showed the significantly lower SGR. The other three feeding strategies (2, 3 and 7 meals per day) showed a similar SGR **(Fig. 21.2.3.1)**. The condition factor index (CF) was similar during the first 90 days irrespective of the feeding strategy tested, but at the end (120 days) the fish fed 1 meal per day showed a significantly lower CF and Hepatosomatic Index (HSI) **(Table 21.2.3.1)**.

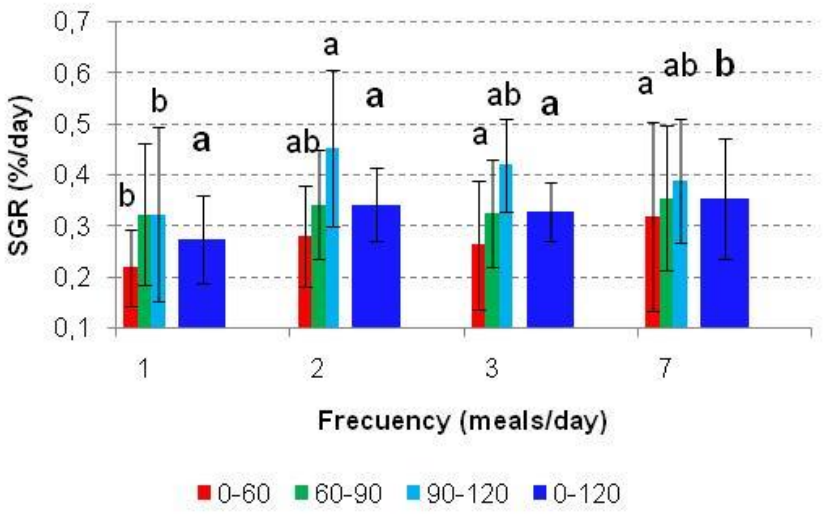


Figure 21.2.3.1. Specific growth rate SGR (% day^{-1}) at the different periods and overall duration (120 days) of fish fed at 1, 2, 3 and 7 meals day^{-1}. Different letter indicates significant differences (P<0.05).

Table 21.2.3.1. Condition factor (CF) (g cm^{-3}) at the different periods and overall duration (120 days) and Hepatosomatic (HSI) and Viscerosomatic Index (VSI) at the end of the study of fish fed at 1, 2, 3 and 7 meals day^{-1}.

Frequency	1 meal d^{-1}		2 meals d^{-1}		3 meals d^{-1}		7 meals d^{-1}	
Period	mean	sd	mean	sd	mean	sd	mean	sd
CF 0	2.116	± 0.134	2.130	± 0.125	2.094	± 0.109	2.145	± 0.120
CF 60	1.919	± 0.089	1.911	± 0.149	1.917	± 0.124	1.945	± 0.156
CF 90	1.920	± 0.106	1.933	± 0.124	1.909	± 0.114	1.961	± 0.129
CF 120	1.826	± 0.111 b	1.889	± 0.134 ab	1.834	± 0.092 ab	1.905	± 0.130 a
HSI 120	0.491	± 0.043 b	0.677	± 0.221 ab	0.726	± 0.067 a	0.687	± 0.131 ab
VSI 120	3.198	± 0.565	3.346	± 0.951	3.204	± 0.275	3.136	± 0.469

Data were presented as mean ± S.D. Different letter indicates significant differences (P<0.05).

The feed intake (% of body weight per day) was significantly lower in the fish fed 7 meals per day in respect to all the other groups, at 60, 90 and 120 days, suggesting a greater feed efficiency for this feeding frequency. In the overall period of study the fish fed 7 meals per day showed the lowest (P<0.05) feed intake of all treatment tested **(Figure 21.2.3.2)**.

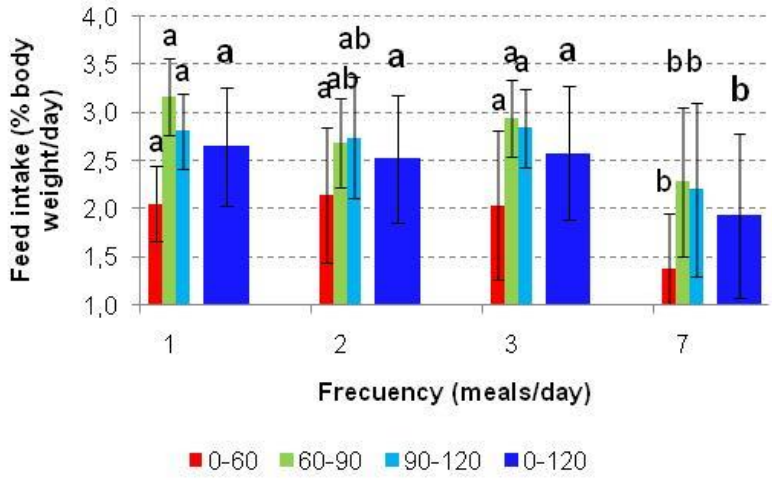


Figure 21.2.3.2. Feed intake (% body weight day^{-1}) at different period and overall duration (120 days) of fish fed at 1, 2, 3 and 7 meals day^{-1}. Different letter indicates significant differences among feeding groups (P<0.05).

The daily feed intake was significantly lower in all fish groups during the first month. Moreover the feed intake at each feeding time (time periods of 8:00-9:00, 10:00-14:00 and 15:00-19:00) did not change in fish fed at 2, 3 and 7 meals day^{-1}. During the following months, the feed intake at each feeding time changed with the number of daily meals offered and the fish tended to ingest more feed at more advanced feeding times when the feeding frequency increased from 2 to 7 meals day^{-1} **(Fig. 21.2.3.3)**.

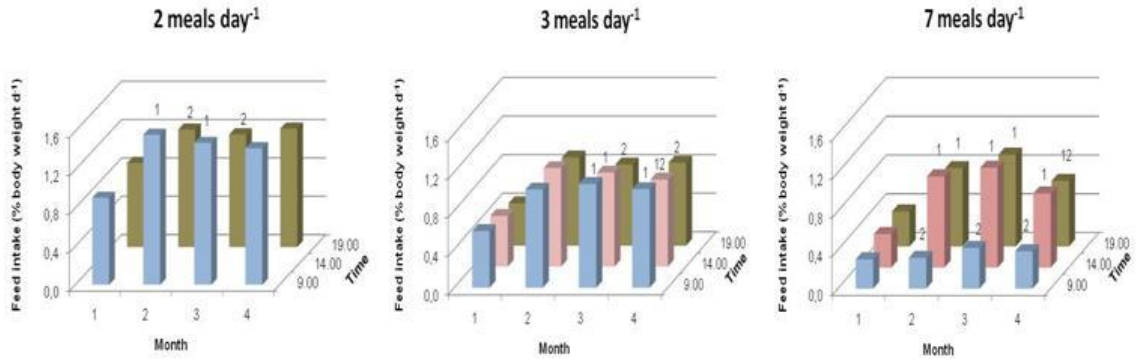


Figure 21.2.3.3. Feed intake (% body weight day^{-1}) at the different months and feeding times (periods) of fish fed at 2, 3 and 7 meals day^{-1}. Different numbers indicates significant differences ($p<0.05$).

Table 21.2.3.2. Effect of feeding frequencies on erythrocytes (x10^5), leucocytes (x10^3), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), glucose (mg/dl). Data collected at 0, 60, 90 and 120 days of the assay.

Initial	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	184.79 ± 53.58	159.97 ± 36.49	164.31 ± 36.20	166.65 ± 53.42
Leucocytes	21.53 ± 5.69	17.11 ± 0.62	21.47 ± 9.92	19.20 ± 3.89
Hematocrit	39.13 ± 2.65	24.00 ± 12.26	31.82 ± 4.79	31.15 ± 16.06
Triglycerides	173.16 ±70.15	198.66 ± 211.53	133.86 ± 61.40	179.09 ± 106.09
Cholesterol	204.56 ± 20.54	182.25 ± 28.56	222.05 ± 77.14	176.37 ± 73.20
Protein	25.03 ± 4.02	30.61 ± 3.26	37.49 ± 16.19	38.87 ± 2.10
Glucose	31.29 ± 13.11	63.18 ± 37.08	90.89 ± 2.38	55.41 ± 15.18
60 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	173.44 ± 32.44	152.55 ± 45.84	196.98 ± 32.31	157.36 ± 18.21
Leucocytes	70.27 ± 56.92	81.48 ± 62.53	108.83 ± 88.94	83.06 ± 65.94
Hematocrit	35.00 ± 0.66	36.18 ± 9.79	35.27 ± 2.91	28.23 ± 7.22
Triglycerides	127.70 ± 37.04	154.89 ± 41.53	95.40 ± 27.65	109.35 ± 75.45
Cholesterol	342.04 ± 79.35	289.77 ± 81.41	305.8 ± 42.55	207.75 ± 82.84
Protein	27.92 ± 5.19	38.07 ± 6.01	38.60 ± 9.11	34.67 ± 10.30
Glucose	58.50 ± 26.61	37.84 ± 14.18	44.84 ± 8.08	42.75 ± 6.88
90 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	229.43 ±20.38	242.33 ±25.05	242.46 ±55.55	242.07 ±97.78
Leucocytes	141.78 ±53.86 a	37.65 ±17.53 b	24.47 ±10.85 b	23.73 ±8.63 b
Hematocrit	37.65 ± 2.38	36.38 ± 6.42	41.31 ± 4.17	34.98 ± 2.93
Triglycerides	142.13 ± 39.44	172.51 ± 16.95	171.84 ± 28.49	157.87 ± 40.41
Cholesterol	243.39 ± 18.13	238.66 ± 12.02	215.4 ± 29.19	223.27 ± 45.23
Protein	36.83 ± 3.03	38.60 ± 0.78	40.15 ± 3.87	35.45 ± 3.79
Glucose	39.98 ± 9.17	53.17 ± 14.62	45.62 ± 22.32	53.41 ± 3.68
120 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	171.77 ±3.56	257.06 ±24.49	251.47 ±23.57	235.85 ±35.45
Leucocytes	119.41 ±52.66	99.74 ±22.64	92.62 ±58.56	62.15 ±1.91
Hematocrit	38.01 ±5.55	39.21 ±0.71	34.98 ±3.37	38.6 ±0.27
Triglycerides	107.65 ±21.67	114.74 ±43.91	114.16 ±29.10	77.75 ±34.04
Cholesterol	224.76 ±27.83 b	292.76 ±14.56 a	266.61 ±14.57 ab	235.39 ±12.28 b
Protein	48.3 ±6.63 a	36.88 ±3.25 a	37.60 ±2.24 ab	31.22 ±3.35 b
Glucose	137.68 ±16.00 a	144.24 ±22.32 a	102.95 ±8.18 b	102.42 ±17.59 b

Data were presented as mean ± S.D. (n=5; 15 fish/group). Different letter indicates significant differences ($P<0.05$).

Hematological and biochemical parameters registered at 0, 60, 90 and 120 days of the trial for the four different feeding frequencies assayed are shown in **Table 21.2.3.2.** At the beginning of the trial, the 12 fish groups were homogeneous in blood biochemical indicators. Both hematological and biochemical parameters obtained in the present experiment are considered to be within the normal range for juvenile amberjack, compared to those of the previous findings (Kawanago et al., 2014; Dawood et al., 2015). At the end of the growth period, significant differences were not found between groups fed with different frequencies. All blood parameters studied remained constant in all groups of fish and only protein, cholesterol and glucose were slightly higher in the 2 meals per day fish group.

During the trial, fish were infected by the monogenean *Zeuxapta seriolae*, which caused mortality of 27% between the fourth and the fifth week. No significant differences were found in the mortality percentage between fish fed with different feeding frequencies. However, significant changes in number of leucocytes were observed along the trial possibly because of infestation.

Antioxidant enzymes were determined at the beginning (Initial) and at the end of the assay in liver muscle, gill and brain from fish fed with different feeding frequencies assayed (**Table 21.2.3.3**). The results showed several differences in antioxidant defenses comparing among feeding frequencies groups for all tissues analyzed. Thus, catalase activity was lower in 1 meal per day group in both liver and gills. Several differences among feeding groups were also observed at GPx and GST for all tissues analyzed.

Immunological parameters from serum of fish fed with different feeding frequencies at 60, 90 and 120 days are shown in **Figure 21.2.3.4**. Bactericidal and peroxidase activities were significantly lower in 1 meal per day fish after 90 days. Also protease activity was lower in this group at the end of the assay (120 days). Data of immunological parameters and oxidative stress enzymes are currently being comprehensively analyzed.

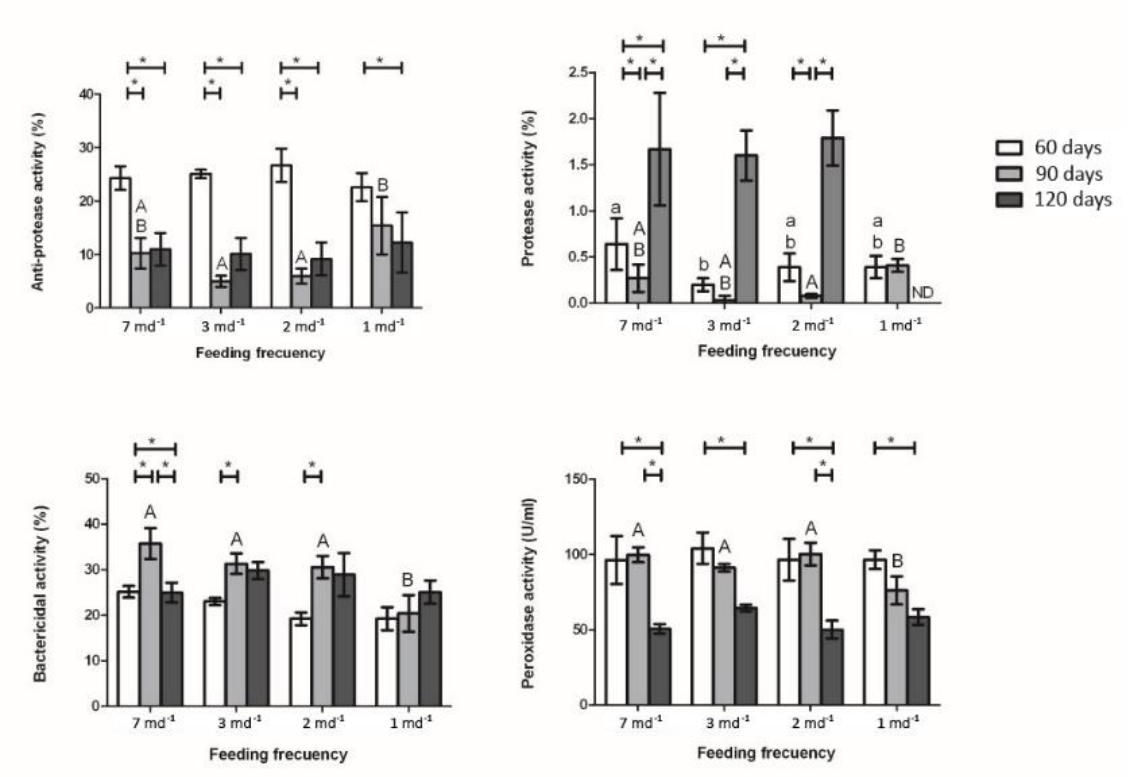


Figure 21.2.3.4. Effect of feeding frequencies on antiprotease (%), protease (%), bactericidal (%) and peroxidase (antioxidant status of liver, muscle, gill and brain from greater amberjack. Data were presented as mean ± S.E.M (n=5-15 fish/group). Different letters denote statistically significant differences (ANOVA, Fisher's LSD post-hoc test $P \leq 0.05$).

Table 21.2.3.3.. Effect of feeding frequencies on antioxidant status of liver, muscle, gill and brain from greater amberjack. Data collected at the beginning (initial) and at the end (120 days) of the assay.

Liver	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	201.9 ± 19.8	213.5 ± 9.8	191.5 ± 13.3	188.6 ± 16.3	182.4 ± 6.1
CAT[1]	63.0 ± 5.8 ^{a}	55.4 ± 4.7^{a}	106.6 ± 6.4^{b}	122.8 ± 14.3^{b}	110.9 ± 3.8 ^{b}
SOD[1]	867.2 ± 40.5 ^{a}	725.7 ± 39.7 ^{ab}	909.8 ± 44.3^{a}	599.0 ± 48.4^{b}	770.0 ± 38.6^{ab}
GPx[2]	28.5 ± 1.0 ^{a}	34.1 ± 2.7 ^{ab}	40.6 ± 2.3 ^{b}	38.0 ± 2.2 ^{b}	33.2 ± 0.5 ^{ab}
GR[2]	85.6 ± 2.3	78.1 ± 2.9	79.8 ± 3.6	80.6 ± 5.6	79.5 ± 3.6
GST[2]	60.1 ± 3.8 ^{a}	84.3 ± 4.1 ^{bc}	90.1 ± 4.0 ^{c}	68.5 ± 3.9 ^{ab}	73.8 ± 3.9 ^{abc}

Muscle	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	117.9 ± 12.3 ^{a}	58.6 ± 3.3 ^{c}	23.0 ± 2.6 ^{b}	15.7 ± 3.0 ^{b}	25.8 ± 2.0 ^{b}
SOD[1]	8.6 ± 1.0 ^{a}	13.0 ± 0.5 ^{b}	13.3 ± 0.4 ^{b}	13.8 ± 0.3 ^{b}	12.7 ± 0.5 ^{b}
GPx[2]	6.5 ± 0.9 ^{a}	11.3 ± 1.2 ^{c}	16.8 ± 1.1 ^{b}	16.6 ± 0.5 ^{b}	14.2 ± 0.9 ^{bc}
GR[2]	130.4 ± 6.5 ^{ab}	127.5 ± 3.1 ^{b}	150.0 ± 6.6 ^{a}	141.7 ± 3.3 ^{ab}	135.8 ± 5.2 ^{ab}
GST[2]	16.3 ± 1.0 ^{a}	3.3 ± 0.3 ^{c}	2.9 ± 0.4 ^{bc}	3.3 ± 0.4 ^{bc}	5.5 ± 0.6 ^{b}

Gill	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	80.4 ± 7.0 ^{a}	46.6 ± 8.7 ^{b}	46.1 ± 9.7 ^{b}	48.5 ± 5.6 ^{b}	29.21 ± 3.5 ^{b}
CAT[1]	12.6 ± 0.4 ^{a}	13.9 ± 0.6 ^{ac}	20.0 ± 0.8 ^{b}	15.8 ± 0.7 ^{bc}	20.8 ± 0.6 ^{b}
SOD[1]	14.7 ± 1.0 ^{a}	19.0 ± 0.6 ^{b}	20.5 ± 1.0 ^{b}	19.5 ± 0.5 ^{b}	21.4 ± 1.1 ^{b}
GPx[2]	241.7 ± 14.2 ^{a}	213.7 ± 13.0^{ab}	201.7 ± 19.1^{ab}	176.4 ± 7.5 ^{b}	228.8 ± 16.1^{ab}
GR[2]	102.0 ± 9.3	98.1 ± 2.0	102.1 ± 2.5	101.0 ± 5.5	93.2 ± 1.6
GST[2]	113.0 ± 10.1 ^{a}	43.1 ± 5.9 ^{b}	36.9 ± 2.7 ^{b}	64.9 ± 10.3 ^{c}	39.1 ± 3.1 ^{b}

Brain	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	178.0 ± 5.7	172.7 ± 4.6	177.4 ± 7.7	167.4 ± 4.7	179.0 ± 8.3
CAT[1]	12.5 ± 0.7	16.6 ± 0.8	12.6 ± 0.7	16.6 ± 0.6	15.5 ± 1.0
SOD[1]	14.5 ± 0.3	15.2 ± 0.3	14.7 ± 0.3	14.8 ± 0.8	16.3 ± 0.2
GPx[2]	18.9 ± 1.0 ^{a}	19.4 ± 0.5 ^{a}	20.5 ± 0.7 ^{a}	20.7 ± 1.0 ^{a}	25.2 ± 0.7 ^{b}
GR[2]	320.0 ± 7.2	318.7 ± 7.1	334.0 ± 7.0	351.3 ± 8.0	326.2 ± 6.0
GST[2]	15.4 ± 1.1 ^{a}	20.1 ± 1.7 ^{ab}	19.5 ± 1.1 ^{ab}	21.5 ± 1.8 ^{ab}	26.6 ± 2.9 ^{b}

FRAP: Ferric reducing antioxidant power; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase and GST: Glutathione S-transferase which units are ([1]) U mg P^{-1} and ([2]) mU mg P^{-1}. Data were presented as mean ± S.E.M (n=5; 15 fish/group). Different letters indicate significant differences (ANOVA, Tukey's test, P<0.05)

Conclusions

In general terms, between the feeding frequencies tested here for *Seriola dumerili* juveniles, the best results in growth and feed conversion rates have been obtained with 7 meals per day. The absence of changes among the hematological and biochemical parameters suggests that greater amberjack juveniles were able to adapt to the different feeding frequencies under the particular culture conditions. However, results from immunological parameters reveal differences in the immune status among fish subjected to different feeding frequencies that could influence the health status of fish.

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).

The trial was undertaken with different size individuals (starting at 5, 200 and 500 g), all trials will be conducted in triplicates. Rearing will be realized in 500-l, for the first two sizes, and 10 m^3 for the third size, tanks at 2 different temperature ranges (a) 14-17°C representing the lower temperatures observed in Mediterranean open sea and (b) 26-29°C representing the upper temperatures observed in Mediterranean open sea. The trial with the 5 g and the 500 g individuals will be conducted at the facility of FCPCT and the one with the 200 g individuals at P1. HCMR. The duration of the trial will be 4 months. Monitoring will include growth performance, feeding activity, gut transit time, digesta sample analysis (protein, fat, dry matter, apparent digestibility, energy) and protease, trypsin, chymotrypsin, lipase enzyme activities. The experiment with smaller fish (5 g) has been completed.

Material and methods

The present study was conducted at P1. FCPCT. The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals and have been approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria.

Experimental fish and Experimental conditions

Fish were selected from the weaning tanks with 5 g body weight, but due to their high susceptibility to stress at this stage, they presented some stress symptoms (lost of appetite, some mortality) and were kept for 15 days (as protocols of fish welfare indicate) and then translated to the experimental tanks. Two hundred and twenty five greater amberjack juveniles of 19.5 ± 4.1 g body weight and 9.8 ± 0.7 cm total body length were distributed in 9 cylindroconical 500 l tanks (25 individuals per tank). The three temperature treatments, 17, 22 and 26 ºC, where assayed by triplicate. Systems with three tanks of a given temperature were controlled by one RAS. Oxygen levels were similar among the different tanks around 7.8 mg l^{-1}. Fish were fed to apparent satiety three times per day during 120 days with a commercial diet (Europa 22, Skretting, Burgos, Spain) with 52% of crude protein and 20 % crude lipids.

Sampling procedures

At the beginning of the experiment and before placing the 25 fish per tank, samples of whole fish (same weight and length than those used in the experiment) were collected and frozen at -20ºC for initial whole body biochemical composition analysis. Data of body weight and length was collected each 30 days after anaesthesia with clove oil, while feeding data was collected every day. Besides, at the end of the experimental period, measurements for morphometric analysis were taken from all experimental fish by photography of all fish. Three fish from each tank (9 per treatment) were sacrificed with anaesthetic overdose to collect liver and intestine for biochemical and histological analysis.

The rest of the animals were used in a gastric evacuation time assay. Animals (18 per experimental tank) were kept fasted during 48 h. After this period, animals were fed until apparent satiation and sampled after sacrifice with anesthetic overdose by dissection. Stomach and intestine were weighted after 2, 4, 8, 12, 18, 24 and 30 h after feeding. Organ content were calculated by weighting the feed content in both stomach and intestine after drying at 40ºC. Data were expressed as mg of meal (dry weight) per 100 g of fish.

Fish growth measurements

Specific Growth Rate (SGR) and feed Conversion Ratio (FCR) were calculated using as follows: SGR = (Ln(final weight)- Ln(initial weight))*100/feeding time (days) and FCR= (total feed fed/total weight gained). The daily growth index (DGI, × 100) as: $100 \times [(W1)^{1/3} - (W0)^{1/3}] \times (days)^{-1}$, where W0 and W1 are the initial and the final fish mean weights in grams. The protein efficiency ratio (PER) was calculated as weight gain (g) / protein ingested $(g)^{-1}$. The daily nutrient gain, (g kg^{-1} ABW day^{-1}) was calculated as: (final body nutrient content − initial body nutrient content) × ABW^{-1} × $days^{-1}$, where ABW was calculated as: (W1 + W0) / 2. Nutrient retention (%) was calculated as (final body nutrient content − initial body nutrient content) x N intake $fish^{-1}$ × 100. The hepatosomatic index (HSI, × 100) was calculated as: 100 × liver weight (g) × whole body weight $(g)^{-1}$ and the viscerosomatic index (VSI, × 100) as 100×viscera weight (g)×whole body weight $(g)^{-1}$.

Morphometric analyses. Body shape

Geometric morphometric analyses were performed to investigate the influence of temperature in the development of the body shape. All experimental fish were photographed using digital cameras (Fuji Finepix S2000HD, resolution 10.0 MP; Canon 50D, resolution 10.0 MP and macro lens F18/100). The body shape of each individual was analysed using a landmark-based method (Rohlf and Marcus, 1993). Twenty homologous landmarks and 2 semi-landmarks on the left side of body were selected (Fig. 21.3.1.1). The coordinates of these landmarks for each individual were acquired using the tpsDig2 software (Rohlf, 2004). A Generalized Procrustes Analysis (GPA) was performed (Rohlf and Slice, 1990; Dryden and Mardia, 1998) on the raw landmarks data to superimpose all specimens to a common location and remove the effects of size and orientation from landmark coordinates. TPS Small 1.28 software package (Rohlf, 2002) was used to evaluate the approximation of the distribution of the specimens in the Kendall's shape space relative to the linear tangent space for each analysed view (Dryden and Mardia, 1998). The correlation coefficient between tangent distances and the Procrustes distances was high (r= 1), indicating that the amount of shape variation was small enough to permit statistical analyses using only the Procrustes distances. Moreover, the arching effect was removed using the Burnaby's orthogonal projection (Valentin et al., 2008; Alós et al., 2014).

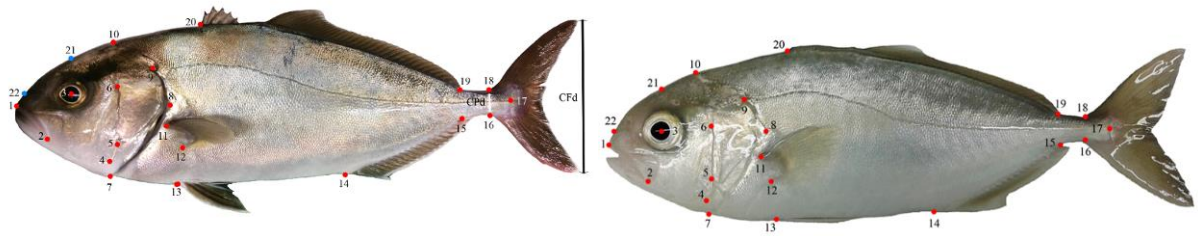


Figure 21.3.1.1. Position and meaning of landmarks (red) and semi-landmarks (blue). 1 anterior tip of the snout; 2 most posterior point of the premaxilla; 3 midpoint of eye; 4 ventral elbow of inter-operculum; 5 the point where praeoperculum, inter-operculum and suboperculum get in contact; 6 dorsal end of the preopercular groove; 7 ventral point of the operculum; 8 limit posterior of the operculum; 9 limit dorsal of the operculum; 10 dorsal margin of head directly above; 11 and 12 upper and lower insertion of the pelvic fin; 13 insertion of the ventral fin; 14 and 15 anterior and posterior insertion of the anal fin; 16 and 18 lower and upper insertion of caudal fin; 17 insertion of lateral line with midpoint of the hypural notch; 19 posterior insertion of the second dorsal fin; 20 anterior insertion of the first dorsal fin; 21 dorsal projection of eye; 22 anterior projection of eye. CFd caudal fin depth; CPd caudal peduncle minimal depth.

As the groups showed significant differences in the furcal length (ANOVA, F= 665, P< 0.0001), hence, a regression between natural logarithmic of furcal length and centroid size was performed (Ravosa and Profant, 2000; Collard and O'Higgins, 2001; Singleton, 2002) to examine the size effect on shape. Centroid size was computed as the square root of the sum of squared distances of a set of landmarks from their

centroid (Frost et al., 2003). Significance was evaluated by using a permutation test against the null hypothesis of independence (10,000 iterations).

A principal component analysis (PCA) was performed on the Procrustes coordinates to determine how the shape varies among groups. The resulting PCs are often termed 'relative warps' (RWs). This is mathematically equivalent to computing a PCA using the Procrustes coordinates of each specimen after GPA when a= 0 (Rohlf, 1993; Meloro et al., 2008). The changes related to size were done by multivariate regression of the PC scores of the Procrustes coordinates (dependent variables) on fish size (independent variable). All data were processed with the MorphoJ ver. 1.06d (Klingenberg, 2011) and PAST ver. 3.07 (Hammer et al., 2001) software packages. A sub-sample of 10 individuals was selected for each temperature to estimate the caudal propulsion efficiency through reduction of drag (CPE) (Webb, 1984). It was defined as the relation between caudal fin depth (CFd) and caudal peduncle minimal depth (CPd) (see **Figure 1**). A Kruskall-Wallis test was used to compare the CPE mean among the three groups followed by Dunnett's multiple comparison test using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Biochemical analyses

Biochemical composition of whole fish at the start and at the end of the trial for the three temperatures was analysed following standard procedures (AOAC, 2000). Besides, liver composition at the end of the experiment was also determined. Ash content was determined by combustion in a muffle furnace at 600°C for 12 h, moisture content was determined after drying at 105°C to constant weight, crude protein by acid digestion using Kjeldahl method (N × 6.25) and crude lipid was extracted following the method of Folch (Folch et al., 1953). All analyses were conducted by triplicate.

Histological analyses

For histology, liver was obtained from each fish (three fish per tank). Tissue samples were fixed in 10% buffered formalin for 1 or 2 days, dehydrated in a graded series of alcohol followed by one of xylene and finally embedded in paraffin wax. Three serial sections (4 μm) were then cut from each paraffin embedded sample and each processed for haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) and analysed at optical microscopy.

Statistical analyses

All data were tested for normality and homogeneity of variance. Means and SDs were calculated for each parameter measured. When required, data arcsine square root transformation was performed, particularly when data were expressed as percentage. Statistical analyses followed methods outlined by Sokal and Rolf (1995).. A One-way ANOVA test was conducted to analyse the effects of the temperature on growth performance, gut transit time and biochemical analyses. Significant differences were considered for $P < 0.05$. Analyses were performed using the SPSS Statistical Software System v20.0 (SPSS, Chicago, IL, USA) and R (version 3.1.0).

Results

Growth performance

After 120 days of feeding fish held at 26º C showed significantly ($P<0.05$) higher body weight compared with fish held at 22ºC. The significant differences between these two groups were significant ($P<0.05$) after 60 days of trial. Fish held at 17ºC showed the lowest ($P<0.05$) final body weight, showing differences with 22ºC and 26ºC group after 30 days of trial (**Figure 21.3.1.2**). Similar results were obtained with the specific growth rate (SGR) (Fig 21.3). In terms of fish length, there were no significant differences between fish held at 22ºC and 26ºC, both groups of animals significantly ($P<0.05$) larger than those held at 17ºC.

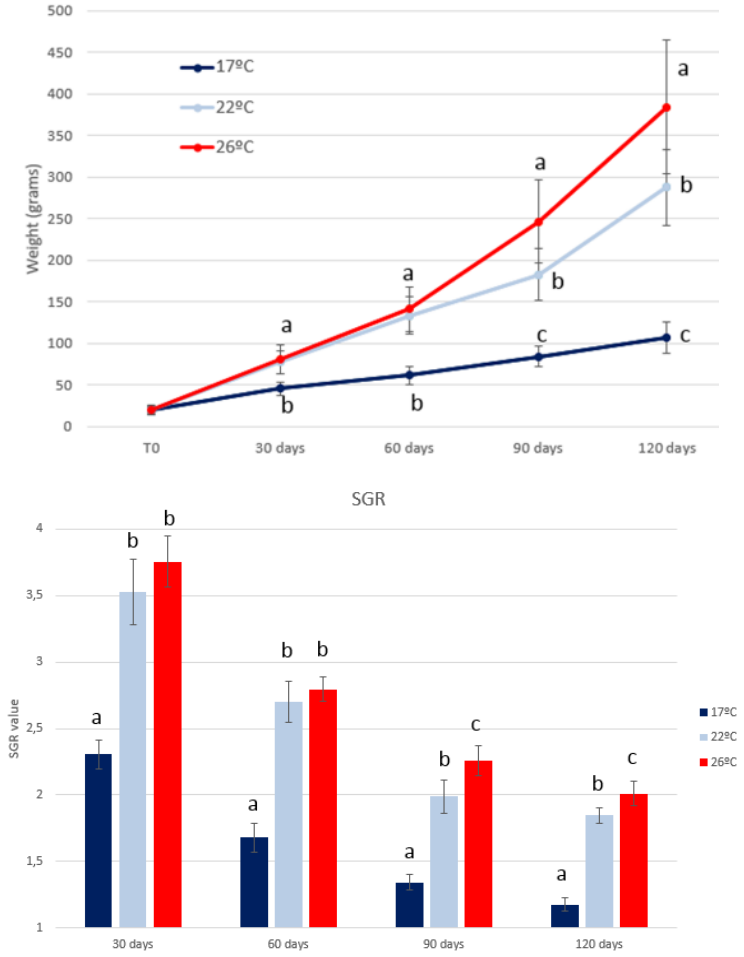


Figure 21.3.1.2. Growth rate and SGR of greater amberjack fingerlings during 120 days of trial at the three different temperatures.

Morphological analyses

Significant differences among groups were found in the centroid size (ANOVA procrustes, F= 6.85, P= 0.0014) and shape (F= 8.79, P< 0.0001). The centroid size was correlated to fish length (P= 0.0005), explaining the 7.65% of variation for allometry accounts, which indicated a change shape linked to fish growth. In the PCA analysis, the firsts 19 PC components explained 91.3% of total variance. The PC1 attained 21.6% of variance and it was strongly related to fish size (r= -0.500; P< 0.0001); whilst, PC2 described 11.5% of variability and it was not correlated to fish size (r= -0.017, P= 0.837). The PC1 showed that the increase of temperature led to elongated shape of fish body, especially of the head (Fig. 21.3.1.3), differencing clearly the specimens reared between 17ºC and 26ºC. However, PC2 did not have the same consistence as before component with the temperature, and all individuals presented similar morphological variability.

The mean values of caudal propulsion efficiency (CPE) differed among the groups (K-S statistic= 16.34, P< 0.001), noting higher propulsion of fishes with a temperature increase (**Figure 21.3.1.4**). The specimens reared to 26ºC showed significant swimming differences with the individuals cultivated to 17ºC (Dunn's Z-statistics= -15.7, P< 0.001) and 22ºC (Dunn's Z-statistics= -10.1, P< 0.05); whilst, it was similar between the individuals reared to 17ºC and 22ºC (Dunn's Z-statistics= -5.6, P> 0.05).

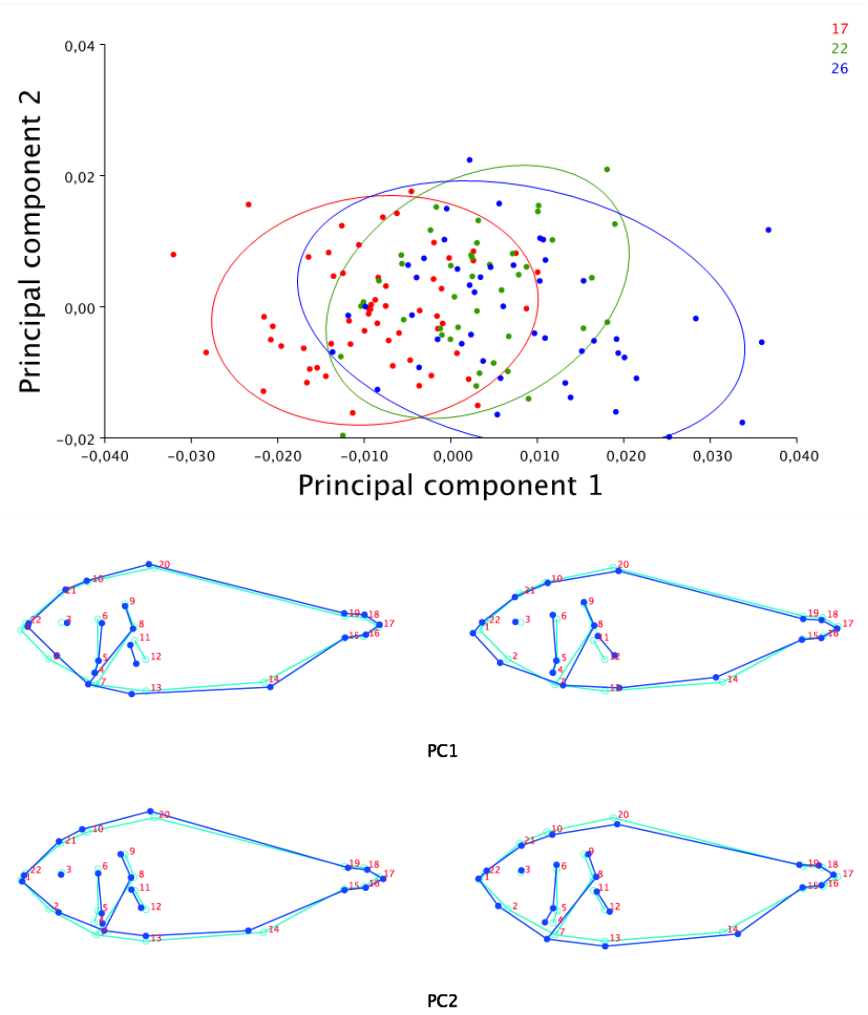


Figure 21.3.1.3. Morphological variations along the x-axis (PC1) and y-axis (PC2). Circles show the 95% of confidence interval. Colours indicate the shape changes producing along axis (negative to positive) respect to a common shape locate in the origin (x= 0, y= 0). Real images of specimens of *Seriola dumerili* reared at 17ºC and 26ºC.

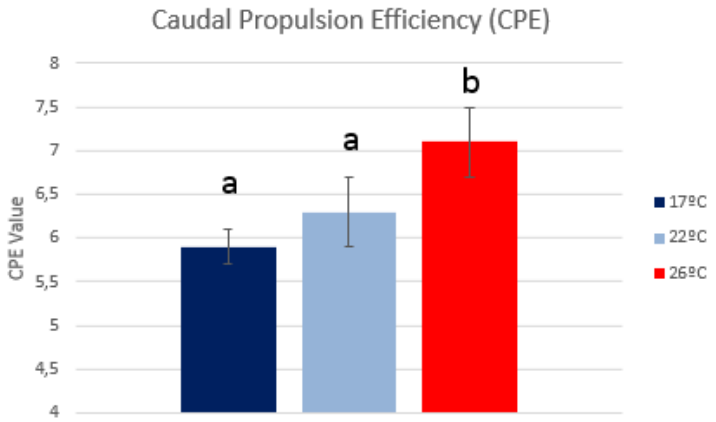


Figure 21.3.1.4. Caudal propulsion efficiency (CPE) value of the three treatments; relation between caudal fin depth (CFd) and caudal peduncle minimal depth (CPd).

Feed utilization

After one month of growth, there were significant (P<0.05) differences on FCR, being this parameter higher for fish held at 17ºC when compared with fish held at 26ºC. For the next sampling point, the differences among fish held at 17º, 22 and 26ºC increased, being significantly (P<0.05) higher for fish held at 17ºC, when compared to fish held at 22ºC and fish held at 26ºC respectively. Regarding total FCR for the whole on-growing period, fish held at 26ºC showed the highest (P<0.05) FCR, being this value below one (**Figure 21.3.1.5**).

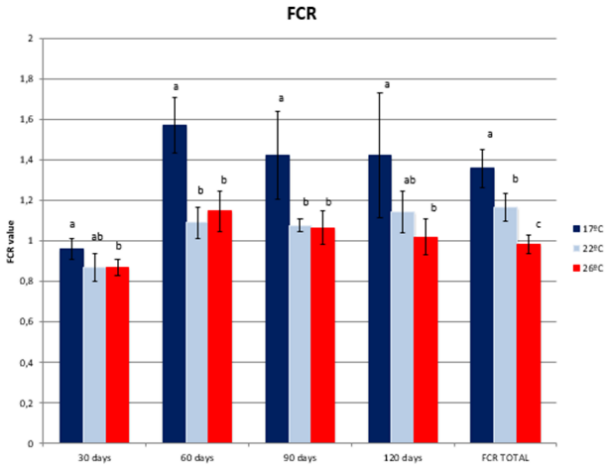


Figure 21.3.1.5. FCR values at each sampling point for the three temperatures and the total FCR.

The amount of feed intake was significantly higher (P<0.05) in fish held at 26ºC when compared to fish held at 17ºC and similar to those held at 22ºC. There were no significant differences in the protein or lipid retention among groups held at different temperatures. However, the protein gain was significantly higher (P<0.05) in fish held at 26ºC when compared to those held at 22ºC, being the protein and lipid gain significantly (P<0.05) lower for those fish held at 17ºC (Fig. 21.3.1.6).

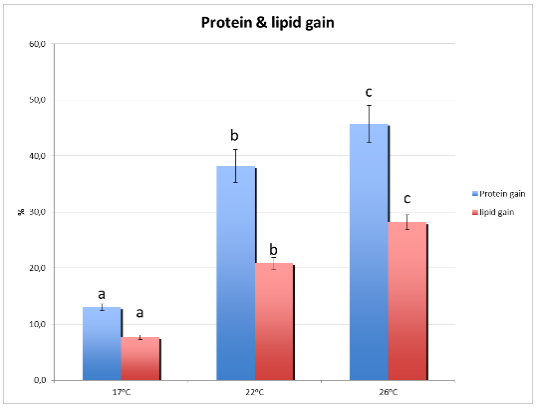


Figure 21.3.1.6 Values of the protein and lipid gain at different temperatures during 120 days.

Gastric evacuation

The gastric evacuation time study showed that those fish held at 22º and 26º C had significantly (P<0.05) more meal in the stomach compared to those held at 17ºC within the first hours after feeding. However, after

8 h of feeding the gastric content of fish held at 26ºC decreased, showing those fish an almost empty stomach 18h after feeding and no gastric content after 24h. This gastric evacuation time contrast with those fish held at 22ºC that showed gastric content after 24h and to those held at 17ºC that showed gastric content even after 30 h of feeding (**Figure 21.21.3.1.7**).

Regarding gut evacuation, differences in the evacuation between temperatures were shown, with 26ºC being the faster treatment in evacuation, significantly ($P<0.05$) lower that gut content of animals held at 22ºC after 8 h. Fish held at 17ºC showed significantly ($P<0.05$) higher gut content 12h and 24h after feeding (**Figure 21.3.1.8**).

Work in progress: 1) Biochemical and histological analyses. 2) digestive enzyme analysis.

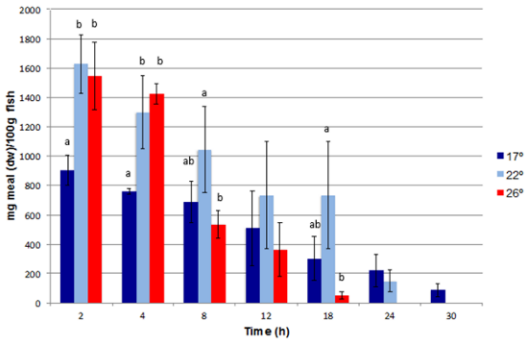


Figure 21.3.1.7. Gastric evacuation time (mg meal dry weight 100 g^{-1} of fish) of greater amberjack juveniles after 120 days held at different temperatures

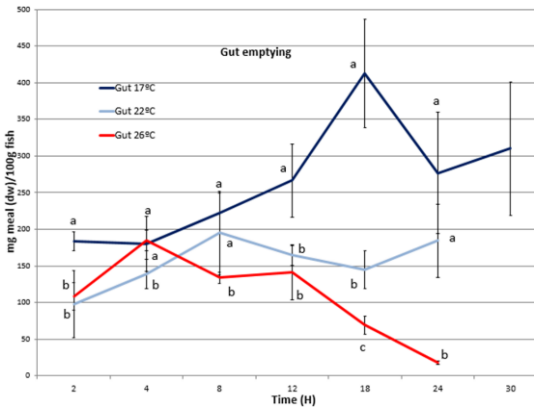


Figure 21.3.1.8. Gut evacuation time (mg meal dry weight 100 g^{-1} of fish) of greater amberjack juveniles after 120 days held at different temperatures.

Action 21.3.2 Definition of optimal stocking density (led by IEO, Salvador Jerez, Virginia Martín, Eduardo Almansa).

Rearing trials at 3 different stocking densities were performed with individual size of 5 g in 500 l-tanks and 150 g in 4000 l tanks (P8. IEO) for a period of 4 months. Monitoring included growth performance, feed efficiency, k index, and quality including morphological aspects and haematological, histological, biochemical and immunological studies. Also analysis of oxidative stress enzymes will be evaluated.

Stocking density has been shown to affect behavioral interactions in several fish species and may ultimately affect growth rates. Therefore, it is important to study the effects of density on the growth of young greater amberjack. In this study, differences in growth performance of juvenile greater amberjack held at three 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.

To achieve this objective, 3 different stocking densities have been tested in greater amberjack juveniles during 2015. The juveniles were divided into 9 groups. Groups were stocked at an initial density of 0.17±0.02 kg m^{-3} (Low density, LD), 0.28±0.01 kg m^{-3} (Medium density, MD) and 0.46±0.07 kg m^{-3} (High density, HD). Fish tagged with a passive integrated transponder (PIT), were fed at the same feeding strategy and the daily feed intake was recorded. Fish were sampled at 0, 30, 60, 90 and 120 days during the trial. In each sampling, blood and mucus samples were collected to evaluate the influence of stocking densities tested on immunological, hematological and biochemical parameters indicative of the health status, nutritional condition and welfare of fish. Samples of fish tissue were obtained for oxidative stress and condition studies at the beginning and the end of the trial. The final stocking density reached for the different treatments at the end of assay (120 days) were 3.66±0.46, 5.74±1.20 and 7.41±0.17 kg m^{-3} for Low (LD), Medium (MD) and High (HD) densities, respectively.

Results

The results showed that at day 30, the Specific Growth Rate (SGR) decreased significantly with the increasing of the density, but no significant differences were found between fish groups at different density assayed in the following periods. However, the SGR in overall period (0-120 days) decreased as stocking density increased. In the overall period, the fish maintained at higher density (HD) showed the lower SGR and dispersion **(Fig. 21.3.2.1)**.

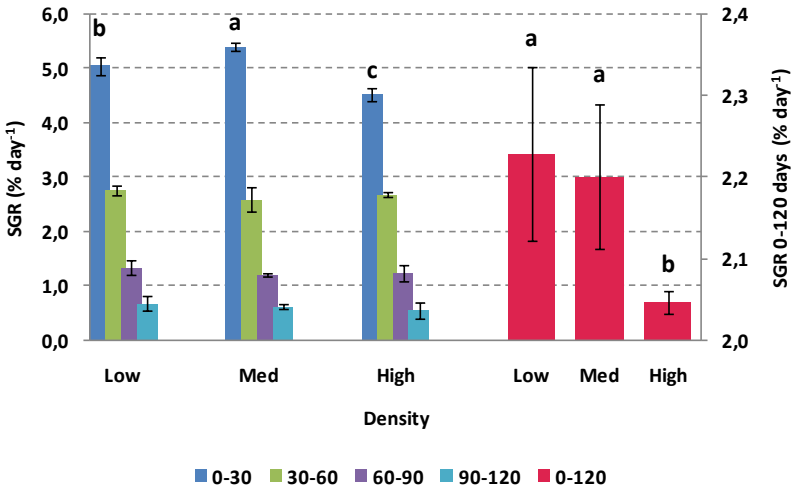


Figure 21.3.2.1. Specific growth rate SGR (% day^{-1}) at the different periods and overall duration (120 days) of fish fed stocked at Low (LD), Medium (MD) and High (HD) density. Different letter indicates significant differences among different stocking densities ($P<0.05$).

Fish stocked at High density showed the higher condition factor (CF index) at 60 days. Fish maintained at Low and Medium density increased their CF index along the assay while in fish stocked at High density decreased their CF resulting in a significantly lower value at the end of assay (120 days) **(Figure 21.3.2.1.** Specific growth rate SGR (% day^{-1}) at the different periods and overall duration (120 days) of fish fed stocked at Low (LD), Medium (MD) and High (HD) density. Different letter indicates significant differences among different stocking densities ($P<0.05$). Hepatosomatic (HSI) and Viscerosomatic Index (VSI) were significantly higher in the High density group **(Figure 21.3.2.2).**

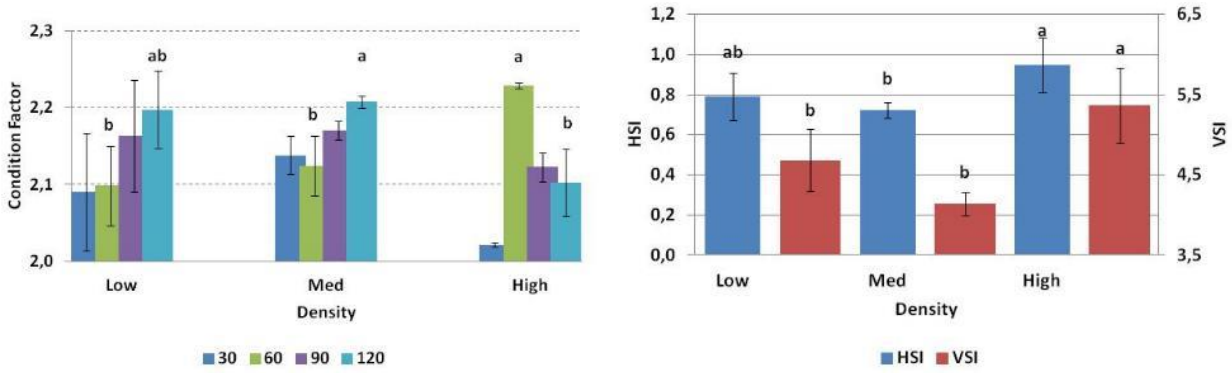


Figure 21.3.2.2. Condition factor (CF) (g cm^{-3}) at the different periods and Hepatosomatic (HIS) and Viscerosomatic Index (VSI) at the end of the study of fish stocked at High (HD), Medium (MD) and Low density (LD). Different letter indicates significant differences among different stocking densities ($p<0.05$).

Feed intake (% body weight day^{-1}) decreased significantly during experimental period in all stocking density assayed (**Figure 21.3.2.3)**. Results of two way ANOVA showed that both factors, time (month) and stocking density influenced the Feed intake being significantly lower at High density than at Low density. This influence was greater during the first two months.

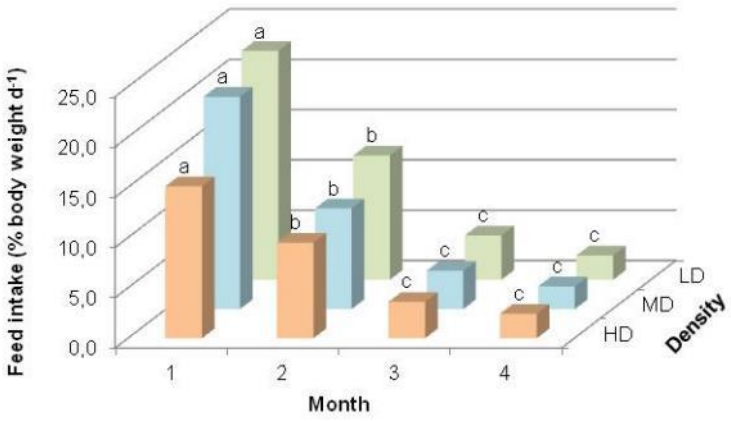


Figure 21.3.2.3. Feed intake (% body weight day-1) of fish stocked at High (HD), Medium (MD) and Low density (LD) during the trial. Different letter indicates significant differences among different periods ($P<0.05$).

The daily feed intake at the different feeding times was lower in fish stocked at High density during the first two months of the trial, although significant differences were registered only during month 2 at 9:00, 13:00 and 15:00 feeding times. During the following two months the feed intake at the different feeding times was similar irrespective of the culture density assayed. At each density, the feed intake at the different feeding times showed slightly changes, but not statistically significant, during the four months **(Fig. 21.3.2.4)**.

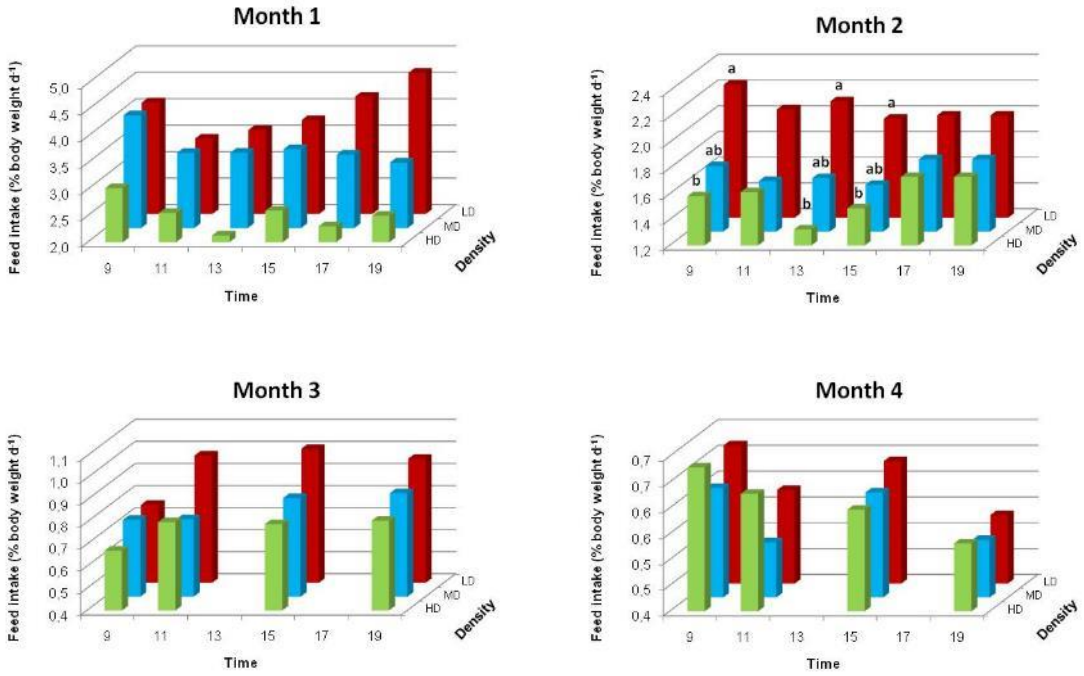


Figure 21.3.2.4. Feed intake (% body weight day^{-1}) of fish stocked at High (HD), Medium (MD) and Low density (LD), during the trial and at the different feeding times. Different letter indicates significant differences among different stocking densities ($P<0.05$).

Conclusions

The results showed that stocking density affects growth rates and feed intake in greater amberjack juveniles. 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.

Deviations from Annex I and their impact:

There were no deviations from Annex I in this WP.

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), Redivo Baptiste (P16); Fontaine Pascal (9), Ledoré Yannick (P9), Ivar Lund (P21), Tu-Linh (P29)			

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)

The WP studied the husbandry requirements during on-growing, with emphasis on the effect on growth, of immune and physiological status (a) environmental parameters, (b) farm conditions and (c) domestication level and geographical origin. During the reporting period, a preliminary experiment was conducted to better define the methodological requirements of a multifactorial stress screening, which was initially planned to start between the months 8 to 12 of the project, and will effectively start on month 17.

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

Task 22.1 Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status (led by FUNDP, Patrick Kestemont).

The objectives of this task were:

(1) To characterize the effects of major husbandry and environmental factors on growth related parameters as well as on physiological and immune responses of cultured pikeperch.
(2) To identify the optimal husbandry and environmental conditions for improving the growth and the survival rates as well as the welfare of pikeperch in intensive culture.

These objectives were achieved through two *in vivo* experiments: (1) a multifactorial screening comparing 16 experimental conditions established from 8 husbandry and environmental factors tested in 2 modalities; (2) a confirmation experiment in which the best experimental conditions characterized as being the optimal modalities were tested whether they are suitable for a high disease resistance after an effective bacterial challenge.

A. Multifactorial experiment

1. Experimental design

The multifactorial approach was based on a fractional factorial design, which has been validated as a relevant screening method in other experimental contexts (Hamre et al., 2004; Gardeur et al., 2007; Teletchea et al.,

2009). Such experimental design has various advantages, such as: (a) evaluating the possible interactions between tested factors, (b) classifying the relative importance of all factors (main effects and interaction groups), and (c) determining the combinations of factors that would be required to improve the rearing system. Eight relevant factors (**Table 22.1.1**) were selected taking into account the current practices in various pikeperch farms, and the few available results concerning the stress sensitivity in percid fish. Two modalities considered as high or low level were applied for each factor, so 16 experimental conditions were compared. Fish were sampled on days 36 and 63 after the start of the treatments to account for short- and long- term responses. Various analytical methods including ELISA and RT-qPCR methods were used for the analyses of physiological and immunological parameters. Based to the results from the methodological refinement assays, it was decided to make further evaluation of physiological stress response by analysing the levels of brain neurotransmitters using HPLC with electrochemical detection (HPLC-EC).

Table 22.1.1: Experimental factors and modalities (= C experimental conditions).

Exp. Conditions (n°)	Light intensity (lux)	Density ($kg.m^{-3}$)	Light spectrum	Photo-period (h)	Water temperature (°C)	Feed Type	Grading	Oxygen saturation (% O_2)
1	10	30	white	24	21	sinking	Y	90
2	100	15	red	10	26	floating	-	60
3	100	15	white	24	21	sinking	-	60
4	100	30	red	10	21	sinking	-	90
5	10	15	red	10	21	sinking	Y	60
6	10	15	white	10	21	floating	-	90
7	100	15	red	24	21	floating	Y	90
8	10	15	white	24	26	floating	Y	60
9	100	15	white	10	26	sinking	Y	90
10	100	30	white	10	21	floating	Y	60
11	100	30	white	24	26	floating	-	90
12	10	30	red	10	26	floating	Y	90
13	100	30	red	24	26	sinking	Y	60
14	10	30	red	24	21	floating	-	60
15	10	30	white	10	26	sinking	-	60
16	10	15	red	24	26	sinking	-	90

2. Results and conclusions

The results showed a significant and positive effect of sinking feed, but various interactions were calculated between the feed type and red light, low light intensity, grading, low temperature and oxygen saturation. Survival rate was mainly improved by low intensity and red light. Light characteristics were also associated to a low stress response in terms of plasma cortisol, glucose, brain neurotransmitters but marked synergy was observed between light intensity and some husbandry factors. But the highest values for the expression of glucocorticoid receptor-1 were observed for fish reared at 26°C and 90% dissolved oxygen. A decrease in the stress response was observed after 2 months, and this was associated to an improvement in the immune status in terms of lysozyme and ACH50 activities. Combining the results on husbandry performances and on stress and immune status (see **Table 22.1.1** and **Fig. 22.1.1**), three experimental modalities were selected as suitable for improving performances for pikeperch in intensive culture. Apart from the sinking feed and grading, these experimental conditions differed mainly by their light characteristics.

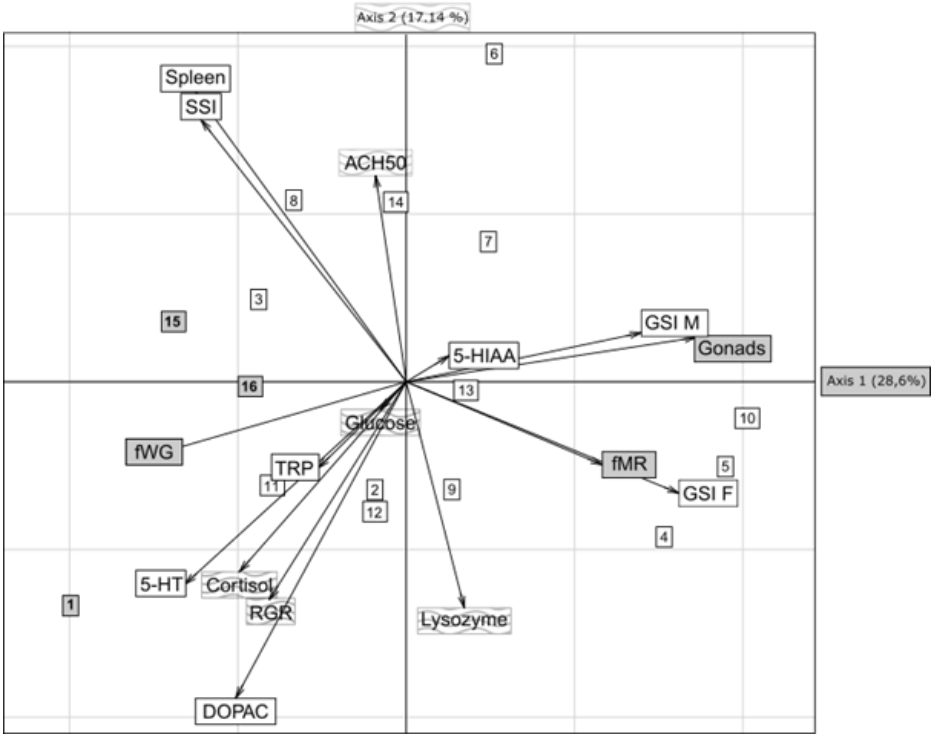


Figure 22.1.1 Projection of global effect of experimental conditions (C1 to C16) on the plans 1-2 of the principal components analysis (ACP). SSI: Spleno-Somatic Index. fWG: Final Weight Gain. TRP: Tryptophan. 5-HT: Serotonin. RGR: Relative Growth Rate. DOPAC: Dihydroxy-Phenyl Acetic acid (dopamine metabolite). 5-HIAA: Hydroxyl-Indol-Acetic Acid (serotonin metabolite). GSI M: Gonado-Somatic Index Male or Female. fMR: Final Mortality Rate. The plans 1-2 of the ACP explained 45.8% of the inertia (total variance) with axis 1 representing the highest variance. The axis 1 was mainly characterized by higher fWG and lower fMR.

B. Confirmation experiment

Experimental design

The results from the multifactorial experiment indicated that two (C1= C15 and C2 = C16) experimental conditions were optimal for increasing the performances and welfare status of pikeperch in intensive culture. These treatments were characterized by low light intensity, sinking feed, high temperature and no grading. It appeared interesting to include the C1 (C3) experimental conditions in the confirmation experiment because these modalities (high stocking density-grading-high oxygen saturation-low light intensity) were close to the culture practices in many pikeperch farms, and fish reared under these conditions exhibited higher husbandry performances in contrast to higher stress response. Fish were exposed to the 3 selected experimental conditions during 36 days in 3 separated circulating systems. At the end of the experiment, plasma glucose, cortisol and lysozyme activity levels were assayed. Fish were then injected with *Aeromonas salmonicida achromogen* for a bacterial challenge and survival rate was daily recorded during 14 days.

Results and conclusions

Values for stress indicators showed that the three selected experimental conditions had comparable low impact on fish stress status. After injections of *A. samonicida achromogen*, fish started to die after two days and all of them died within 7 days (**Fig. 22.1.2**). The survival probability curves were the highest for the first

rearing system (C2) but comparable to those for the rearing system (C1), and significantly differed compared to the system (C3) that showed the lower disease resistance ($p < 0.01$).

Therefore, the comparison of the 3 rearing systems indicated that long light duration may be detrimental to the welfare of pikeperch since all systems were set at 10 lux of red or white light, and the third system differed by its long photoperiod of 24 h and grading, but it is still necessary to validate such hypothesis using large facilities as in commercial farm conditions.

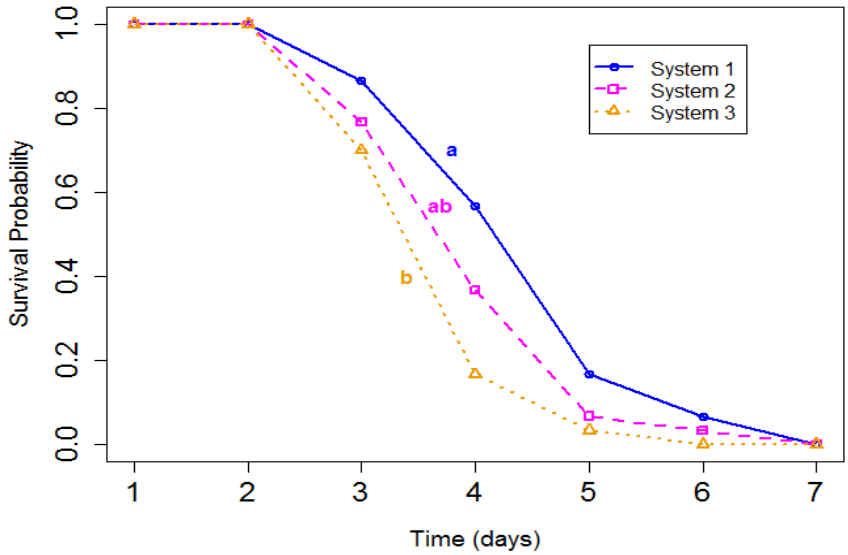


Figure 22.1.2 Mortality of pikeperch (*Sander lucioperca*) injected with *A. salmonicida achromogen* (10 millions of bacteria 100g fish^{-1}). Significant differences between curves are indicated by different letters (a, b). Systems 1, 2 and 3 represented C15, C6 and C1 in the multifactorial experiment, respectively.

C. General conclusions

The results from the research program of task 22.1 indicated that the type of feed is the main directive factor for the variability in the husbandry performances for pikeperch. But strong synergy was shown with feed type and mainly light characteristics (light intensity, photoperiod and light spectrum). Physiological stress and immune responses were also affected by light characteristics but in association with stocking density and sometimes with temperature level. Neurotransmitters appeared as reliable stress indicators for pikeperch, and where affected mostly by the interactions between light intensity and temperature. Surprisingly, grading did not appear for a high stress impact except for its effect on some neurotransmitters such as 5HIAA, a metabolite of serotonin.

The results demonstrated low stress sensitivity in most of experimental conditions in which fish exhibited the highest husbandry performances. Moreover, some results indicated a positive relationship between physiological status and immune competence. Validation experiment indicated that only the selected experimental conditions by the multifactorial investigation had comparable efficiency in terms of low impact on fish stress and immune status. However, fish reared under only two experimental conditions showed higher disease resistance after induced infection by *A. salmonicida*. The latter 2 combinations of husbandry and environmental modalities were selected as optimal rearing conditions for pikeperch, and will be further validated in Asialor commercial farm conditions (WP22.2).

The full results from all these experiments have already been reported and submitted as ***Deliverable 22.1. Effects of multiple variables on stress, immune response and growth performances and recommendations of optimal conditions for pikeperch grow out***.

Task 22.2 Characterization of pikeperch growth, immune and physiological status in farm conditions (led by ASIALOR, Tu-Linh).

This task has not started yet, and is depending on the availability of fish and materials at P29. ASIALOR. Since light characteristics may be important for pikeperch juveniles, it has been decided to maintain fish during 8 to 10 months under the two, defined as, optimal experimental modalities but testing only red vs white light spectrum, since other factors modalities induced less variability.

Various biomarkers including husbandry parameters (survival and relative growth rate), stress indicators (plasma cortisol and glucose levels and brain hormones) and immune parameters (lysozyme activity, activity of the alternative complement pathway and the expression of some key immune genes in kidney) will be assessed.

Task 22.3 Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity (led by FUNDP, Patrick Kestemont).

This task has not started yet. But we are already looking how and where we will collect the different populations to be tested based to the results from the microsatellite characterization of domesticated pikeperch broodstock (see ***Deliverable 4.1 Genetic analysis of domesticated pikeperch broodstocks***).

Deviations from Annex I and their impact:

Milestone and deliverable dates were delayed because (1) the adaptation of the P9. UL facilities to the multifactorial protocol requirements took more time than expected and (2) it was also necessary to perform two preliminary experiments in order to better define some methodological aspects appropriated to the multifactorial stress screening since there is limited information on stress response for pikeperch. Despite that delay, the results from those preliminary assays facilitated the full achievement of ***Deliverable D22.1***, and will not have any effect on the WP.

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 progress towards objectives and details for each task (13-30 Mo):

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 submitted as ***Deliverable D23.1 Cost effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status***. In addition the results have been published in the journal Aquaculture 462, 92–100. The aim was to evaluate a weaning protocol for wild flathead grey mullet fry based on the transition from live prey to inert diets with different levels of fish meal substitution (0, 50 and 75%) in diets for grey mullet fry in terms of growth performance, survival, proximate composition and digestive processes.

The different levels of fish meal (FM) substitution with a blend of different plant protein sources (corn gluten, wheat gluten and soy protein concentrate) did not significantly affect the weaning (days 0-30) and the early on-growing period (days 30-60) for flathead grey mullet fry (**Table 23.1.1**).

Table 23.1.1 Final body wet weight (BW, mg), standard length (SL, mm), Fulton's condition factor, specific growth rate in BW (%/day) and survival (%) of wild flathead grey mullet fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources). Data is presented for the two distinct phases in which the study was divided: weaning of fry with the abovementioned diets (days 0-30) and the early on-growing phase (days 30-60).

Day 30 (end of the weaning)					
	BW (mg)	**SL (mm)**	**K**	**SGR_{BW} (%/day)**	**S (%)**
FM diet	390 ± 15	2.5 ± 0.01	2.6 ± 0.06	1.9 ± 0.1	78.6 ± 5.1
PP50 diet	385 ± 12	2.4 ± 0.02	2.7 ± 0.05	1.8 ± 0.1	73.4 ± 3.1
PP75 diet	375 ± 18	2.4 ± 0.02	2.7 ± 0.05	1.9 ± 0.1	71.1 ± 4.2
Day 60 (end of the trial)					
	BW (mg)	**SL (mm)**	**K**	**SGR_{BW} (%/day)**	**S (%)**
FM diet	707 ± 17	3.2 ± 0.02	2.17 ± 0.05	2.1 ± 0.05	74.6 ± 3.1
PP50 diet	661 ± 10	3.1 ± 0.02	2.18 ± 0.04	1.9 ± 0.07	70.4 ± 4.2
PP75 diet	681 ± 20	3.2 ± 0.02	2.17 ± 0.03	1.8 ± 0.09	69.1 ± 3.1

Although all groups of fish showed a normal distribution in BW, fish fed the PP50 and PP75 diets showed a more homogeneous distribution than that of fish fed the FM diet (**Figure 23.1.2**). In this sense, fish fed PP50 and PP75 diets showed higher Kurtosis values (0.79 -0.44) than those fed the FM diet (0.14), whereas skewness values were quite similar among three of the tested diets (0.36, 0.48 and 0.35 for FM, PP50 and PP75 diets, respectively). These results may be of practical significance since this may reduce the task of fish grading during the rearing process, as production lots will be more homogenous, although the link between the experimental diets with different levels of FM substitution and the more homogenous distribution in body weight at the end of the trial remains unclear.

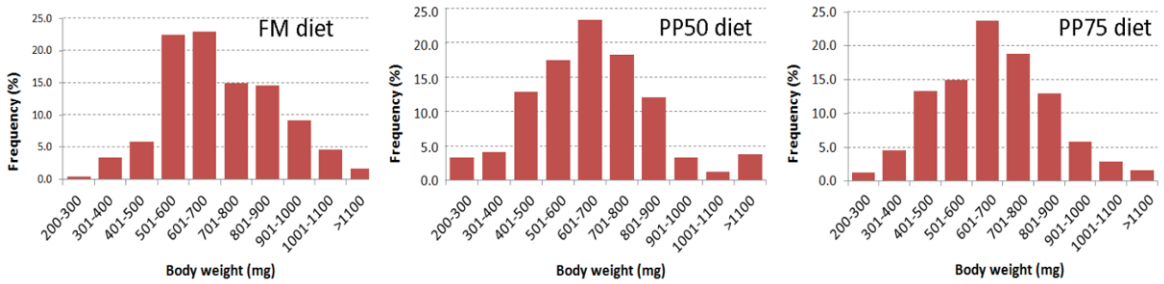


Figure 23.1.2 Frequency of body weight distribution of wild flathead grey mullet fry weaned onto experimental diets with different levels of fish meal substitution (FM, no fish meal substitution: PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

This study concluded that FM substitution between 50 to 75% by a mixture of different plant protein sources (corn gluten, wheat gluten and soy protein concentrate) in wild flathead grey mullet fry weaned onto compound diets, did not affect their growth performance and survival. There were no differences in the proximate composition, fatty acid and amino acid profiles among fish fed the different experimental diets. Similarly, the digestive capacities of fish measured by the activity of different pancreatic and intestinal enzymes was not affected by diet composition. The diets did not affect the tissue organization of the liver

and intestine, nor their antioxidative stress defences. Overall, the results indicated that weaning diets for wild flathead grey mullet harvested for restocking in aquaculture ponds for on-growing may be formulated with a high level if not complete replacement of fish meal with alternative plant protein sources.

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 this task hatchery produced F1 juvenile grey mullet (P4. IOLR) will be supplied to P25. DOR. These fish will be stocked at two different densities (0.5 and 1 juvenile m^2) in two earthen 6,000 m^2 ponds and fed an improved grey mullet extruded feed, (WP3 Nutrition). This feed will be custom-produced for all grey mullet tasks in the WP23 by P31.IRIDA. In parallel, a 1 year density study (4 and 6 juveniles m^2) using the project's grey mullet feed will be carried out on F1 juveniles (P4. IOLR) in 4 circular cement ponds (30 m^3), where each density will be tested in duplicate tanks. Fish performance will be evaluated in terms of FCR, PER, SGR, overall weight gain and survival. In addition, analyses of lipid class and fatty acid composition of selected tissues (liver, muscle and gonads) will be carried out by P4. IOLR. However, a number of changes were implemented due to limited tank availability and problems of delivery of the feed produced by P31. IRIDA.

Methods and Materials

On 25/3/15 two cement (19 m^2) and one polypropylene (3.5 m^2) ponds were stocked with F2 fish from eggs spawned on 31/10/14 at different densities (55, 29 and 286 fish m^{-2}, respectively). The fish were anesthetized and weighed singly and stocked in the three tanks. The weight distribution of a representative sample of 68 fish with an average weight of 8.23 ± 0.23 g is shown in **Figure 23.2.1a** and demonstrated an approximate normal weight distribution. The experimental tanks were in an open system fed by filtered (10 µm) and ambient sea water (40‰). Unfortunately, due to unexpected Israeli customs bureaucracy and demands, the arrival of the extruded mullet feed from P31. IRIDA was endlessly delayed and did not arrive in time for the growth trial (feed arrived only in May 2016 when a similar experiment will be carried out during 2016-2017) at the P4. IOLR and P25. DOR. However, the stocked fish were mostly fed with a similar formula pelleted IOLR feed (the P31. IRIDA feed formula is based on the P4. 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.

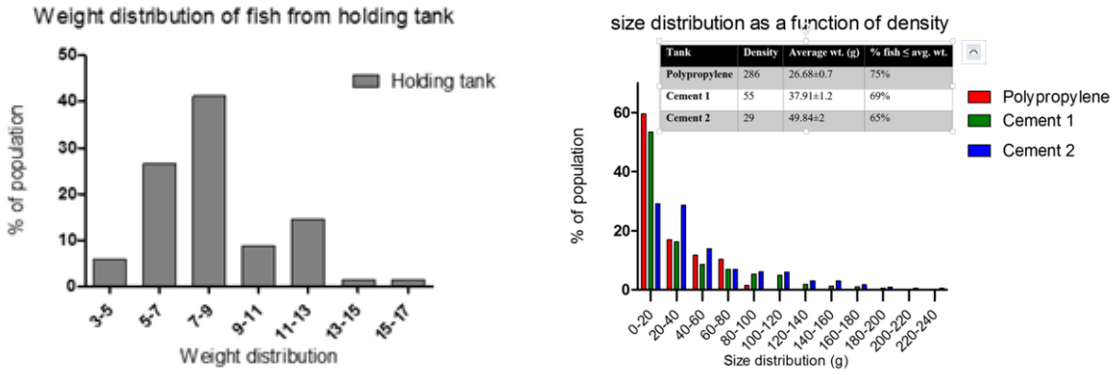


Figure 23.2.1 The (a) weight distribution of a representative sample of 68 fish with an average weight of 8.23 ± 0.23 g and the (b) size distribution as a function of density in the tanks at the end of the trial.

Results and discussion

Although this 1 year growth trial was not conducted using the P31. IRIDA diet, but a similar diet produced at the P4. IOLR facility, there was a clear indication of stocking density on size distribution. As there was almost no mortality, the reported density effect was almost certainly due to a density driven interaction between individuals. 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 (**Figure 23.2.1**). 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. The skewness of the population might be reduced with an improved diet as might be the case in the planned 2016-2017 trial in Israel with the poultry meal replacement with fish meal in the P31. IRIDA diet.

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).

The trial which has started in P26. GEI's farm since last week of July 2015 is ongoing without significant obstacles. Wild-caught mullet are fed a species specialized diet produced by P31. IRIDA and reared into two different densities (4 and 6 individuals per m^2). Intermediate growth was monitoring by weighing a sample of fish from each of the 6 cement tanks. Feed intake and survival were monitoring daily from the beginning of the trial until May 30, 2016.

Methods and Materials

Survival, growth, and feed intake during the 9-month feeding period.

The tanks were continuously supplied with artesian bore water with a dissolved oxygen level around 8 ppm, which is considered optimum and water temperature at 18°C. The photoperiod followed the natural cycle of the season. The feeding was performed 2 times per day at visual satiation (09:00 & 15:00 h) six days a week and the daily feed intake was recorded. Monitoring of fish health and feed consumption (**Table 23.3.1**), as well as recording of water physicochemical parameters was performed daily. Up to this point of rearing, no major problems or technical faults have been encountered.

Results

The percentage survival during the 9 month feeding was 89 ± 3.3% and 88 ± 1.3 for the predefined densities, 4 and 6 individuals per m^2, respectively. On May 30, 2016 an intermediate monitoring of fish growth was carried out at the P26. GEI farm by P1. HCMR's staff. Appoximately 15 fish were randomly sampled from each tank, anaesthetized using phenoxyethanol (0.25 mg l^{-1}) and fish weights were recorded (**Table 23.3.1**, **Figure 23.3.2**). The mean fish weights were found to be 50 g ± 10 and 42 g ± 6 for the predefined densities of 4 and 6 individuals per m^2, respectively.

Table 23.3.1. Feed consumption (kg) per tank from 24/9/15 up to 30/6/2016.

T1 **(4/m²)**	***T2*** **(4/m²)**	***T3*** **(4/m²)**	***T4 (6/m²)***	***T5 (6/m²)***	***T6 (6/m²)***
10,24	9,64	8,79	10,20	9,46	9,31

Figure 23.3.2. Intermediate weighing of flathead grey mullet fry at P26. GEI facility on May 30, 2016.

Discussion

Although this feeding trial is on-going and the growth results are preliminary and based on a sub-sample of the pond, there appears to be a tendency of higher weight with lower density, which agrees with the studies in Israel (**Task 23.3**) and Spain (**Task 23.4**). The project is following the projected timeline

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).

Methods and Materials

Wild grey mullet fingerlings arrived to the facilities of P18. CTAQUA in February 2015. A total of 1,500 fish of 1 g average body weight, were stocked in a recirculation system (RAS) with 15 tanks of 125 l each. The RAS was comprised of units for mechanical filtration, biofiltration, protein skimmer and UV treatment. Fish were acclimatized during one week, mainly for salinity adaptation. Salinity upon arrival was 10 ppt and during the seven days of acclimation it was gradually changed to 35 ppt. The fingerlings were fed manually 4-5 times per day until they were moved to the farm earthen ponds. During the first fifteen days feed type was the one supplied by the fish provider. After this period, the fingerlings were fed a commercial feed for seabream fingerlings.

Water quality parameters were controlled twice per week, except temperature and dissolved oxygen checked daily. Mortality as well as fish welfare was checked daily as well.

Fingerlings remained in the RAS during 4 months until they reached an average body weight of 3.5 g. During this period, 10% mortality was observed and its cause was not identified since feeding behavior was very good and water quality of the RAS system was adequate for the species. During the last two weeks of June, grey mullet fingerlings were acclimatized to the lower salinity in the farm (12‰) and to the feed they would receive in the field. This feed was provided by P31. IRIDA, a diet specifically formulated for grey mullet (based on P4. IOLR formula) of 1.5 mm diameter.

During the first days of July 2015, the grey mullet was moved to the farm, located in Trebujena, province of Cádiz (South Spain). A total of 1.344 fish were moved. Initially two very similar ponds were going to be used for the feeding trial but due to farm production needs they provided two different ponds, (pond L3 and pond L4) that were prepared by the farm staff to receive the fish. Pond dimensions were the following:

- L3: 1100 m^2; in this pond the density of 0,5 individuals m^2 was used; 544 fingerlings were seeded.
- L4: 800 m^2 ; in this pond the density used was 1 individuals m^2; 800 fingerlings were seeded.

The ponds used for the experiment are shown in **Figure 23.4.1**. Both have elongated shape with the inlet gate and outlet gate on the extremes. The fish have been fed with automatic belt feeders and also manually

at the first time of the morning to check fish feeding behavior. Fingerlings seeded are shown in **Figure 23.4.2.** For the samplings, fish have been weighed individually.

Figure 23.4.1. View of the two ponds L4 (top) and L3 (bottom) used for the trial.

Figure 23.4.2. Fingerlings of 3.6 g average body weight introduced in the experimental ponds.

Results

At the writing of this report (Mo 31), two partial samplings have been performed. Sampling in farm conditions is rather difficult due to the special characteristics of this type of culture. The fish were collected with a net dragged along the length of the pond and this can cause considerable stress **(Figure 23.4.3).**

Figure 23.4.3 Fish collection on the side wall of the pond for fish sampling.

From the two samplings done so far, the results indicate that there is a difference between the two pond densities used. It has to be mentioned that it has not been possible to collect all the fish, since grey mullet has the ability to jump at a considerable height above the net. Results are shown in **Table 23.4.1.**

The trial is still in progress. Another sampling and modification of the culture conditions is planned for later during summer, when the temperatures are lower and fish suffer less during handling and weighing. At that moment, all the fish from pond L4 will be collected and moved to pond L3 to continue growing for 3 months more.

Table 23.4.1 Data on grey mullet samplings done during the trial.

DATE	POND	INITIAL FISH NUMBER	N (sampled fish)	ABW (g)	Density (fish/m^2)
01/07/2015	L3	544	-	3.60	0.5
	L4	800	-	3.60	1.0
04/02/2016	L3	544	57	92.65	0.5
	L4	800	88	33.17	1.0
21/06/2016	L3	544	28	163.55	0.5
	L4	800	183	62.94	1.0

Discussion

Although this feeding trial is on-going, the results so far clearly indicate a growth advantage in the lower stocking density (0.5 fish m^2) and is consistent with the results in the Israel and Greek growth trial, which also showed a density effect (**Task 23.2**). In addition, the much higher weight gain in Spain compared to Israel may be tied to the replacement of poultry meal with fish meal and the fact that the P31. IRIDA diet was extruded and more water stable than the pelleted P4. IOLR diet.

Deviations from Annex I and their impact:

In Task 23.2 there was a deviation from Annex 1 due to unexpected Israeli customs bureaucracy and demands, which delayed the arrival of the extruded mullet feed from P31. IRIDA and did not arrive in time for the growth trial started in 2015. Now that the sell-by-date has been passed, a new feed shipment has been produced and will arrive in May 2016 and will be used to carry out a similar study in 2016-2017 at the P4. IOLR and P25. DOR facilities.

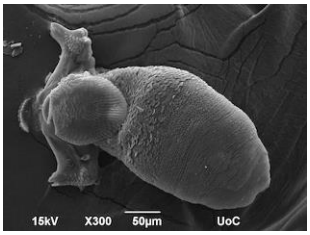
Group Work Packages

Fish Health

In the area of **Fish health**, a study of Systemic Granulomatosis (SG) of meagre showed that vitamin D did not affect the development of SG. Histological assessment of all fish gave new insights into the development of the disease including the possible implication of rodlet cells and the unique inflammatory response of the fish. In addition we have seen that both high inclusions of Phosphorus and astaxanthin have beneficial effects concerning the severity of SG. In the study of Chronic Ulcerative Dermatopathy (CUD), 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 that 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. We also carried out work on the characterization of the immune system. In meagre, several incidences have been recorded with an outbreak of monogeneans in broodstock and of mycobacteriosis in cage cultured fish being the most significant.

Work with greater amberjack included further mesocosm trials for the development of rapid detection methods for epitheliocystis, and screening of gill samples from different Greek fish farms. During the current reporting period work also included a) morphological studies on the incidence of monogenean parasites 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. Primers for detection of 11 immune genes have been optimized for qPCR, and are 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 have been undertaken 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 has been performed with juveniles, with *Bacillus oceanisediminis* and *Aeromonas* spp. being detected. Challenge trial were also undertaken 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 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.

We have managed to express the nodavirus capsid protein in all three expressions systems. 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 still remain to be done.



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

6. Identify the causes of systemic granulomatosis (SG), and chronic ulcerative dermatopathy,
7. Investigate anti-parasite treatments in juvenile meagre,
8. Undertake preliminary characterisation of immune genes and study specific immune responses post-vaccination,
9. Evaluate the occurrence of *Nocardia* infections in meagre and develop an autogenous vaccine,
10. 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 progress towards objectives and details for each task (13-30):

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.

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 study took place in P1. HCMR between July 2014-and October 2014. 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 but also specific molecular biomarkers related to Vitamin D.

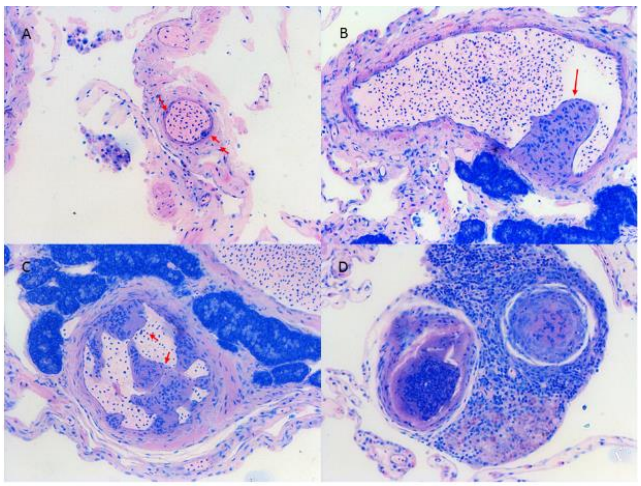


Figure 24.1.1. Blood vessel implication is evident in the manifestation of the disease. In this figure various sections of blood vessels from the peritoneal membranes and the liver of affected fish are shown. There are specific growths composed of inflammatory cells at the endothelium of the vessels which are indicated with red arrows. In more progressed stages (C and D) these growths seem to block the lumen of the vessel.

The results showed that Vitamin D did not affect the development of granulomatosis in any of the levels provided. Nonetheless, histological assessment of all fish gave new insights into the development of the disease including the possible implication of rodlet cells and the unique inflammatory response of the fish. In addition, serum biochemistry that was conducted for the first time provided the baseline level of various parameters of the affected meagre in comparison with other fish species.

The full details concerning this task have been submitted 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

About 1 g meagre, produced in May 2015 at the facilities of P1. HCMR were used for the feeding trial. In total, 1,350 fish were weighed and placed into 27 500-l cylindrical tanks at a density of 50 fish per tank (0.47 kg m^{-3}). Three replicates were allocated to each diet. The 9 diets were prepared by Skretting (P20. SARC). The feeding trial lasted 4 months (1 July 2015- 27 October 2015). 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 but also specific molecular biomarkers like CYP27.

The results showed that Phosphorus affected the development of granulomatosis with the higher dose resulting in a statistically significant reduction of SG (**Figure 24.1.2**). This was more evident in the livers and kidneys which are the organs most affected by the disease (**Figure 24.1.3**). High P levels were also beneficial for the overall growth of the fish and was also related to higher CYP27 expression. Calcium on the other hand did not affect the development of the disease. The optimum levels of Ca/P were determined.

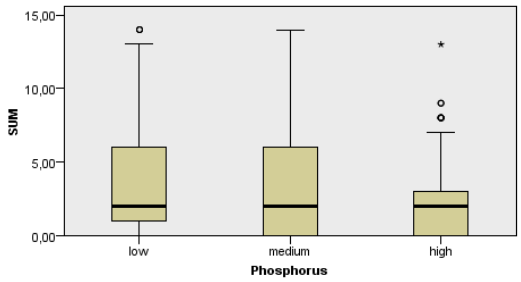


Figure 24.1.2. Boxplots of meagre's total score of granulomas at the end of the experiment fed with various P levels. Outliers are presented as circles and extreme scores as asterisks. The medians of the groups with high and low P content are significantly different (H(2)=10.077, p=0.006).

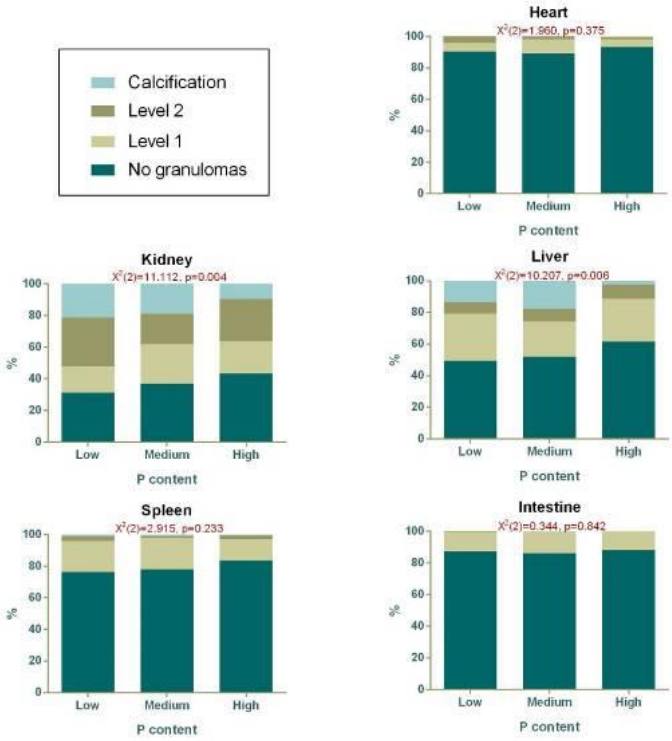


Figure 24.1.3. Percentage of the fish fed with various P levels in each of the 4 categories of the granulomas scoring system (no granuloma, Level 1: granulomas visible only with microscopy, Level 2: granulomas visible macroscopically and tissue calcification) for every examined tissue. Kruskal-Wallis test results are indicated with red letters.

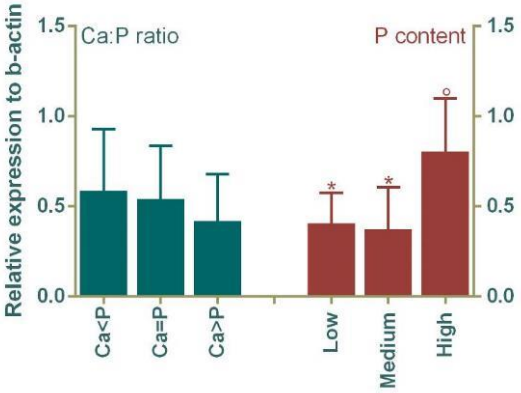


Figure 24.1.4. Relative expression of CYP27A in liver of meagre fed diets with different levels of Ca:P (blue columns) and different levels of P (red columns) in the end of the feeding trial. Values are means ± SD. Different symbols indicate the statistically significant differences between the diets with different P content ($p<0.05$).

The full details concerning this task have been submitted in ***Deliverable 24.2 The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre*.**

Trial 3. (HCMR)

This task is scheduled to begin in summer 2016.

Trial 4. (FCPCT-Daniel Montero) Effects of vitamins E, C, plus astaxanthin.

Material and methods

Experimental diets

Based on an isocaloric (16% lipid) and isoproteic (50% protein) fish meal and fish oil based diet, six experimental diets were prepared by adding different levels of vitamin E, C and astaxanthin. Diet 0 (no addition of vitamin E, C and astaxanthin), Diet A (500 mg·kg^{-1} A), Diet EC (300 mg·kg^{-1} E, 100 mg·kg^{-1} C), Diet ECA (300 mg·kg^{-1} E, 100 mg·kg^{-1} C, 500 mg·kg^{-1} A), Diet EECC (700 mg·kg^{-1} E, 600 mg·kg^{-1} C), Diet EECCA (700 mg·kg^{-1} E, 600 mg·kg^{-1} C, 500 mg·kg^{-1} A) (**Table 24.1.4.1)**. A subsample of each diet was taken and stored at -80ºC for the subsequent biochemical analysis.

Table 24.1.4.1. Composition of experimental diets (% dry weight). (*continues in the next page)*

	O	**A**	**EC**	**ECA**	**EECC**	**EECCA**
Ingredients						
Wheat	15.97	15.97	15.97	15.97	15.97	15.97
Wheat gluten 12C	16.50	16.50	16.50	16.50	16.50	16.50
SPC 12C	16.64	16.64	16.64	16.64	16.64	16.64
Faba beans whole 12C	5.00	5.00	5.00	5.00	5.00	5.00
FM North-Atlantic	35.00	35.00	35.00	35.00	35.00	35.00

12C						
Fishoil North-Atlantic	10.26	10.26	10.26	10.26	10.26	10.26
DL-Methionine	0.20	0.20	0.20	0.20	0.20	0.20
L-Lysine	0.02	0.02	0.02	0.02	0.02	0.02
Min mix 2/04 med jod	0.10	0.10	0.10	0.10	0.10	0.10
Plurivel 70%	0.21	0.21	0.21	0.21	0.21	0.21
Lutavit E-50	0.00	0.00	0.03	0.03	0.07	0.07
Lutavit C Aquastab 35%	0.00	0.00	0.01	0.01	0.06	0.06
Vit.mix no VK	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin K	0.0035	0.0035	0.0035	0.0035	0.0035	0.0035
Astaxanthin 10%	0.00	0.05	0.00	0.05	0.00	0.05

Fish and feeding

The meagre juveniles for the trial were obtained by induced spawning of a broodstock at the ECOAQUA facilities (Taliarte, Canary Island, Spain). The initial mean weight was 79.6 ± 0.34 g, the juveniles were transferred to 18 fibre glass tanks of 500 l with 50 fish per tank at an initial stocking density of 7.9 $kg \cdot m^{-3}$. All tanks were covered with a net to prevent leaks. The temperature and dissolved oxygen concentration were measured twice a week with values from 17.6 to 21.6°C and 5.8 to 6.6 mg l^{-1}, respectively. The meagre juveniles were fed 3 times per day (8:00, 11:30, 15:00), 6 days per week (Monday-Saturday). All the uneaten feed of each tank was collected daily and dried to calculate the daily feed intake. Dead fish were recorded daily and survival was determined.

Growth performance and feed utilization

The trial lasted 135 days and samplings were done every 45 days. For 24 h before each sampling, fish were not fed. Fish were anesthetized with clove oil and were measured for the estimation of growth parameters. Fish samples for histology, gene expression, enzymatic analysis and biochemical were taken at the end of the trial.

The 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.

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 - fish eviscerated fish weight)/fish weight.

Histology

At the beginning of the trial, 50 meagre juveniles and at the end 23 fishes per diet were sampled for macroscopic evaluation of granulomas and samples of liver, kidney, heart and spleen were taken for histology analysis. These samples were fixed in 4% formalin, 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), Ziehl-Neelsen (ZN), the Fite-Faraco method and Gram stain.

The severity of granulomatosis was scored in each organ. A quantitative method was developed to classify the severity of granulomas in each organ. The score depends of the organ, because the number of granulomas in each organ is variable. The severity was classified in liver, kidney and heart according to the following criteria shown in **Table 24.1.4.2**.

Table 24.1.4.2.- 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

Gene expression

Liver, kidney and heart were taken aseptically from 10 fishes in the 90 days sampling and stored at -80 °C. Total RNA was extracted using TRI© Reagent (Sigma). Purity was assessed by spectrophotometry (A260/A280), followed by a visual quality assessment via agarose gel electrophoresis on 2% agarose gels stained with GelRed ™ Nucleic Acid. The cDNA was synthetized using SYBR® Green RT-PCR Reagents Kit.

The relative transcript abundance of Interleukin-1b, Interleukin-10, Tumor Necrosis Factor alpha and Cyclooxygenase were determined by quantitative (q) RT-PCR. Expression level of each gene was normalized to the corresponding expression of β-Actin or Elongation factor. Primers used for these genes were obtained by P3. IRTA and P5. UNIABDN, and have been reported in ***Deliverable 24.3 Cloning of key marker genes of innate and adaptive immune responses in meagre***. It is the first time that these genes are measured in meagre tissue (liver, kidney and heart), so it has been necessary to determine the amplification conditions for gene and tissue. In addition, the design of "specific" primers for glutathione peroxidase, superoxide dismutase, catalase and heat shock protein genes is in progress.

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. The variables that satisfied the normality and homogeneity a parametric one-way (ANOVA) and Tukey test were used. 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 was used for the variables with normality and a Mann-Whitney test for the non-parametric. A significance level of 0.05 was used.

Results

Combination of different levels of dietary vitamin E, C or astaxanthin did not affect fish growth performance, feed utilization and biometric parameters of juveniles meagre in 104 days of feeding (**Table 24.1.4.3**). In this feeding period, meagre juvenile grew from ~ 79.6 g to 264.3 g. No significant difference among the final weight, length, weight gain, specific growth rate (SGR), survival, fish condition factor (K) and hepatosomatic and visceral indexes were found. A good food conversion ratio (FCR) was obtained in all the diets (0.84-0.87) but there were not any differences among the diets. After the 104 days of feeding, the number of fish per tank was changed from 50 to 25 to adjust the density. The mean initial weight was ~ 264.3 g and 59 days after (day 163) the mean weight was ~402.9g. There were significant differences in the final weight being higher in the diets O, ECA and EECCA. The FCR ranged (0.91-1.01), and SGR (0.77-0.86) (**Table 24.1.4.4**).

Table 24.1.4.3. 0-104 days growth performance ($p<0.05$).

0 - 104 experimental days						
Diets	Weight (g)	Weight gain (g)	Length T (cm)	FCR	SGR	Survival (%)
O	268.09±44.26	188.50±5.50	28.20±1.58	0.86±0.01	1.15±0.02	100±0.00
A	258.30±43.35	178.97±7.89	28.07±1.39	0.87±0.02	1.12±0.02	100±0.00
EC	262.78±45.55	183.25±2.56	27.81±1.57	0.87±0.02	1.14±0.03	98.00±2.00
ECA	265.11±42.30	185.95±11.56	28.22±1.48	0.87±0.02	1.15±0.02	96.67±4.16
EECC	260.67±38.52	180.48±11.27	28.21±1.33	0.87±0.03	1.12±0.03	97.33±4.62
EECCA	271.01±42.83	188.57±3.60	28.15±1.56	0.84±0.00	1.16±0.03	98.00±2.00

Table 24.1.4.4. 104 - 135 days growth performance ($p<0.05$).

104 -135 experimental days									
Diets	Weight (g)	Weight gain (g)	Length T (cm)	FCR	SGR	HSI	VIS	K	Survival (%)
O	413.44 ± 69.63 ^{bc}	128.87 ± 9.82	33.42 ± 1.79	0.92 ± 0.04	0.85 ± 0.04	1.68 ± 0.20 ^{b}	3.21 ± 0.23	1.10 ± 0.07 ^{b}	100±0.00
A	386.19 ± 73.70 ^{a}	114.05 ± 15.76	32.76 ± 1.92	0.98 ± 0.09	0.80 ± 0.08	1.68 ± 0.22 ^{b}	3.13 ± 0.31	1.09 ± 0.07 ^{b}	100±0.00
EC	394.31 ± 69.63 ^{ab}	113.83 ± 1.44	32.91 ± 1.96	1.01 ± 0.03	0.77 ± 0.03	1.52 ± 0.25 ^{ab}	3.09 ± 0.31	1.10 ± 0.07 ^{b}	100±0.00
ECA	414.72 ± 74.81 ^{bc}	130.88 ± 4.68	33.40 ± 2.09	0.91 ± 0.04	0.86 ± 0.06	1.70 ± 0.36 ^{b}	3.25 ± 0.45	1.10 ± 0.08 ^{b}	100±0.00
EECC	393.89 ± 65.49 ^{ab}	116.801 ± 8.21	33.17 ± 1.76	1.00 ± 0.14	0.80 ± 0.07	1.45 ± 0.23 ^{a}	2.96 ± 0.27	1.07 ± 0.05 ^{a}	93.33± 1.15
EECCA	414.72 ± 73.68 ^{c}	126.72 ± 4.03	33.32 ± 1.94	0.92 ± 0.04	0.83 ± 0.04	1.63 ± 0.30 ^{ab}	3.11 ± 0.33	1.10 ± 0.06 ^{b}	93.33± 1.15

Histology

At the end of the feeding period, only 10 fish presented macroscopic granulomas, not being related to any particular dietary treatment. In the microscope observation for the evaluation of the tissues could be observed different stages of development of the granulomas. These granulomas begin with an accumulation of macrophages (**Figure 24.1.4.1a**), which progressively produce a necrotic centre (**Figure 24.1.4.1b**), and in some cases are surrounded by a layer of fibroblasts and inflammatory cells (**Figure 24.1.4.1c**). In all cases, the Zielh-Neelsen and Fite -Faraco stains were negative.

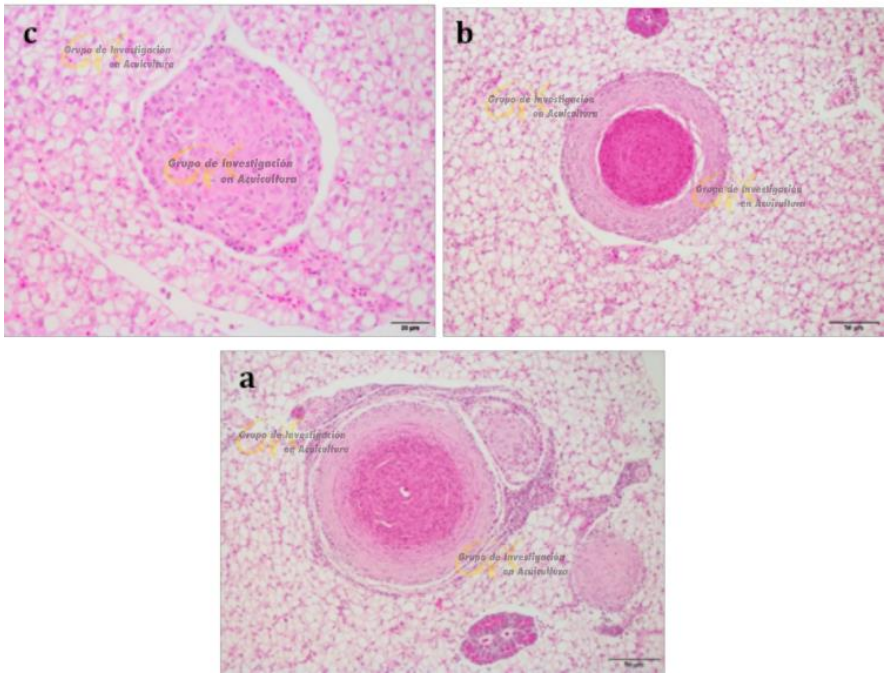


Figure 24.1.4.1. Different stages of granuloma development in liver. **a)** Irregular aggregate of macrophages. **b)** granuloma with necrotic center. **c)** Well develop granuloma with necrotic center surrounded by a layer of inflammatory cells.

The most affected organ was the liver (up to 96% of fishes), followed by the kidney (~ 41%) and heart (~ 6%). In no cases was spleen affected. Significant differences were found in the number of fish that had granulomas (**Table 24.1.4.5**), with a tendency to have less granulomas when astaxanthin is added to low levels of vitamin E and C, and when high levels of vitamins E and C with or without astaxanthin are included in diets for juvenile meagre (**Table 24.1.4.5**).

Table 24.1.4.5. Percentage of affected fish with microscopy granulomas ($p<0.05$).

Diets	% Fish affected with microscopy granuloma
O	96.33 ± 7.57^{ab}
A	96.00 ± 6.93^{ab}
EC	100.00 ± 0.00^{b}
ECA	81.57 ± 12.42^{a}
EECC	79.67 ± 10.97^{a}
EECCA	87.33 ± 1.15^{ab}

Similar results, although without significant differences, were obtained in the severity of granulomas **(Table 24.1.4.6)** and in the number of fish with a severity scored with 3 points in liver and kidney **(Figure 24.1.4.3),** decreasing both values when astaxanthin is added to low levels of vitamin E and C, and when high levels of vitamins E and C with or without astaxanthin are included. Analysis of gene expression, enzymatic and biochemical are currently in progress**.**

Table 24.1.4.6. Average of granuloma severity ($p<0.05$).

	Granuloma severity		
Diets	Liver	Kidney	Heart
O	1.43 ± 1.24	0.74 ± 1.01	0.00 ± 0.00
A	1.65 ± 0.83	0.70 ± 1.02	0.04 ± 0.21
EC	1.61 ± 0.99	0.74 ± 1.01	0.13 ± 0.34
ECA	1.39 ± 0.99	0.65 ± 0.88	0.30 ± 0.88
EECC	1.13 ± 0.97	0.74 ± 1.01	0.13 ± 0.63
EECCA	1.30 ± 0.88	0.39 ± 0.66	0.17 ± 0.65

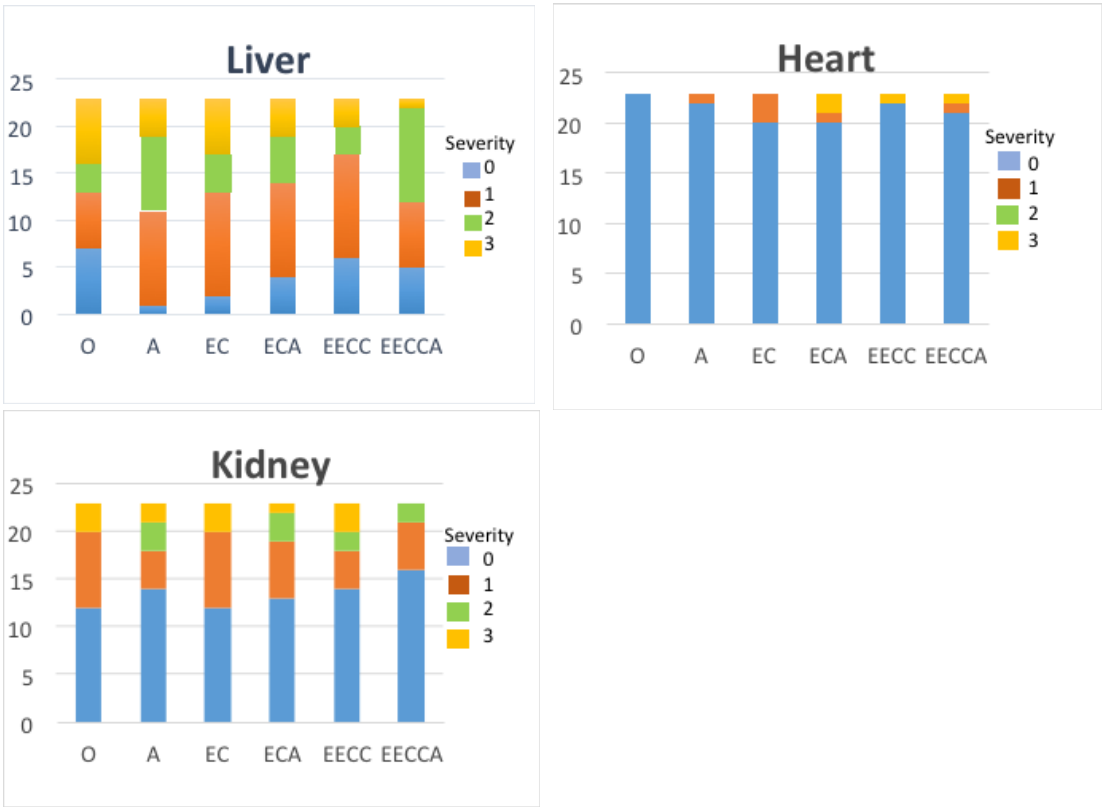


Figure 24.1.4.3. Number of fish in each severity stage in liver, kidney and heart fed different diets ($p<0.05$).

Trial 5 (FCPCT)

The effect of Se, Mn and Fe will be examined in SG prevention. Diet formulation and experimental design are currently in progress

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 in the 1st Periodic Reportt.

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 Cronic 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 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 was 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																					
Histochemisty																					
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	CGGCGATAACTTCTACTACAAAGG
	R	GCCAGCAAGCACATACCA
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 b-actin. 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 histochemisty: 3 fish from each tank were fixed in 2% paraformaldehyde in phosphate buffer (pH 7.4) at 4°C for 1 day and then stored in phosphate buffer with an additional 6.8% sucrose at 4°C. 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.

SEM, histochemistry and histology are in progress.

Preliminary 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 sea water (**Figure 24.2.1**). The average length and weight of the fish of the different water sources are presented in **Figure 24.2.1**. The growth performance of the fish was not affected by the different source of water ($p>0.05$).

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.

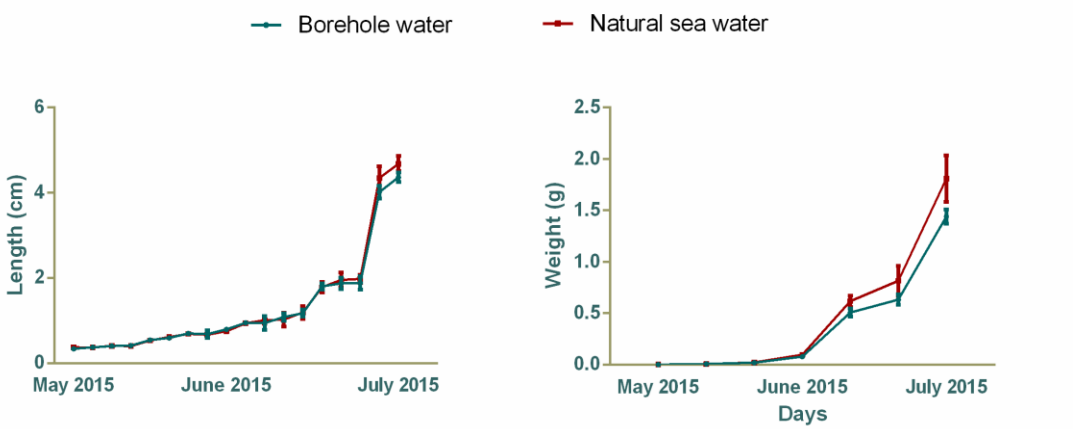


Figure 24.2.2. Average length and weight of meagre reared in borehole and natural seawater. The values are mean±SD.

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 O2 levels did not different between the two sources (**Figure 24.2.3**).

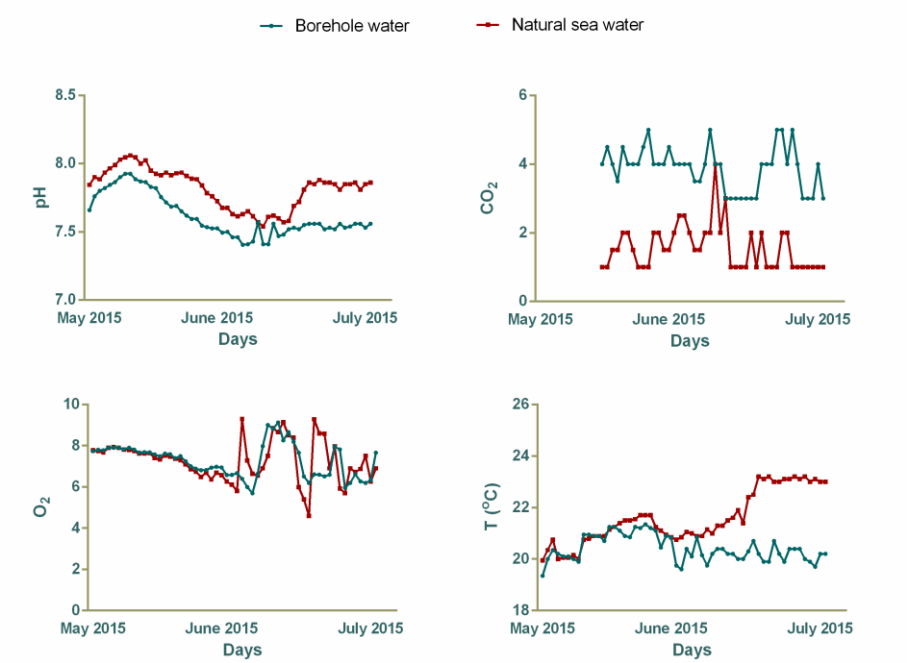


Figure 24.2.3. Physicochemical analysis of two different sources of water.

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, the expression of CathK and TRAP was significant higher in the head of fish reared in borehole water than in fish reared in natural sea water. The expression of vATPase did not exhibit significant differences between the two water sources (**Figure 24.2.4**).

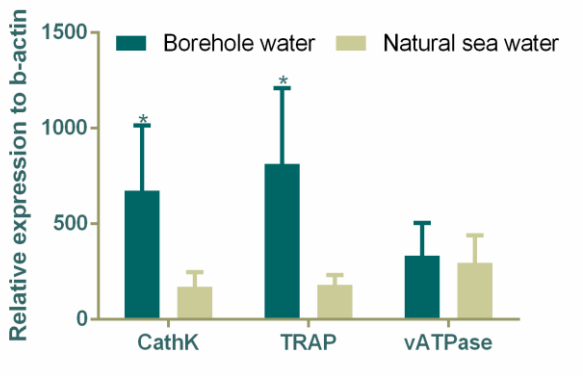


Figure 24.2.4. Relative expression of CathK, TRAP and vATPase of meagre's head reared in borehole and natural sea water at the end of the rearing trial (56dph). Values are means±SD (*) indicate a statistical significant differences between the two water sources ($p<0.05$).

From the comparative histological analysis of meagre reared in borehole and natural seawater no differences were observed until 40 dph. The onset of the disease was found to be about on 46th dph. **Figure 24.2.5** shows a supraorbital canal of meagre reared in natural seawater and in borehole water. In meagre of natural seawater the supraorbital canal was completely developed while in meagre from borehole water we observed erosion, ulceration and loss of the basal membrane while the neuromast was exposed to the external environment.

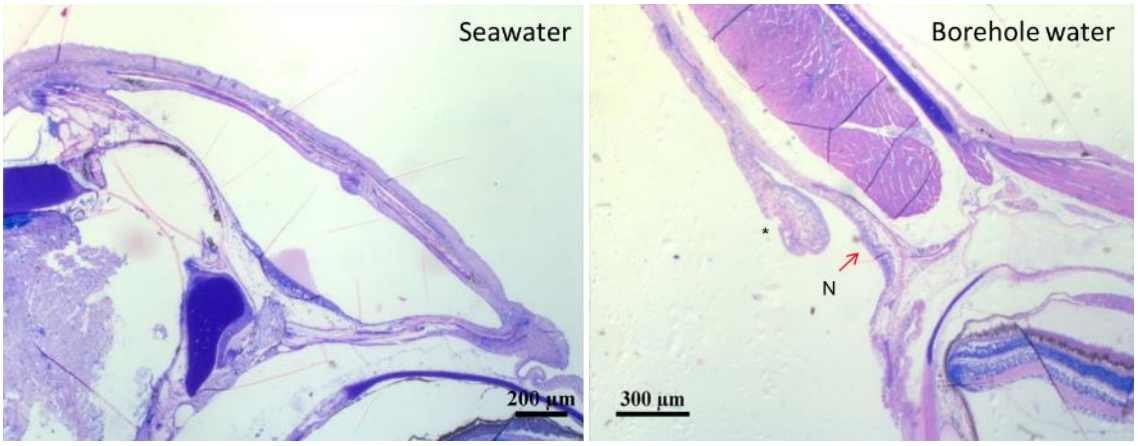


Figure 24.2.5. Supraorbital canal of meagre (46 dph) reared in natural sea water and borehole water. Meagre from natural seawater had normal supraorbital canal while in meagre from borehole water there was loss of the basal membrane, hyperplasia (*) while the neuromast (N) was exposed to the external environment.

The preliminary 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 osteoclast which are in close proximity with the environment like the osteoclasts of the lateral line canals. In such 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 preliminary results are in agreement with this hypothesis since there is a big overexpression of the genes which are related to the osteoclast activity in the fish grown in borehole water. Analysis is still ongoing.

Task 24.3. Anti-parasitic treatments (led by IRTA, Ana Roque).

Currently one of the most frequently detected health problems in meager is the presence of external parasites, namely on the gills. A preliminary analysis of last year results indicate that meagre does not feed well on medicated feed and histological slides from internal organs showed degradation of liver and intestine. An experiment was designed to investigate when this degradation starts.

Material and Methods

A system with four tanks was set up with 12 fish in each tank. Juveniles were around 100g wet weight and each was fed a different diet (control, diet with mint oil, diet with cinnamon oil and diet with praziquantel) at 2% of their weight. Once a week 3 fish were sacrificed and sampled for histology from each tank. The experiment lasted 4 weeks. Analysis is still in progress.

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 objective of this task was to isolate *Nocardia* spp., which have been reported as a possible threat for meagre aquaculture. During the first years of the project we have examined a large number of fish of varying sizes using both microbiological but also molecular techniques. Fish have been collected from various localities during the reporting period. Healthy, moribund and fish exhibiting disease signs were sampled belonging to a range of developmental stages. A summary of the samplings is presented in the **Table 24.4.1**. Several samples have been obtained earlier and analysis have been performed during the DIVERSIFY project.

Table 24.4.1. Samples processed for Nocardia isolation.

Sampling Date	Locality	# fish	Mean W (g)	Mean L (cm)
11/9/2013	HCMR	20	Pre ongrowing	
7/10/2013	HCMR	20	Pre ongrowing	
16/3/2014	HCMR	20	Pre ongrowing	
10/4/1014	Galaxidi	1	307,7	29,5
29/9/2014	Souda	20		
5/5/2015	Atalanti	2	2,445	
10/8/2015	HCMR	9	4	6
15/10/2015	Siteia	10		
20/10/2015	Siteia	10		
26/10/2015	Siteia	10		
27/10/2015	Aegean	5	518	37,5
26/2/2016	HCMR	1	breeder	breeder
1/3/2016	Souda	4		
11/3/2016	Galaxidi	8	471,5	33
6/4/2016	Galaxidi	7	39,4	15

Tissue sampling included mainly the kidney and depending on the case the liver, spleen, heart, brain, ascitic fluid etc. Tissues used for molecular analysis were preserved in -20^{o}C. Tissues used for histology were preserved in 10% buffered formalin (PBF). The PBF preserved samples 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, Ziel-Neelsen (acid-fast bacteria) and Grocott stains (fungi) and examined under a light microscope.

For the isolation of *Nocardia* spp. general (BHI 2% NaCl and BHI 0,5% NaCl) and selective for Mycobacteria, also recommended for *Nocardia* spp. (*Löwenstein-Jensen*, LJ) solid media were used. Cultures were performed mainly from the kidney using aseptic techniques. Plates were incubated at 25^{o}C and were observed for more than three weeks.

For the detection of *Nocardia* spp. from tissues and growth on culture media the genus specific primer pair: NG1 (5'-ACCGACCACAAGGGG-3') and NG2 (5'-GGTTGTAACCTCTTCGA-3') (Laurent et al., 1999) was used to amplify a 596 bp part of 16S rRNA gene. *Nocardia seriolae* NCIMB 13256 was used as positive

control. For the detection of *Mycobacteria* the taxon specific primer pair 246 (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1522 (5'-AAGGAGGTGATCCAGCCGCA-3') (Böddinghaus et al., 1990) was used to amplify approximately 1400 bp of 16S rRNA gene. The primer pair My1 (5'-GGA AAG GTC TCT TCG GAG-3') and 1522 to amplify approximately 1400 bp of 16S rRNA gene of *M. marinum* (Ucko et al., 2002). A *Seriola dumerili larvae from HCMR previously found positive for Mycobacteria was used as positive control.*

Sampling of P1. HCMR 10/8/2015

Nine healthy fish were sampled in total. Macroscopic examination of internal organs revealed that all fish exhibited granulomas mainly the in kidney. No bacterial growth was observed in the cultures. Tissue from liver and kidney were used for PCR detection of *Nocardia* spp. **Figure 24.4.1**. Presence of Mycobacteria was also assessed. Samples were **negative for both pathogens.** *Histopathological examination revealed that all samples* ***were negative for Nocardia spp.***

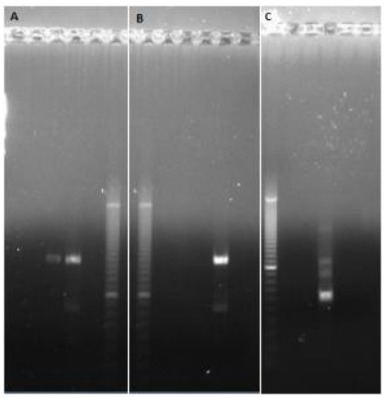


Figure 24.4.1. Example of PCR for detection of *Nocardia* spp. The first two wells represent samples. The second two wells represent the positive controls for *Nocardia* spp. and Mycobacteria respectively. The last well represent the blank control sample. A ladder of 3000 bp was used to compare products' sizes in all PCRs. A) PCR for detection of Mycobacteria. B) PCR for detection of *Mycobacterium marinum*. C) Nested PCR on 16S gene for the detection of *Nocardia* spp.

Sampling Aegean Sea (Greece) 27/10/15

Five fish exhibiting epidermal lesions were sampled. All exhibited abscesses and granulomas in the kidney to different extents though (**Figure 24.4.2**). The PCR from bacterial growth on LJ was negative for *Nocardia* spp. but gave a positive result for presence of Mycobacteria. *Histopathological examination* of spleen, kidney and liver *revealed that all samples were negative for Nocardia spp.*

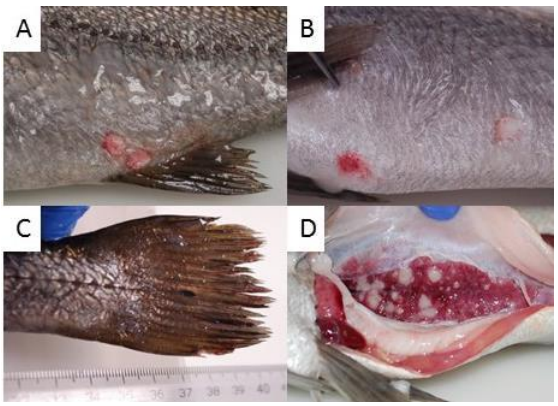


Figure 24.4.2. Fish exhibited granulomatous lesions on the skin (A and B), fin erosion (C) and abscesses and granulomas in the kidney resembling mycobacterial infection

Although positive PCR products for *Mycobacterium marinum* were obtained, the bacteria could not be further recultured in subsequent attempts and could not isolated in pure culture. The PCR products will be sequenced in order to identify and further characterize the pathogen.

Sampling at HCMR 26/2/2016

Sampling was conducted of a breeder fish after death. The aetiology of death has not been defined yet. Samples for histology included tissue from the gills, kidney and liver. Fish tissues for molecular analysis included kidney and ascitic fluid. Culture for bacterial pathogens was conducted from kidney, ascitic fluid and swim bladder. Cultures were conducted directly from the tissues, and from homogenized tissues in serial dilutions. Dilutions were also used for the cases of mixed growth on isolation plates in order to separate the microorganisms.

Microscopic examination was conducted from fresh kidney and gill tissues and ascitic fluid. The fish exhibited granulomas affecting mainly the kidney but the manifestation of the diseases was light to moderate. A PCR was conducted for detection of *Nocardia* spp. from tissues and growth on culture media with the primers described above. Samples used for PCR are presented below:

1. Ascitic fluid
2. Growth from kidney on LJ
3. Growth from homogenized kidney on LJ
4. Growth from kidney on LJ (recultured on LJ)
5. Kidney tissue
6. Growth from kidney on BHI2% NaCl (from dilution)
7. Growth from swim bladder on BHI2% NaCl
8. Growth from kidney on BHI2% NaCl
9. Growth from kidney on BHI2% NaCl
10. Growth from kidney on BHI2% NaCl
11. Growth from kidney on BHI2% NaCl (from dilution)
12. Growth from kidney on BHI2% NaCl (from dilution)
13. Growth from kidney on BHI2% NaCl (from dilution)

PCR for detection of *Nocardia* spp. was negative. PCR for Mycobacteria gave positive results for most of the samples. *Histopathological examination* of spleen, liver and gills *revealed that all samples were negative for Nocardia spp.*

Sampling at Galaxidi Marine Farms at 11/3/2016 and 6/4/2016

The analysis of this sampling is still in progress. All fish exhibited granulomas affecting mainly the kidney. The swim bladder was enlarged and all fish exhibited exophthalmia. In large fish granulomatous skin lesions were observed in some cases while the epithelium of the swim bladder was covered by granulomatous material (see **Figure 24.4.3**).

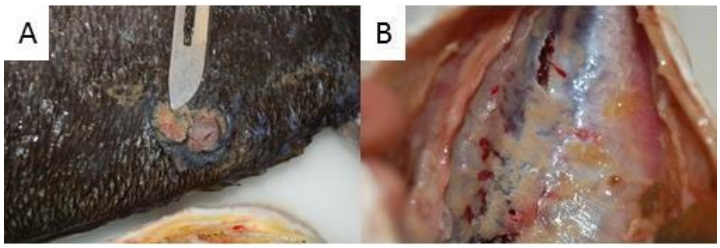


Figure 24.4.3. A. Granulomas at the epidermis and the underlying muscles. B Diffused granulomas at the peritoneal membrane covering the swim bladder.

Further research on other pathogen taxa such as Mesomycetozoa and Fungi have been planned in order to assess the different pathologies observed in the different cases. ***Nocardia* spp. has not been detected yet in any of the incidents described above, neither by PCR or by staining of tissue sections**. Results are summarized in the table below.

Table 24.4.2. Results concerning the isolation/identification of Nocardia

Sampling Date	Locality	Isolation of Nocardia	Histo samples	PCR Nocardia
11/9/2013	HCMR	(-)	(-)	(-)
7/10/2013	HCMR	(-)	(-)	(-)
16/3/2014	HCMR	(-)	(-)	(-)
10/4/1014	Galaxidi	(-)	(-)	
29/9/2014	Souda	(-)	(-)	
5/5/2015	Atalanti	(-)	(-)	
10/8/2015	HCMR	(-)	(-)	(-)
15/10/2015	Siteia	(-)	(-)	
20/10/2015	Siteia	(-)	(-)	
26/10/2015	Siteia	(-)	(-)	
27/10/2015	Aegean Sea	(-)	(-) Myco (+)	(-)
26/2/2016	HCMR	(-)	(-)	(-)
1/3/2016	Souda	(-)	(-)	(ND)
11/3/2016	Galaxidi	(-)	(-)	
6/4/2016	Galaxidi	(-)	(-)	

Sub-task 24.4.2. Preparation of an autogenous vaccine (HCMR, Pantelis Katharios).

Since no strains of *Nocardia* have been isolated to date this task cannot be performed. Instead we are planning to change this task and include a commercial vaccine for *Vibrio* that will be tested in meagre (See later "Deviations". This change will affect Task 24.6, which is led by Aberdeen.

Task 24.5. First characterisation of the immune system (led by UNIABDN, Chris Secombes).

At the outset of this work, specific genes were agreed upon as being markers of immune system development and also representative of different immune functions. The genes that were targeted were unknown for meagre and for this reason recovery of the sequences of interest was needed prior to designing the specific quantitative PCR assays for the completion of this task. Alignments of homologs of the target genes were prepared using sequences from extant species already annotated in GenBank. These alignments were used to identify regions of the genes that were conserved among the various species represented in the alignments, to allow primer design. Next, tissue samples collected from gills, spleen, kidney and intestine were used for RNA extractions. These samples for RNA isolation were prepared from post-weaning sub-adult individuals to optimize recovery of all targeted genes including those for which expression is initiated later in development. Following cDNA synthesis and PCR, amplified gene fragments were cloned and sequenced.

Using the meagre sequences obtained, new primers were designed to have approximately the same melting temperatures (Tm ºC), to optimize the efficiency of primer binding and allow multiplexing of different reactions on the same qPCR plate. In total sequences were obtained from 5 endogenous control genes and 22 target genes for evaluation of the immune system functions and ontogenetic development.

For analysis of immune genes during development samples were collected during grow-out of larvae of meagre by P3. IRTA. Duplicate samples were taken at each time point with one set collected in RNA later for the purpose of molecular biological analyses and the second set collected in neutral buffered formalin for histological analyses. The analysis of immune gene expression is on-going. More details concerning this task have been reported in ***Deliverable 24.3 Cloning of key marker genes of innate and adaptive immune responses in meagre*.**

Task 24.6. Monitor specific immune responses (led by UNIABDN, Chris Secombes).

The vaccination/challenge experiment to be performed in this task is at the planning and experimental design stage, with a view to begin in the summer/autumn 2016. Meantime progress has been made with the antibody production to meagre IgM. The full length sequence was obtained in Task 24.5 and appropriate peptides selected, by measuring peptide immunogenicity, hydrophobicity, and accessibility, for which to raise antibodies against (*e.g.* Using IEDB Antibody epitope prediction software, **Fig. 24.6.1**).

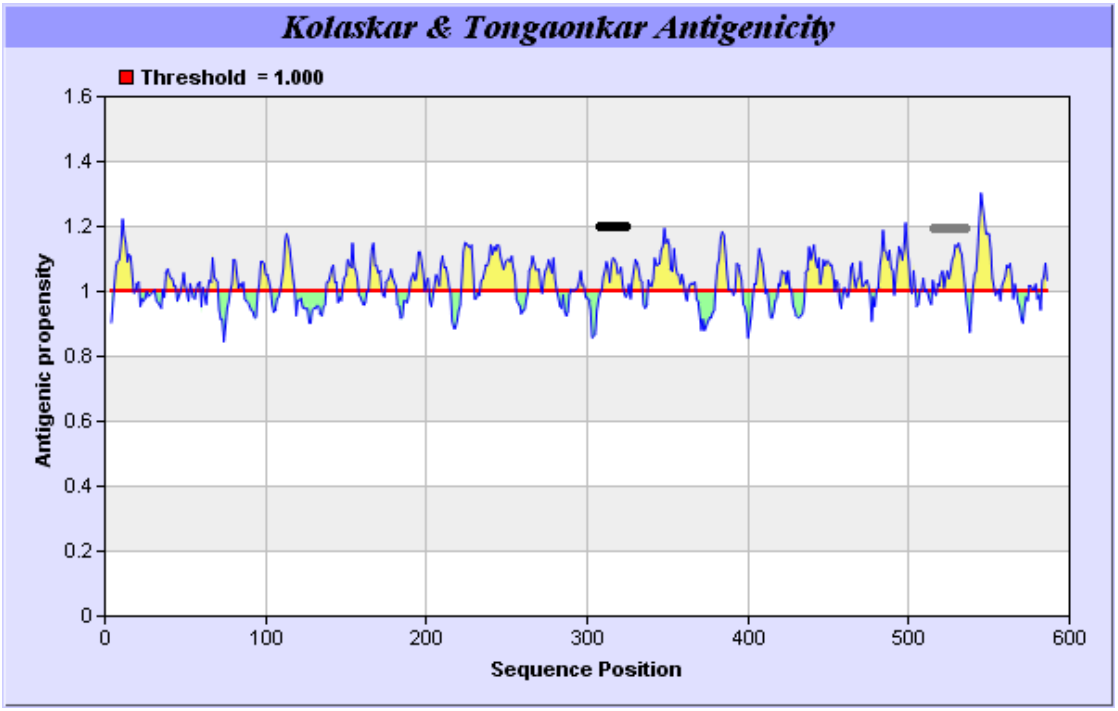


Figure 24.6.1. Graph showing the antigenicity, accessibility and hydrophobicity of the full length meagre IgM sequence. The black dash indicates peptide Z68, and the grey dash indicates peptide Z69.

Two peptides were synthesised (Z68 and Z69), and conjugated to Ovalbumin for immunisation of Balb-c mice using standard procedures. Serum was collected post-immunisation and used in an ELISA assay with peptides conjugated to Bovine Serum Albumin to coat the plates. The results (see **Figure 24.6.2**) indicate that there is a strong polyclonal response to both peptides, with an OD over double the negative control considered a positive response.

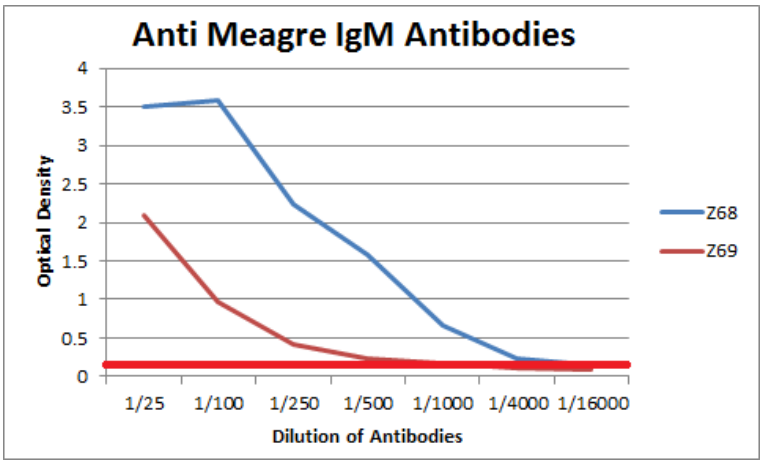


Figure 24.6.2. Results from ELISA using target peptide and sera from the tail bleed of mouse. The red line is equal to double the negative control, which indicates a positive result for polyclonal antibodies.

The spleens from these mice have been harvested for hybridoma generation. The hybridoma cells have been cloned in 96 well plates and a preliminary screening for positive monoclonal antibodies (moAb) has been performed. Several wells with a similar or higher OD than the positive control (sera from the mouse polyclonal Ab) have been found, indicating strong candidates for B-cells producing highly specific moAb to meagre IgM.

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, four samplings were made and analyzed with no mortality associated. No pathogenic bacteria have been isolated. Besides, Nodavirus determination in a meagre population within P2. FCPCT facilities was also conducted with negative incidences of Nodavirus in the population.

We are currently tuning *Photobacterium damselae* subsp. *piscicida*, Nodavirus and Nocardia challenges in 2016.

For the development of the challenges against pathogens we will use meagre of an average weight of 40 g, inoculated intraperitoneally with doses ranging from 10^3 to 10^7 cfu/ml and we will monitor mortality. Fish will be kept up to 15 days after the last mortality. For Nodavirus infection we will make an intramuscular dose with 10^8 TCID50/ml. All challenges will be made when fish are in the right size.

Material and Methods for Nodavirus detection in meagre population:

PCR for NODAVIRUS. Nodavirus for detecting PCR primers were used **Table 24.7.1** at a concentration 10 µM (Invitrogen).

Table 24.7.1. Primers used to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
NODA 1 reverse	CGAGTCAACACGGGTGAAGA	427 pb
NODA 2 forward	CGTGTCAGTCATGTGTCGCT	

Amplification was performed in 25 µl volume containing 5 µl cDNA, 2.5 µl 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of MgCl2, 1 µl of each primer, 0.125 µl Taq polymerase (Bioline) and the rest of DEPC water to a total volume of 25 µl for each reaction. The protocol is described in Table **24.7.2.**

Table 24.7.2. Protocol cycles for the second phase.

TEMPERATURE ºC	TIME	Nº OF CICLES
94	4'30''	1
94	30''	35
58	30''	
68	30''	1
68	10'	

The PCR products were stored at 4 ° C or -20 ° C until display in agarose gel electrophoresis 2%. The above PCR product was used for a Nested-PCR for this virus. To this end the pair of internal primers that amplify the PCR product of primers obtained from Table 1 and which generate a product of 159 bp amplification (**Table 24.7.3**) was used.

Table 24.7.3 Primers used for a Nested-PCR to detect Nodavirus. (Bp = base pairs).

PRIMERS	SEQUENCE (5'-3')	FRAGMENT SIZE
R31 reverse	AGTGTCTCCAGCTTTCTTC	159 pb
F21 forward	GATTTCGTTCCATTCTCTT	

Primers were used at a concentration of 50 mM (Invitrogen). Amplification was performed in 25 µl volume, containing 3 µl of Nodavirus PCR product, 2.5 ml µl of 10X buffer, 0.5 µl of mixture dinucleotide phosphate (20mM) (Bioron), 0.75 µl of MgCl2, 1 µl l of each primer, 0.125 ml µl of Taq polymerase (Bioline) and the rest of DEPC water to a total volume of 25 µl, per reaction (**Figure 24.7.1**).

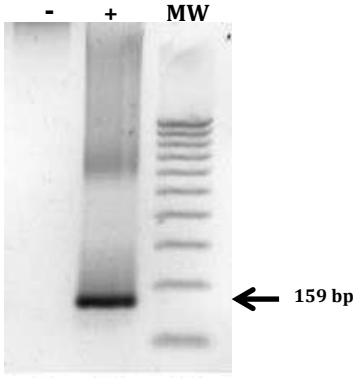


Figure 24.7.1. Amplification of Nodavirus PCR product. Positive control for 159 base pairs.

Control of Nodavirus in broodstock and juvenile meagre

Samples of different animals from the P1. FCPCT facilities were analysed. From broodstock, samples from nervous system from any dead breeders during the project and frozen for analysis were analysed. From juveniles, samples of nervous system from previously stressed animals (250 g body weight) were taken after sacrifice with anaesthetic overdose. The results show a population of virus-free broodstock (**Figure 24.7.2**), and a population of juveniles with no presence of virus (**Figure 24.7.3**). No Nodavirus was found in the P2. FCPCT meagre population.

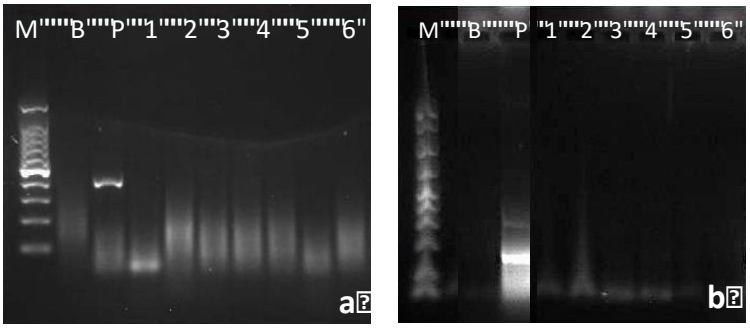


Figure 24.7.2. Analysis of nervous system samples from broodstock population: a) PCR, b) Nested-PCR.

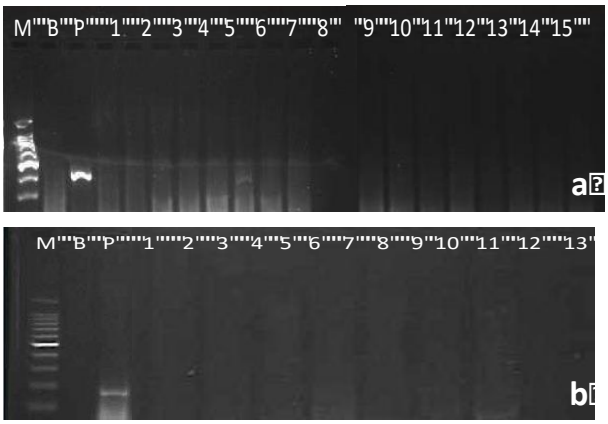


Figure 24.7.3. Analysis of nervous system samples from previously stressed juveniles: a) PCR, b) Nested-PCR.

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. Below we report briefly two incidences of diseases that have been recorded and will be presented in detail in the manual.

Diplectanum sciaenae in broodstock of meagre

An outbreak of diplectanid monogeneans occurred in the facilities of IRTA in Spain. The Spanish research team sent samples to HCMR and the parasite was studied thoroughly using histology and SEM. The parasitosis is a recurrent issue and has already resulted in significant losses of valuable broodstock. Under this task we have managed to identify the parasite as *Diplectanum sciaenae* and to provide identification tools. The work has now been published in the journal *Veterinary Parasitology: Regional Studies and Reports* (**Figure 24.8.1**), and images of the parasite can be seen in **Figures 24.8.2 and 24.8.3**.


Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

ELSEVIER

Short Communication

Diplectanum sciaenae (Van Beneden & Hesse, 1863) (Monogenea) infecting meagre, *Argyrosomus regius* (Asso, 1801) broodstock in Catalonia, Spain. A case report

K.B. Andree [a], A. Roque [a], N. Duncan [a], E. Gisbert [a], A. Estevez [a], M.I. Tsertou [b], P. Katharios [b,*]

[a] IRTA, C/ al Poble Nou, Km 5,5 43540 Sant Carles de la Ràpita, Tarragona, Spain
[b] Institute of Marine Biology, Biotechnology and Aquaculture, HCMR, Former American Base of Gournes, Heraklion 71003, Crete, Greece

Figure 24.8.1. The title of the article published that describes the meagre parasite.

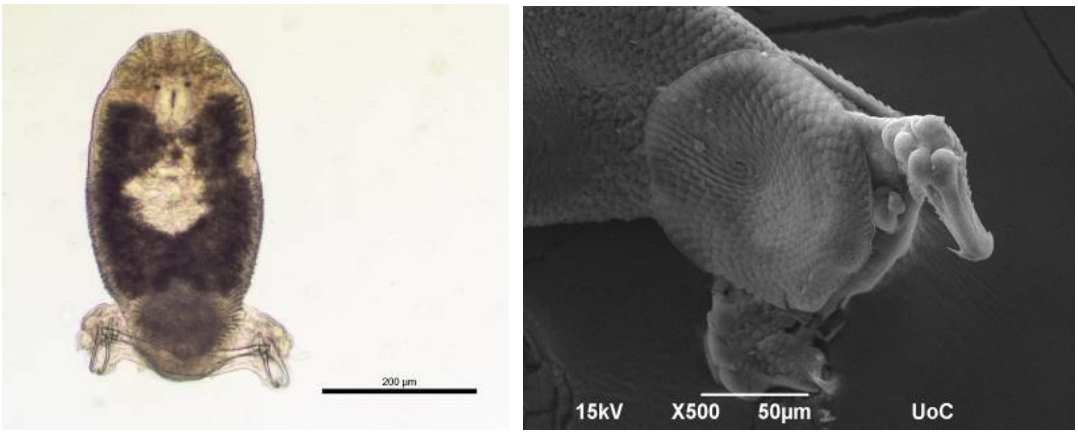


Figure 24.8.2. Micrograph of *Diplectanum sciaenae* from meagre broodstock (left). SEM micrograph of *Diplectanum sciaenae* haptor, which is the attaching apparatus of the parasite (right).

Mycobacteriosis in meagre

The affected meagre were obtained from a fish farm located in the Aegean Sea, Greece. The fish exhibited lesions resembling granulomas in the internal organs and mainly in the kidney. These lesions were considered to be different in texture compared with the lesions of SG and, therefore, the samples were sent for further investigation at P1. HCMR by the fish vet of the farm.

Five fish exhibiting epidermal lesions were sampled. All exhibited abscesses and granulomas in the kidney but to different extents (**Figure 24.8.4**). PCR from bacterial growth on LJ was **negative for *Nocardia* spp.** but gave a positive result for presence of Mycobacteria. *Histopathological examination* of spleen, kidney and liver *revealed that all samples* ***were negative for Nocardia spp. but had lesions typical of Mycobacteriosis (Figures 24.8.4 and 24.8.5).***

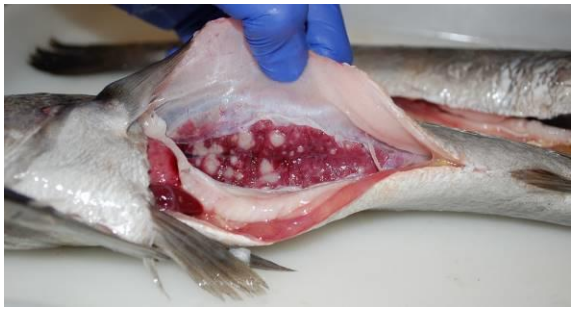


Figure 24.8.4. Fish exhibited granulomatous lesions and abscesses, and granulomas in the kidney resembling mycobacterial infection.

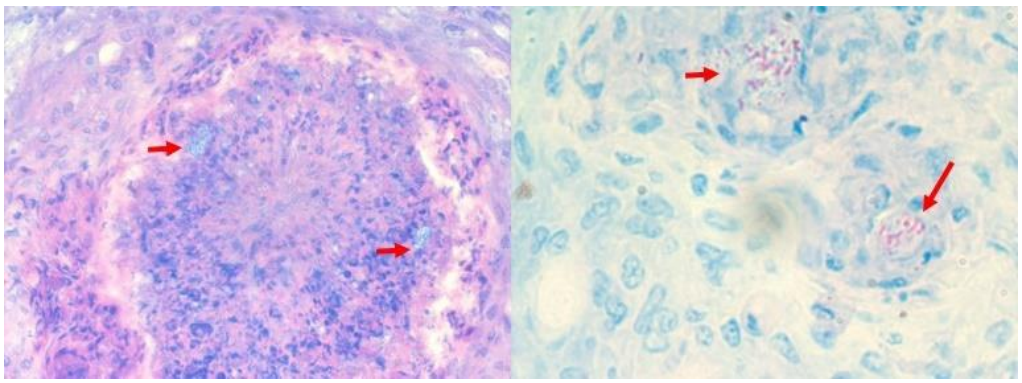


Figure 24.8.5. Histological sections from affected livers showing the typical granulomas related to mycobacterial infections. In the left picture the arrows point to "pocket-like" areas of the granulomas filled with bacteria. On the right is the same lesion at higher magnification with the bacteria stained positively with Ziehl Neelsen stain.

Although positive PCR products for *Mycobacterium marinum* were obtained which was in accordance with the histological assessment, the bacteria could not be further recultured in subsequent attempts and could not be isolated in pure culture. The PCR products will be sequenced in order to identify and further characterize the pathogen.

References

Böddinghaus, B., Rogall, T., Flohr, T., Blöcker, H., Böttger, E.C., 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. 28 , 1751–1759.

Laurent, F.J., Provost, F., Boiron, P., 1999. Rapid Identification of Clinically RelevantNocardia Species to Genus Level by 16S rRNA Gene PCR. J. Clin. Microbiol. 37 , 99–102.

Ucko, M., Colorni, A., Kvitt, H., Diamant, A., Zlotkin, A., Knibb, W.R., 2002. Strain Variation in Mycobacterium marinum Fish Isolates. Appl. Environ. Microbiol. 68 , 5281–5287. doi:10.1128/AEM.68.11.5281-5287.2002.

Deviations from Annex I and their impact:

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 D24.12, which will now be "Determination of the efficacy of vaccination of meagre against Vibriosis", and D24.13, which will now be "Description of immune gene expression post-immunisation and challenge of meagre with a *Vibrio* vaccine".

All changes will be official requested in the next amendment (3rd) of the ANNEX 1.

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):

In the 1st Periodic Report we piloted a number of systems and undertook first studies looking at the disease issues affecting greater amberjack culture. These included:

- 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 under different conditions 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 progress towards objectives and details for each task (13-30 Mo):

In the current reporting period progress has been made against all tasks and is outlined in detail 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 have been 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 have been 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 has been performed with juveniles in **Task 25.5**, with *Bacillus oceanisediminis* and *Aeromonas* spp. being detected. Challenge trial were also undertaken 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.

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 cultures 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 prematurely at 12 dph due to a massive mortality of unidentified cause. 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. 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.

2015 experiments

Two parallel larval rearing trials were made in P1. HCMR using the mesocosm larval technology. **Table 25.1.1** contains the information concerning these trials and the samples obtained.

A volume of 10 l of water were taken from the water column of the tanks at each sampling, in triplicate. Samples were taken using a special sampler. Samples were fractionated. Particularly, they were sequentially filtered through decreasing pore diameter filters: **250 μm, 120 μm, 53 μm** και **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 was 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 meagre and 13 for greater amberjack in 2015.

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

The filtrates were placed in 50 ml falcon tubes and centrifuged immediately at 5000 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 the appropriate volume of preservative, PBF 10% and RNA later respectively,

while the filtrates for future use were stored at -80ºC. Filtrates from the 0.22 μm filters were preserved in glycerol 25% at -80ºC.

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 56ºC) and DNeasy Blood & Tissue Kit was used for the DNA extraction. DNA of the microorganisms from the filter (0.22 μm diameter) was similarly extracted whereas DNA from the cultivated bacteria was extracted via a boiling process. One third of the filter (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.

Specific pairs of primers were used for each pathogen. The primers and PCR conditions used are presented in detail in **Table 25.1.2.**

Table 25.1.2. Data of the pairs of primers and the PCR conditions for the detection of pathogens, and the microorganism target.

Pathogen	Primer	Primer's sequence (5'-3')	Annealing temperature	Extension duration	Product size
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			

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 during the experimental larvae rearing in 2014 (See 1st Periodic Report). Possibly, this problem was related to bad quality of the eggs and some technical issues that also appeared (*e.g.* regulation of temperature of the tanks, shading of the tanks etc.), since there was no isolation of pathogens from the fish. For the experiment in 2015 the technical issues were solved resulting in a 33-day rearing duration with the survival and development of the larvae being similar to that referred to in the literature.

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 (*e.g.* inclusions in gills and skin) were not found in the fresh preparations nor in histology. Positive signal for *Chlamydiaceae* and *Endozoicomonas* spp. appeared for some of the filtrates. A positive signal for *Chlamydiaceae* was observed in different filtrates of both experiments and also in algae samples from the microalgae cultures that was used for the rearing process during the early developmental stages of the larvae. A 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 *Ichthyocystis* spp in the samples analyzed.

Apart from the experimental larvae rearing, that were performed for the isolation, for the observation and the understanding of the biology and the life cycle of Epitheliocystis agents, at the P1. 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 underwent initial 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). The qPCR, fluorescent in situ hybridization (FISH), TEM and genomic analysis were performed in Zurich. The typical morphology of the cysts of Epitheliocystis in gilthead seabream gills is shown in **Fig. 25.1.1**.

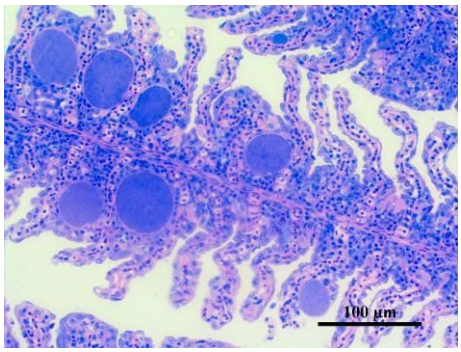


Figure 25.1.1. Typical appearance of Epitheliocystis. Intracellular inclusions in the gill epithelium of the secondary lamellae can easily be observed. T he sample is from a sea bream farm in Argolida, Greece.

Two different morphological types of cysts were observed in gill histology. The majority of the cysts were big with a diameter 80-100 μm surrounded in a fine, thin membrane whereas there were a few others of a 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. The 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 undertaken 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 β- or in γ-proteobacteria. These bacteria have a mainly intracellular life cycle and their cultivation *in vitro* has not been accomplished yet.

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).

From 2015 until now a total number of 67 gill samples from 25 sea bream with confirmed Epitheliocystis infection have been collected from 9 different regions of Greece. Twenty two are preserved in 96% ethanol

and 7 in RNA later for molecular analysis, 25 are preserved in PBF 10% for histology and 15 are preserved in glutaraldehyde for Electron Microscopy analysis.

A PCR screening of the samples confirmed that the microbial agent responsible for Epitheliocystis is *Candidatus* Ichthyocystis spp while Chlamydia are also present in most of the samples as well. PCR products giving a positive signal for *Candidatus* Ichthyocystis spp and *Chlamydia* (*Endozoicomonas* spp. was not detected in any of the samples) will be sequenced and compared to the identified sequences. Then, a phylogenetic analysis will reveal the phylogenetic relationship among the bacteria detected.

Candidatus Ichthyocystis spp has not been detected in Crete so far. In HCMR facilities, Epitheliocystis has affected larviculture using a 'Mesocosm' technique many times. The disease has emerged twice in *Diplodus puntazzo* larviculture and in *Seriola* and *Dentex* culture. From the incidents referred above, molecular identification was successful for the second incident of Epitheliocystis outbreak in *Diplodus* when the main pathogen related to the disease was *Candidatus Endozoicomonas cretensis* n. sp. One of the main problems is that most of the time, in the same incident, more than one microbial agent 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 the fact that the disease did not emerge 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 to the disease, since the primers used for *Chlamydia* detection were universal primers for *Chlamydia* signature primers. On the other hand, samples that gave a positive signal for Endozoicomonas will be analyzed further. The PCR products will be sequenced and then compared to the sequences of the pathogen *Candidatus Endozoicomonas cretensis* n. sp.

We intend to repeat the rearing trial in 2016 using greater amberjack eggs (Mo 31-32). The experiments will be based on the same methodology described here.

Task 25.2. Promoting resistance to parasitic incidence on greater amberjack (led by FCPCT, Daniel Montero).

Four different tasks have been conducted within the last year to achieve the proposed objectives to promote resistance to parasitic incidence on greater amberjack. 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.

A.- Morphological study on the incidence of monogenean parasite in Greater Amberjack skin. Optical and electron microscopy studies have been conducted.

Material and Methods:

For this study juvenile animals (342.9 ±52.9g) reared in the facilities of P2. FCPCT were used and distributed in 6 cylindroconical 500 L tanks with 5 fish in each tank. Fish were fed to apparent satiety during 30 days with a high protein commercial diet (Vitalis, Skretting). Animals were divided in two experimental groups, one group received an antiparasite prevention treatment protocol, that consisted of freshwater baths of 4 min to kill the oncomiracidia and the adult stages of the parasite, and also a thorough cleaning with bleach of the tanks and nets to kill the eggs that could have got entangled to miscellaneous parts of the tanks. For the other experimental group, no antiparasite prevention treatment protocol was applied. The final sampling came 30 days after the start of the trial, the animals were sacrificed and a dissection was conducted to take the skin of two different regions, cranial and dorsal. This points were selected because of their difference in the prevalence of fixation of the parasite, and are also a common place where the parasite gets attached. Tissue samples were fixed buffered formaldehyde 4% for posterior histological studies.

For the microscopic analyses a Glycol Methacrylate embedding (Technovit 7100;Kulzer) was conducted, this way plastic blocks produce an increase of the resolution power of the images. For the observation of the goblet cells, the sections were stained with Alcian-PAS, using GIEMSA for staining contrast instead of hematoxylin. The microscope for the observations was an OLYMPUS CX41RF with an adapted camera OLYMPUS U-MDOB3.

Results

1. Comparison between dorsal region and cranial region epithelium. Dorsal region present a thinner epidermis than cranial region (**Fig. 25.2.1**). More goblet cells were observed at the epidermal layer in the dorsal region. The dermis layer in the dorsal region is thinner than in the cranial region.

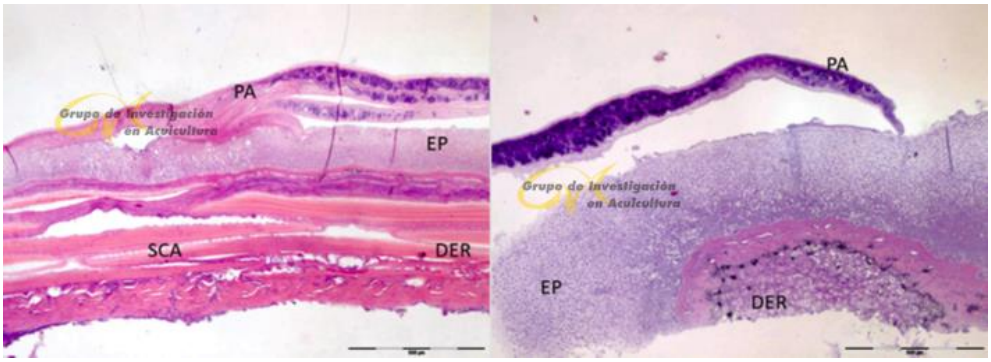


Figure 25.2.1. Dorsal (left) and cranial (right) region. PA: parasite; EP: epidermis; SCA: scales; DER: Dermis.

At the cellular level after parasite infection, a hydrophic degeneration was observed around the parasite site of adhesion (**Fig. 25.2.2**). Vacuoles present a water imbalance and occupy almost all the cytoplasm, moving the nucleus and all the cellular organelles to the cell extremes. This hydropic vacuole degeneration could cover bigger areas and produce spongiosis with intercellular edema. At the tissue level, a clear disruption of the epidermal layer was observed (**Fig. 25.2.3**). The haptor with the attachment structures produce an overpressure in the region where the parasite gets fixed, which results in a disruption of the typical structure of the tissue and the cells.

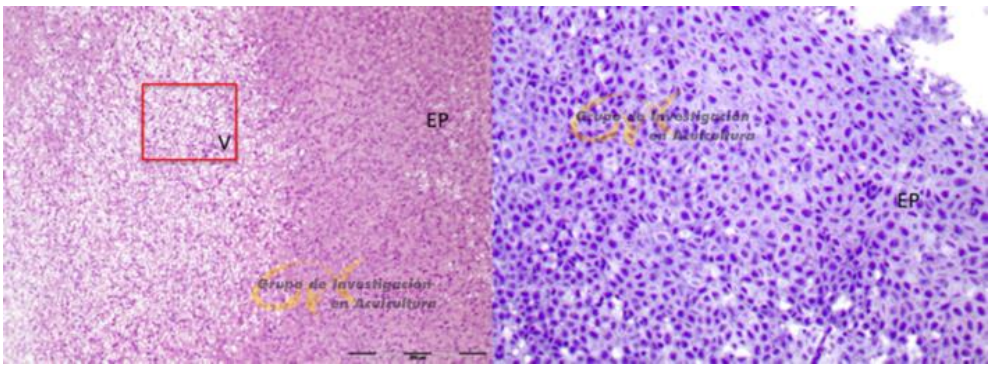


Figure 25.2.2. Vacuolization in the epidermis of *S. dumerili*. High level of hydropic degeneration that results in spongiosis (Left) *vs* epidermis of a non-parasitized animal (right). V: Vacuolization; MCLT: Mononuclear cells lymphocytic type.

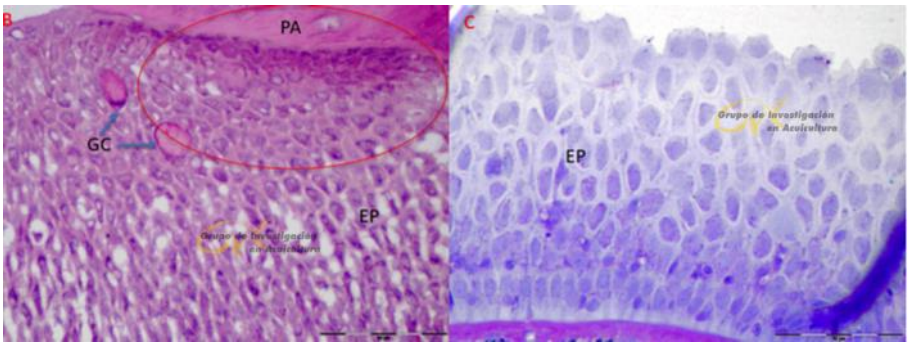


Figure 25.2.3. Disruption of the tissue and the cells (left) compared with a non-parasitized animal (right). PA: parasite; EP: epidermis; GC: Goblet cells.

A mechanical damage produced by the attachment structures, the hooks (hamulus) and the anchors, was also observed (**Fig. 25.2.4**).

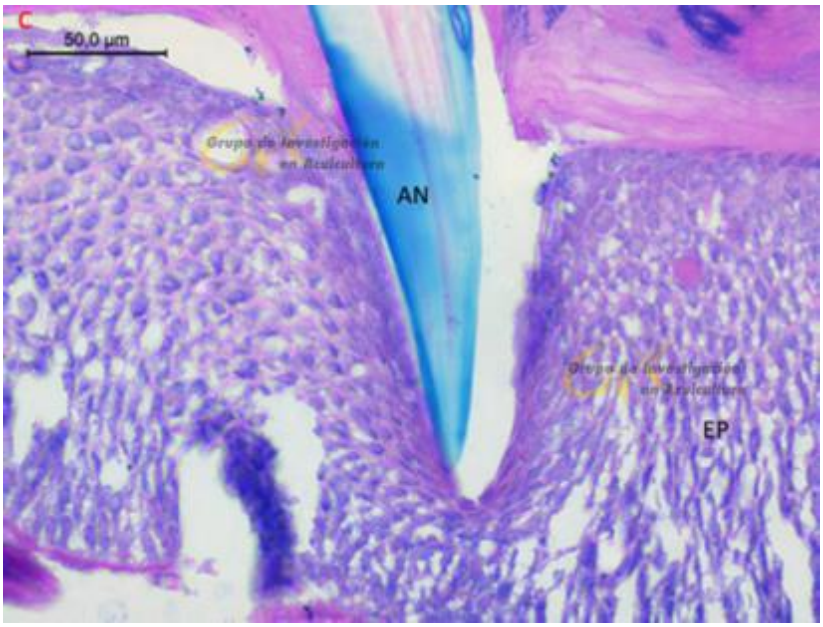


Figure 25.2.4. Mechanical damage that the attachment structures produced at the epidermis level. AN: Anchor; EP: Epidermis.

The incidence of parasite infection also induced an increase in the number of goblet cells (**Fig. 25.2.5**).

Work in progress: 1. Evaluation of the number of goblet cells. 2. Electron microscopy evaluation.

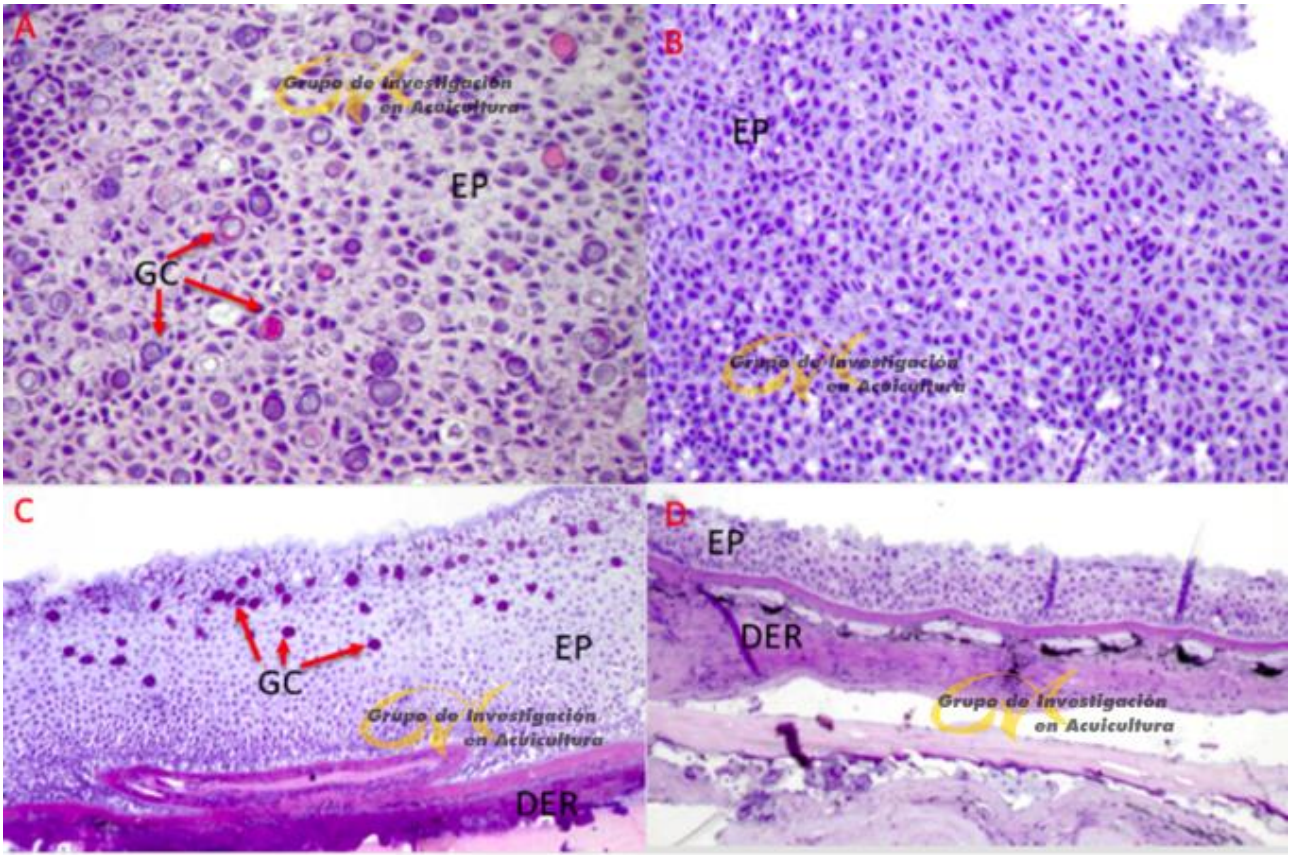
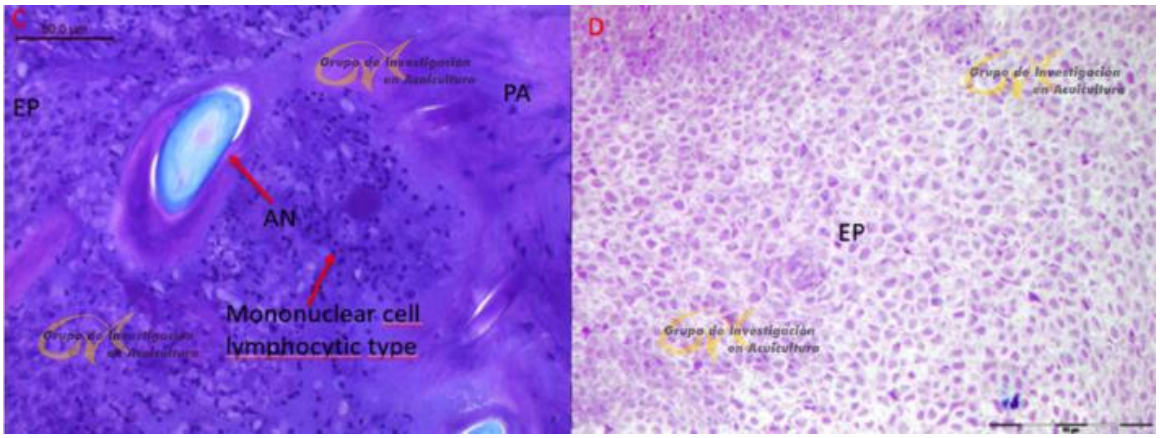


Figure 25.2.5. Longitudinal section of the epidermis layer of a parasitized (A) and non-parasitized (B) greater amberjack. Transversal section of a parasitized (C) and non-parasitized animal (D) with different amount of goblet cells. GC: Goblet cell; EP: Epidermis; DER: dermis.

At the immunological level or immune defence barrier some modifications when the parasite gets attached to the host were clearly observed (**Fig. 25.2.6**). Mononuclear cell lymphocytic type mobilization was observed near the regions where the anchors and hooks are introduced in the host. This immune cell mobilisation is a focal extravasation of lymphocytes related with blood vessels, producing a perivascular dermatitis.

(*Figure continues in the next page*)



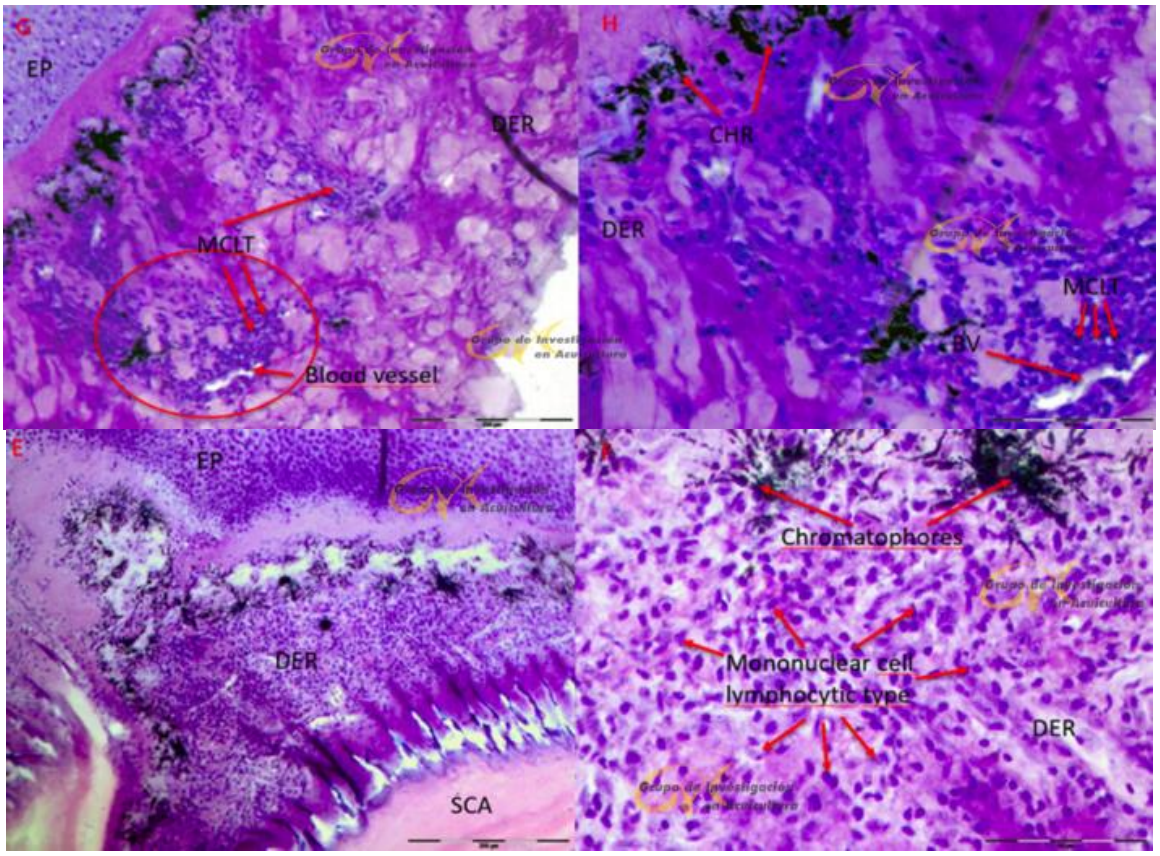


Figure 25.2.6. Mononuclear cell lymphocytic type infiltrations (MCLTI) rounding the anchors and hooks (C), compared with a non-parasitized animal (D). Massive MCLTI in the dermis layer (E,F), and focused extravasation related to blood vessels (G,H). MCLT: mononuclear cell lymphocytic type; CHR: Chromatophores; BV: Blood vessels.

B.- Determination of environmental conditions that can modulate Greater Amberjack resistance to parasitic infection

Within this task, the parasitic incidence on greater amberjack was studied in terms of preferred site for parasite infection. In addition, some studies of greater amberjack skin and immune system at different temperatures (in connection with Task 21.3 Development of appropriate husbandry practice - Action 21.3.1) were also conducted. Samples of gill leucocytes from temperature experiment were provided to UNIABDN for analysis. A extra experiment was conducted with different stimulations (Poly I:C, LPS) on greater amberjack juveniles at P2. FCPCT facilities at MBS. Samples were processed at UNIABDN.

Material and methods, temperature trial.

Experimental fish and Experimental conditions

Two hundred and twenty five greater amberjack juveniles of 19.5 ± 4.1g body weight and 9.8 ± 0.7cm total body length were distributed in 9 cylindroconical 500 l tanks (25 individuals per tank). The three temperature treatments, 17, 22 and 26ºC (±0.5), where assayed in triplicate, with each of the three tanks at a given temperature controlled by one RAS. Oxygen level was similar among different temperatures (around 7.8). Fish were fed to apparent satiety three times per day during 120 days with (52/22) a commercial diet (Europa 22, Skretting, Burgos, Spain) with 52% of crude protein and 20 % crude lipids. After 4 months, the

fish were sampled for skin, gut and liver histological studies. Blood was obtained by caudal sinus puncture to obtain serum (after centrifugation) for immunological studies (serum lysozyme, bactericidal and peroxidase activities). Serum samples were kept at -80ºC until analysis. In addition, samples for plasma cortisol were also obtained after centrifugation and kept at -80ºC until analysis. Skin mucus samples from different temperatures were also obtained to determine the variation of mucus immune potential due to culture temperature (**Fig 25.2.7.**).

Work in progress: 1. Immune potential of skin mucus analysis in progress: lysozyme, peroxidase and bactericidal activities. 2. Plasma cortisol evaluation in progress. 3. Morphological studies at different temperatures in progress.

Figure 25.2.7. Mucus sampling protocols.

C) Formulation of a diet supplemented with mucus stimulation products.

A commercial diet supplemented with mucus stimulation products has been formulated and prepared to conduct a trial with greater amberjack juveniles. The experimental diets have been supplemented by a blend of mannan-oligosaccharides.

Work in progress: 1. Experimental design in progress. Experiment to be done during 2016.

D) Standardization of Monogenean cultures

Eggs of *Neobenedenia* were collected from the adults (**Fig. 25.2.8**) using two methods: A) a 53 µm net was placed in tanks with infected greater amberjack juveniles, where the adult parasites produce eggs that get entangled with the nets and gives the possibility to collect them. B) Removal of adult parasites with tweezers was also conducted, placing them in petri dishes with filtered seawater and waiting for a few hours until the adults starts producing eggs. Eggs obtained (**Fig. 25.2.9**) were incubated at 20ºC for 7 days in filtered seawater.

Work in progress: 1. Standardizing protocols currently in progress.

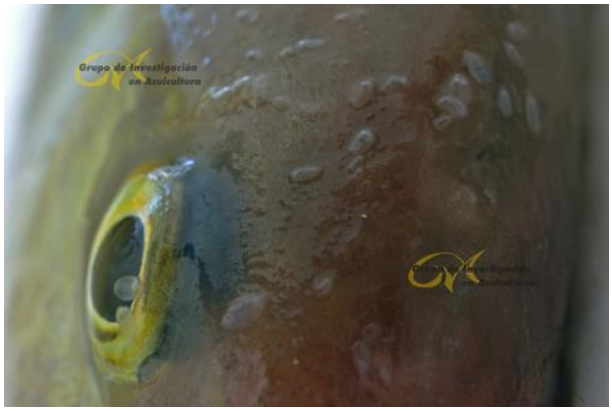


Figure 25.2.8. *Neobenedenia girellae* adults attached to the head of greater amberjack.

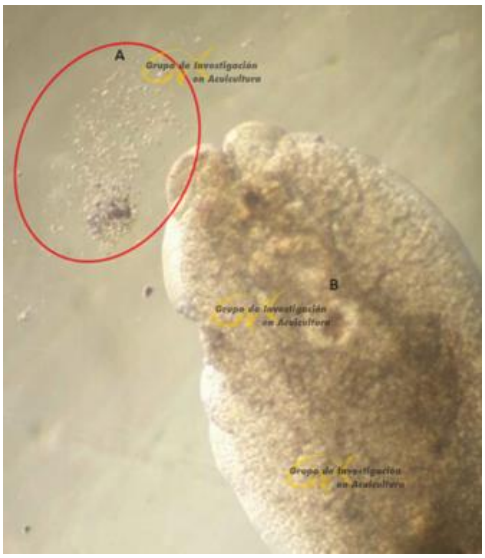


Figure 25.2.9. A: eggs produced by an adult of *Neobenedenia girellae.* B: Adult parasite.

Task 25.3. Identification of immune markers (led by UNIABDN, Chris Secombes).

Gene discovery and QPCR primer design

Partial sequences have been identified for all of the target immune genes, which are focused on mucosal immunity. The QPCR primers have been produced from the partial sequences for these genes and validated by QPCR (**Table 25.3.1**). Further gene discovery is ongoing in an attempt to clone a few pro-inflammatory genes (eg IL-1β) to allow assessment of certain PAMP/IS treatments, and is additional to the programme.

Table 25.3.1. Expression primers established for Amberjack immune genes.

	GENE	QPCR PRIMER (SENSE)	QPCR PRIMER (ANTI-SENSE)
ENDOGENEOUS CONTROLS	EF-1α	TGC CAT ACT GCT CAC ATC GCC TG	ATT ACA GCG AAA CGA CCA AGA GGA G
	β-ACT	TCT GGT GGG GCA ATG ATC TTG ATC TT	CCT TCC TTC CTC GGT ATG GAG TCC
CYTOKINES	IL-10	CTC AAG AGT GAT GTC ACC AAA TGT AGA AAC T	AGC AAA TCC AGC TCG CCC ATT
	IL-17AF	GGTGGCCCCAGAGGATCTCC	GGAGGACCAAAACCTGGTAGTAGATGG
	IL-17D	CGGTCTACGCTCCCTCCGTG	GCGGCACACAGGTGCATCCC
	IL-22	GCC AAC ATC CTC GAC TTC TAC CTG AAC	TGG TCG TGG TAG TGA GTC ACA TTG C
ANTI MICROBIAL PEPTIDES	Defencin	ATGAGGCTGCATCCTTTCCATG	AGAAAATGAGATACGCAACACAAGAAGCC
	Hepcidin	GATGATGCCGAATCCCGTCAGG	CAGAAACCGCAGCCCTTGTTGGC
	Piscidin	ATC GTC CTG TTT CTT GTG TTG TCA C	CGC TGT GGA TCA TTT TTC CAA TGT GAA A
INNATE IMMUNITY	Mx	GGCTACATGATTGTGAAGTGCAGGG	CTTCCAGTCGAGGCAGAGATTTCTCAATGT
	iNOS	TGTTTGGCCTTGGCTCCAGGG	GCCCAAGTTCTGAATGACTCCTCCTG
ANTIBODIES	IgM	CTCTTTGATAGGAATACCGGAGGAGAG	CAACTAGCCAAGACACGAAAACCC
	IgT	TGGACCAGTCGCCATCTGAG	GGGAAACGGCTTTGAAAGGA

Ways to induce mucosal defences in greater amberjack tissue

To better understand how the amberjack immune molecules are induced during infection/vaccination, fish were stimulated *in vivo* and *in vitro* with three selected PAMPs (poly I:C, LPS and flagellin), with PBS exposed fish acting as controls.

For the in vivo study 10 fish in each stimulation group were injected intraperitoneally with the stimulant and the head kidney, gill, spleen and intestine sampled after 24h. For the in vitro study head kidney and spleen tissue was collected and cell suspensions prepared. The cells were then stimulated and sampled after 4h, 8h and 12h. To date QPCR has been performed for the *in vivo* samples for each of the discovered genes. Analysis of the *in vitro* stimulated samples is still underway.

The data indicate that polyI:C, LPS and Flagellin can all stimulate the expression of amberjack immune genes, as shown in **Figure 25.3.1**, where gills show enhanced iNOS, Mx and hepcidin expression after polyI:C stimulation, enhanced hepcidin and piscidin expression after LPS stimulation and enhanced hepcidin and piscidin expression following flagellin stimulation.

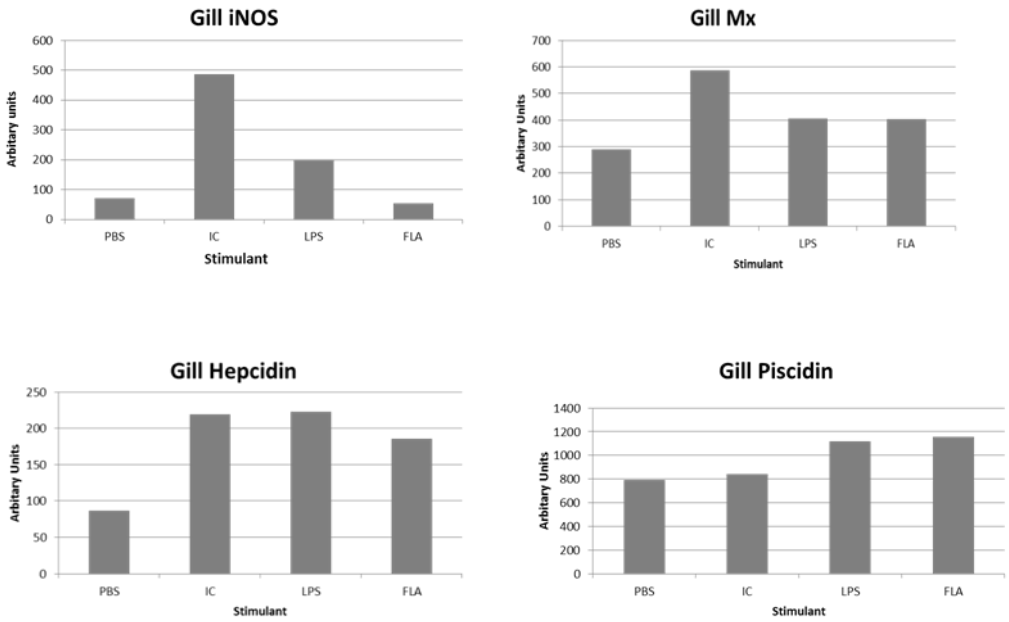


Figure 25.3.1. Gene expression analysis of gill tissue 24h following injection (ip) with polyI:C (IC), LPS, or flagellin (FLA).

Greater amberjack mucosal defences

With the increases seen in antimicrobial peptide expression following bacterial PAMP stimulation (LPS, flagellin) above, it is clear they may represent an important mucosal defence. Hence, we will study their activity in relation to defining mucus defences. A full length sequence has been obtained for a defencin, a piscidin and a hepcidin transcript in greater amberjack and the signal peptide, propeptide and active peptide identified. **Figure 25.3.2** shows the piscidin sequence, with the calculated mature peptide properties given in **Table 25.3.2**. The piscidin mature peptide has also been modelled (**Fig. 25.3.3**) and will be synthesised and it's bioactivity confirmed. We plan to do this with all three antimicrobial peptides as part of deliverable D25.2: Mucus defences of greater amberjack analysed and immune potential characterised.

```
atgaggttcatcgtcctgtttcttgtgttgtcactggtcgtcctcatggctgaacctggg
 M  R  F  I  V  L  F  L  V  L  S  L  V  V  L  M  A  E  P  G
gaaggtttcattcaccacattatcaaaggaatttttcacattggaaaaatgatccacagc
 E  G  F  I  H  H  I  I  K  G  I  F  H  I  G  K  M  I  H  S
gcgatcaacaggaggagacacggaatgacagagctagagcaggagcagtttgaccgagat
 A  I  N  R  R  R  H  G  M  T  E  L  E  Q  E  Q  F  D  R  D
cgggctgattttgtctag
 R  A  D  F  V  -
```

Figure 25.3.2. Nucleotide and deduced amino acid sequence of the amberjack piscidin transcript. The predicted mature is shaded.

Table 25.3.2. Properties of the amberjack piscidin mature peptide. Note that is has a net positive charge important for antibacterial activity.

Peptide properties	
Sequence	FIHHIIKGIFHIGKMIHSAINRRRH
Length	25
Isoelectric point	12.79
Mass	3030.7145
Net Charge	5+
Hydrophobicity	+22.76 Kcal/mol^{-1}

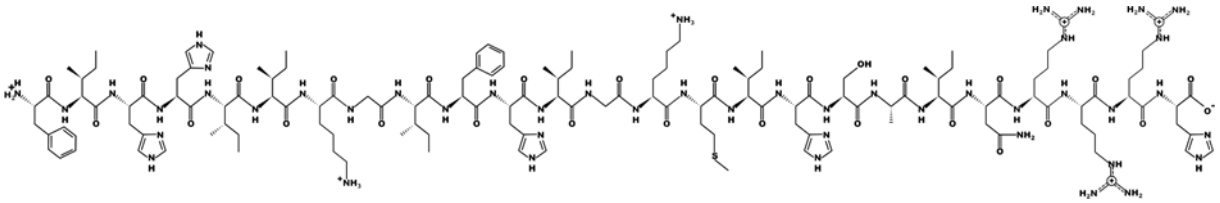


Figure. 25.3.3. Peptide structure of the amberjack piscidin, as modelled in PepDraw.

Task 25.4. Effectiveness of stocking density and anti-oncomiracidia attaching substances in the control of monogenean parasites (led by IEO, Juana R. Cejas).

Some preliminary work has been done and relevant information obtained that will be useful for the planned trial to study the efficacy of baths with anti-oncomiracidia substances. The collector device, previously designed at the IEO facilities, has been used for weekly monitoring of the infestation level by monogeneans in grow out fish of 180 greater amberjack juveniles (262.1 ± 55.5 g) distributed in 12 indoor tanks during

120 days. The mean initial density of the tanks was 3.8 kg m^{-1} and the first fish mortality was recorded after four weeks.

It was found that the juveniles were infested by a gill fluke that, which identified using molecular methods (by sequencing the 28S gene) as *Zeuxapta seriolae*, doing the first sequencing of the Cytochrome Oxidase I (COI) region. Eggs from others monogenean, such as the skin fluke *Neobenedenia melleni*, were not recorded. During this grow out period the total mortality registered was 27% and the highest number of dead fish coincided with the peak of egg number **(Fig. 25.4.1)**.

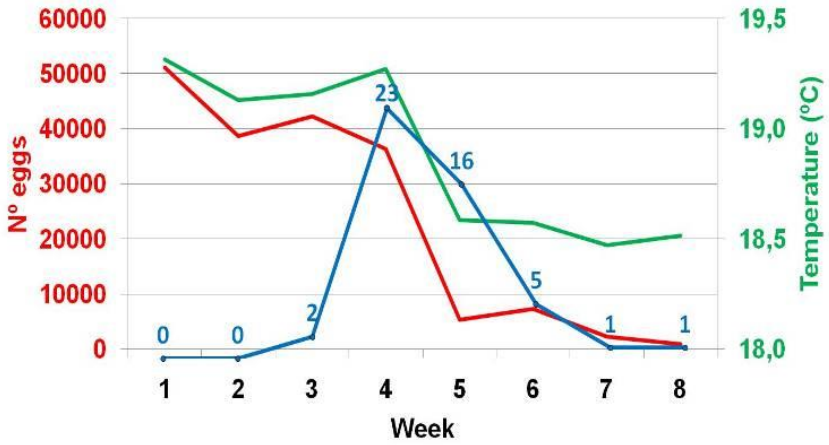


Figure 25.4.1 Mean (weekly) number of *Z. seriolae* eggs collected (red), number of dead fish (blue), and temperature (green) during the first eight weeks.

In a second grow out trial with greater amberjack juveniles, stocked initially at three different densities (0.17±0.02, 0.28±0.01 and 0.46±0.07 kg m^{-3}) and distributed in 9 indoor tanks (3 tanks per density), the weakly monitoring using the collector device during 120 days showed the presence of *Zeuxapta seriolae* and *Neobenedenia melleni* eggs. However their presence did not coincide in time **(Fig. 25.4.2)**.

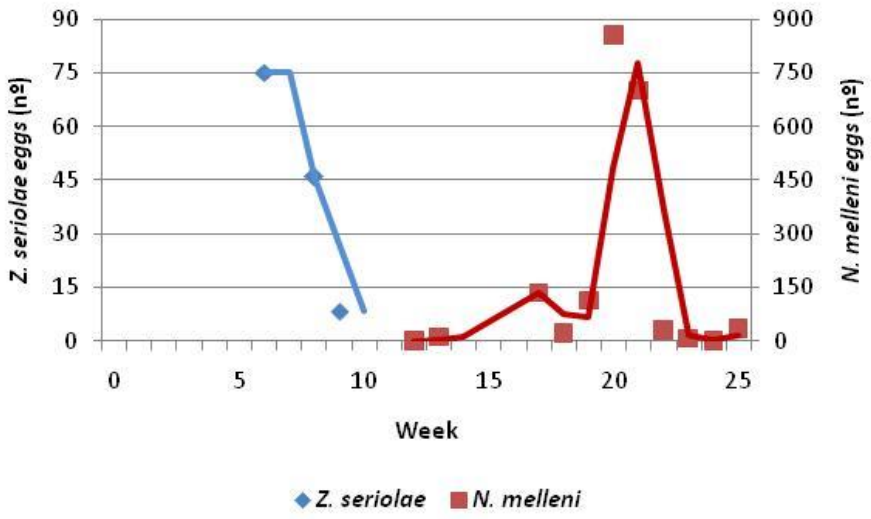


Figure 25.4.2 Mean (weekly) number of *Z. seriolae* (blue) and *N. melleni* (red) eggs collected during grow out trial.

A low number of *Zeuxapta seriolae* eggs were recorded about six weeks after initiating the trial and disappeared three weeks later, while *Neobenedenia melleni* eggs were recorded twelve weeks after start of the assay. During this grow out period no mortality was registered.

We are currently conducting further studies monitoring several greater amberjack groups to determine the effect of water temperature and/or photoperiod as well as culture density on the presence of eggs from the different monogenean species. All these data will provide information about the biology and behaviour of these parasites and may help the development of protocols against monogeneans in greater amberjack culture.

In this regard, a preliminary assay testing the effect of different substances (sucrose, glucose and mannose), as pro or anti *Zeuxapta seriolae* attaching substances, based on the parasite potential lectin substrate affinities, has been done. A total of 4-6 individual samples from the external arch and 2-4 from the second and third gill arch were obtained. The parasites were filtered by one nylon mesh and two gill-attached parasites were observed during 5-6 h after they were incubated with different media (glucose-seawater, sucrose-seawater and mannose-seawater).

The parasites were very active under glucose and sucrose seawater media during this period. However, the parasites incubated in the mannose-seawater medium (0.25 M) showed inactivity and were released from gill tissue after 2-5 min. Moreover, the gill cells showed a high viability after 18 h **(Fig. 25.4.3)**.

Figure 25.4.3. Greater amberjack gill arch and gill fluke *Zeuxapta seriolae*.

The current studies are focusing on the use of mannose as an anti monogenean treatment and the determination of optimal substrate concentration to remove the parasites with minimal negative effects on fish.

Task 25.5. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in amberjack (led by FCPCT, Daniel Montero).

Four sampling points were done during the period, two of them with associated mortality of greater amberjack juveniles. The first results obtained was the Isolation and identification of two different bacteria: *Bacillus oceanisediminis* and *Aeromonas spp* (identification currently in progress).

In addition, a challenge test against *Listonella anguillarum* was also conducted. No mortalities occurred in concentrations lower that the one causing death by anaphylactic shock. A second challenge test against *Photobacterium damselae sb. Pisicida* was also conducted with mortalities seen and the isolation of a strain of bacteria from Greater Amberjack.

Specific protocols for each technique used:

- Tuning PCRs from infected fish

For this technique we took 20 mg of tissue (anterior kidney) and performed a DNA extraction (DNeasy Blood & Tissue Kit, QIAGEN), and proceeded to performing the PCR using the following steps:

PCR for *PHOTOBACTERIUM DAMSELAE* SUBSP *PISCICIDA* (410bp)

Primers:

PDSPFW 5' -AGGGGATCCGATTATTACTG-3'

PDSPRV 5' -TCCCATTGAGAAGATTTGAT-3'

PCR reaction mixture containing 10 μl of the bacterial suspension, 5 μl of 10x PCR buffer (100 mM Tris–HCl, 20 mM $MgCl_2$, 500 mM KCl pH 8.3), 0.2 mM of each dNTP, 1 μM of each primer, 1.25 U of Taq DNA polymerase and double-distilled water up to a final volume of 50 μl. DNA denaturation was carried out at 94ºC for 5 min and then a total of 35 PCR cycles were run under the following conditions: DNA denaturation at 94ºC for 30 s, primer annealing at 55ºC for 30 s and DNA polymerization at 72ºC for 1 min. After the final cycle, reactions were terminated at 72ºC for 7 min.

PCR for *LISTONELLA ANGUILLARUM* (429 bp)

Primers:

VAFW 5'-ACAT CATCCATTTGTTAC-3'

VARV 5'-CCTTATCACTATCCAAATTG-3'

PCR amplification was performed on colonies of bacteria and DNA purified from each strain as templates. The PCR was carried out in a 50 μl reaction mixture that contained 250 μM of each deoxyribonucleoside triphosphate (dNTP), 10 pmol of each primer, 5μL of 10_ Taq buffer with $MgCl_2$, 0.5 U of Taq DNA polymerase (Takara Bio, Japan), and distilled water up to 50 μL.

The PCR conditions were: 95 ºC for 10 min, followed by 25 cycles of 95 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 30 s, and finally, one cycle of 72 ºC for 7 min.

- Tuning and *Photobacterium damselae* subsp. *piscicida* and *Listonella anguillarum* challenge.

For the development of the challenges against pathogens we use amberjack with an average weight of 120 grams, experimentally inoculated intraperitoneally with doses from 10^3 to 10^7 cfu/ml and we monitor deaths and keep the fish up to 15 days after the last death.

In the case of Listonella we noticed the total absence of deaths, showing amberjack is a refractory species to Listonella infection. For Photobacterium fish die from 48 h with a single dose of 10^7 cfu / fish. Identification of Aeromonas detection is currently in progress.

Task 25.6 Diagnostic-recommendation manual for greater amberjack health (led by HCMR, Pantelis Katharios).

During the reporting period an incidence of kidney tumor from greater amberjack broodstock was doscivered. One of the greater amberjack broodstock fish held in the facilities of P1. HCMR in Crete exhibited difficulties in swimming, related to a possible spinal deformity. The deformation of the fish was

first evident to P1. HCMR staff in summer 2015. In November 2015, it was decided to euthanize the fish and remove it from the broodstock.

The fish was euthanized with an overdose of anaesthetic. The fish weighted approximately 25 kg. Dissection was made immediately. A huge renal mass was observed that was pushing the spine (**Figure 25.6.1A**) and was the cause of the deformation observed when the fish was alive. The mass contained many various-sized cysts which were filled with air (**Figure 25.6.1B and C**). The mass was removed and samples were taken for histology. Samples were also kept in ethanol, -80ºC and RNA later. Apart from the renal mass, samples from other organs including the liver, spleen and intestine were also taken and preserved. Microbial cultures were made in different nutrient media including TSA, TCBS and BHIA.

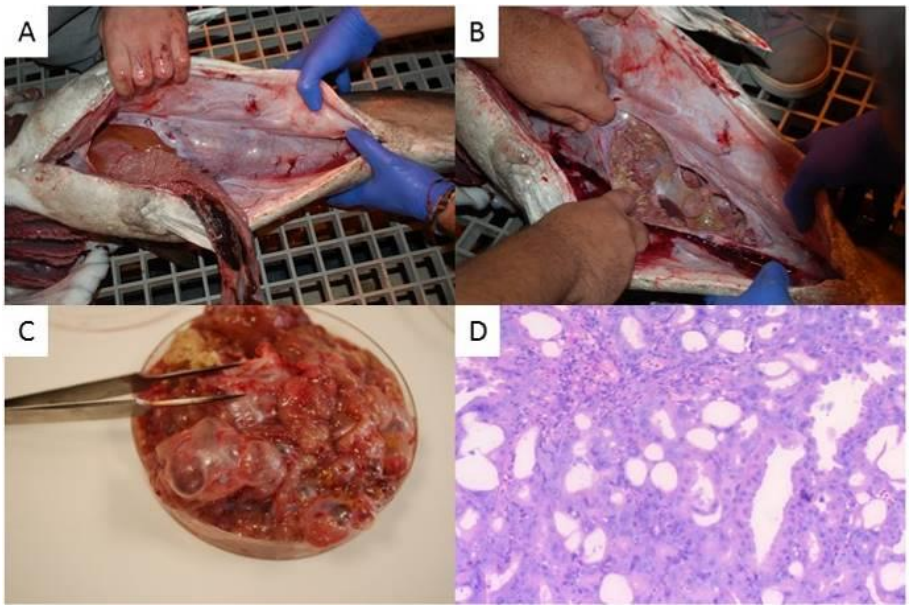


Figure 25.6.1. A. Enlarged kidney in the greater amberjack broodfish. **B.** The renal mass contained gas-filled cysts. **C.** Numerous cysts could be observed in the removed mass. **D.** Histological section of the mass showing the proliferative tissue and the various cysts.

Histological evaluation of the samples was made by Dr. Maja Rueten from the University of Zurich, Department of Veterinary Science. The epithelial cells formed irregular tubules and even solid cell nests. The cytoplasm amount was small to moderate, the nuclei were small to large, with irregular chromatin pattern and nucleoli of different sizes. The diagnosis of the neoplastic lesion was renal cystic adenocarcinoma (**Figure 25.6.1D**). All other tissues examined were normal.

One Vibrio strain was isolated in both TCBS and TSA. The strain was identified as *Vibrio campbellii* with BIOLOG GENIII and as a *Vibrio* sp using 16s rRNA sequencing. Preliminary phylogenetic analysis grouped this strain in the harveyi clade. Although the bacterium can be considered an opportunistic pathogen it is not related with the tumor. Cystic adenocarcinomas are quite rare in fish and can be caused by toxins or can be spontaneous. Further analysis of the incidence is in progress.

Deviations from Annex I and their impact:

There were no deviations to the Annex I in this WP.

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 progress towards objectives and details for each task (13-30 Mo):

Task 26.1 Production of VNN capsid protein (led by IMR).

Full description of the work and results in this task has 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***, submitted in November 2015. The remaining task of assessment of microalgae for expression of VNN protein is in progress, and the results from this first assessment will be ready by the end of June 2016.

Objective: The objective of this task was to assess the two eukaryotic systems -microalgae and a protozoan (*L. tarentolae*) for the production of nodavirus (Viral Neural Necrosis, VNN) capsid protein, to be used for the development of an oral vaccine for Atlantic halibut (*Hippoglossus hippoglossus*).

Background: Recombinant capsid protein from nodavirus expressed in *E. coli* has been shown to induce protection when formulated in a vaccine and administrated by injection. However, bacterial cells do not glycosylate the expressed protein, as do higher eukaryotes. It might be that other expression systems (**Fig. 26.1.1**) may provide antigens more like the native viral proteins produced after viral infection. By expressing the capsid protein of nodavirus recombinantly in different systems it should be possible to find out if post-translational modifications influence antigenicity and thereby its ability to induce protection when used as antigen in a vaccine.

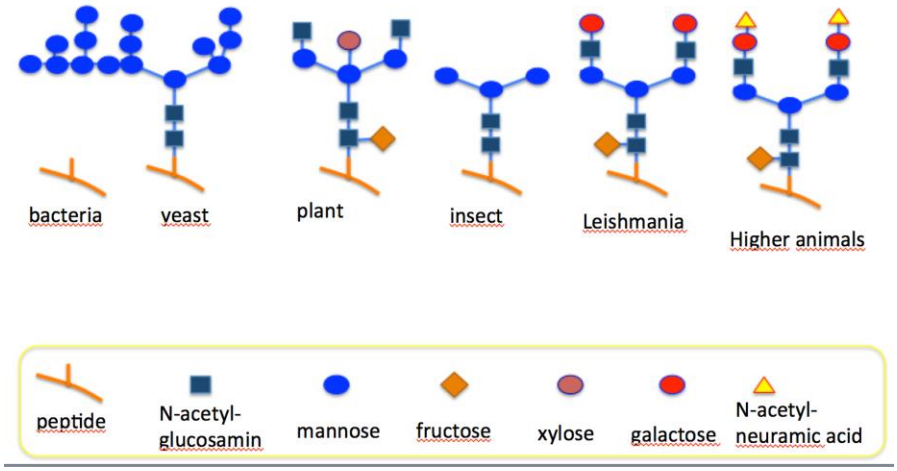
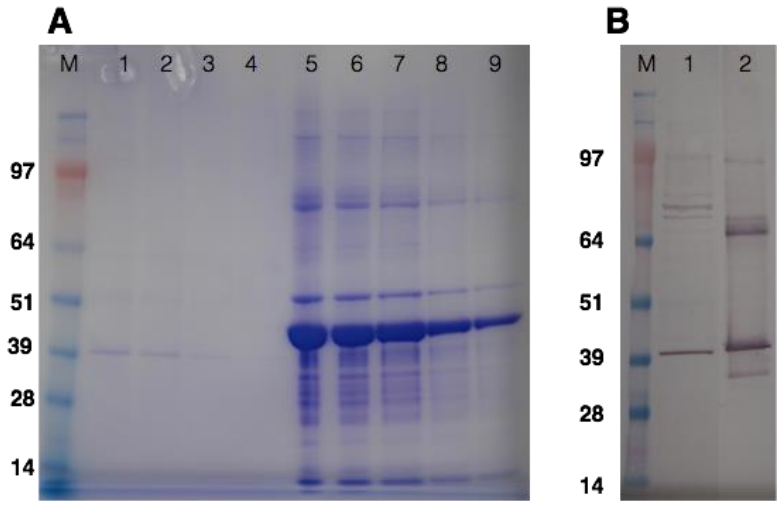


Figure 26.1.1. Glycosylation of proteins in different organisms, showing the extent of glycosylation.

Description: The aim was to express the protein antigen in systems having different post-translational modifications and test if that could influence the antigenicity of the proteins. An already existing collaboration with a research group working with plants was in place to allow working with microalgae. Hence, one of these systems was originally planned to be microalgae. However, at the time DIVERSIFY began, our collaborators had already started cloning the gene into tobacco plants for transitional expression, and thus tobacco plants were used instead of microalgae.

Materials and methods: Deliverable 26.1 gives a detailed description of the design of primers, and cloning. The expressed proteins by all three systems, were analysed by western blot using rabbit anti AHNV capsid antibodies (Sommerset et al., 2005) diluted 1:1000 as primary antibody.



Results and Discussion: Expression of recombinant capsid proteins was achieved in all the three systems tested. Recombinant proteins were isolated as inclusion bodies from the *E. coli* strain BL21 (DE3) after induction with IPTG.

Figure 26.1.2 shows a PAGE gel with serial dilutions of the recombinant protein expressed in *E. coli* and Western blots of samples from expression in *Leishmania tarentolae* and tobacco leaves.

Figure 26.1.2. **A**) Picture of a 4-12 % Commassie Blue stained NuPage gel with 2x serial dilution of recombinant protein purified from plant (lane 1-4) and from *E.coli* (lane 5-9). **B**). Western blot using rabbit antibodies raised against recombinant capsid protein from Nodavirus. Lane 1, recombinant capsid protein expressed in *L.tarentolae*. Lane 2, recombinant capsid protein expressed in plant.

Conclusion: We have managed to express the nodavirus capsid protein in all three systems. 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 still remain to be done.

Task 26.2 Monitor and assess immune response and protection (led by IMR).

To achieve oral vaccination the antigen has to be presented in a way that the target fish will ingest it. Halibut larvae are fed with *Artemia* at the early life stage. If we manage to get uptake of the antigen to Artemia, we anticipate it can act as a vector for oral uptake to the halibut larvae.

This task has two subsequent objectives. The first part of the task is to test the delivery of vaccine candidates produced in Task 26.1 to *Artemia* and further to Atlantic halibut larvae.

The plan for this is as follows:

During June 2016, *Artemia* will be bathed in seawater containing: 1. *E. coli* with GFP marker, 2. *E. coli* without GFP, but with VNN expression, 3. *L. tarantolae* with GFP marker, 4. *L. tarantolae* without GFP, but with VNN expression, 5. VNN in the form of inclusion bodies expressed by *E. coli*. In addition, if the expression in microalgae is successful, this will also be included in the trial. Moreover, we are in the process of re-connecting to our earlier collaborator to explore if including VNN expressed by tobacco plant will be possible in the same trial.

Artemia will be bathed for a period of approximately 30 min, washed for surface proteins, and later analysed for possible uptake of VNN using Western blotting. The GFP marker will help microscopical analyses for general uptake of *E. coli* and *L. tarantolae* as a control to determine if these organisms are taken up at all by Artemia. We are also in the process of expression of the recombinant antigen in microalgae *Chlamydomonas reinhardtii*. However, this is a challenging expression system as this organism has a very high GC-content and therefore a biased usage of codons.

Based on these analyses, one of the candidates will be employed for delivery of the VNN protein to Atlantic halibut larvae, and the uptake in larvae will be assessed. This part is planned during Autumn 2016.

The second part of this task regarding monitoring and assessment of the immune response and protection in the Atlantic halibut juveniles will be based on the results of the first part of this task, and is also planned to be carried out in Autumn 2016.

Deviations from Annex I and their impact:

There has been no deviation so far. However, the wet lab challenge facility that is required for the activities in deliverable 26.3 has been closed for over 2 years now. The activity has thus not yet been carried out, and there is some uncertainty about whether it will be possible to carry it out during Autumn 2016. This might possibly delay the deliverable 26.3 to month 48. This has been indicated in the previous report.

Group Work Packages

Socioeconomics

All objectives of WP27 regarding the organizational and institutional context have been finished in the 2nd Reporting Period. In the performance of these tasks the researchers learn that sector experts have difficulty to identify the market opportunities since not all species are well-known in all markets. However, the analysis shows that most countries have a stimulation program to consume more fish. These species could benefit from such a program.

Task 27.3 showed that the main obstacle for acceptance of new products in the chain is that buyers and category managers of supermarkets are no longer experts of the category they cover. Therefore the difference between local for local and global sourcing is difficult to explain.

Task 27.3.1 learned that most chances for growth in the aquaculture market are in sustainability and convenience. What is anyhow necessary is positioning of the species towards other animal proteins, since these are dominant in some of the EU markets.

In the development of new products (task 28.1), we faced that the consumers and researchers were so creative in defining new products that a selection had to be made. Fish seems to be a food category that doesn't have a saturated market yet. New product introductions in the market (2482 in 2014) already explain that retailers (the main innovators) also see possibilities in this food category for growth. For us it was difficult to select the most promising and to let go other interesting and innovative new product ideas. Of the selected products prototypes have been produced that have been sensory tested in task 29.2. The sensory tests results are now analysed and will be published very soon.

The segmentation study (task 29.1) has identified a clear group of consumers that could be early adopters of these aquaculture species in the five selected countries and the market size of these segments.

In this GWP, all tasks have been performed according to DOW objectives. However, especially the research regarding the institutional and organizational context shows that in the past there has been hardly any consumer research done in positioning of products. Given the dominance of salmon, trout, *Pangasius* and carp in the EU market all other products have to be positioned around these market leaders. On the contrary, in the meat market there is much more variance in the assortment.

In planning we have a delay of a few months, which we hope to catch up in 2016. These delays were mainly due to availability of experts (WP 27), no availability of data (WP27) or logistic problems (WP 29 sensory test).

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), Athanasis 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 been focussed on identifying the Institutional and organizational context in which the new species can be introduced. The macro-environmental context analysis learns 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. What is most difficult for new species is production development in accordance with the market development. 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 progress towards objectives and details for each task (13-30 Mo):

Task 27.1 External environmental analysis (led by DLO, Gemma Tacken)

Completed during the 1st Reporting Period. The full description of the work and results has 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 dynamics in the fish supply chain***.

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)

In this task the key forces of competition in the European market for DIVERSIFY's six species were analysed. A Porter Five Forces analysis has been performed for each species. This exercise analyses the level of competition within the industry and allows for business strategy development. It draws upon industrial organization (IO) economics to describe the forces that determine the competitive intensity and, therefore, suitability of the product for a market. This analytical work is related to its principal innovator, Dr. Michael E. Porter of Harvard University (1985). The five forces are *Bargaining power of suppliers, Bargaining power of buyers/customers, New entrants, Substitute products* and *Intensity of competitive rivalry* (**Figure 27.2.1.1**).

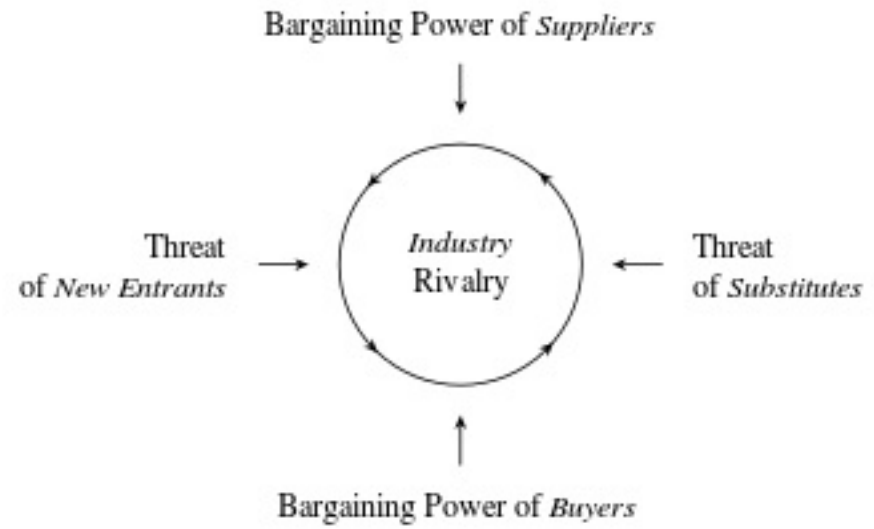


Figure 1. Graphical representation of Porter's Five Forces model (Porter 1985; Porter 1998).

Using Porter's Five Forces model of Competitive Position Analysis, an analysis has been carried out for the selected species. For each step of the value chain, current suppliers, customers, substitutes, rivalry and potential entrants into the market are described. The analysis of these Five Forces provides strategic insight into the market structure and competitive situation on the market of the selected species, including different products and markets. Each of DIVERSIFY species has the potential to improve its position in the markets and to offer added value. Their biological and economical potential can stimulate the growth of the European aquaculture industry. For each species, economic potential is analysed in relation to observed socioeconomic bottlenecks.

On the supply side, strategic questions are to be addressed, as for example to what market the producers should aim, production size and other issues as quality and product diversification, in order to obtain a competitive position.

The main challenge on the demand side for the DIVERSIFY species is to compete with current substitutes. Price will be a crucial factor, besides quality and service. The new species will face competition on price from cheap imports mostly from third countries outside the EU, and from established aquaculture species that for many years have developed their position.

Market orientation should decide on the assortment of products for sale and the range of species, but this differs between Mediterranean countries and Northern Europe. In addition, other marketing mix questions, such as the use of packaging and brands need to be analysed. When it comes to products, origin and distribution patterns, the European market is to be considered as diverse, with great variations between countries. Each national market has its own characteristics, though some common traits may be found in neighbouring countries.

This competitive analysis reflects that most of the DIVERSIFY species are not well known today as farmed fish, although some are as wild captured fish. In all cases, farmed production is relatively small in relation to the wild catch. Meagre and Atlantic halibut are the only candidate species with a significant farmed production sold in the European market. However, the other species are relatively unknown.

Regarding market development, much has to be done on brand awareness and recognition. Competing species are well known, as for example tuna, hake, sole, turbot, *Pangasius*, carp, European sea brass and gilthead sea bream. This competitive analysis shows that if the objective of the candidate fish species is to penetrate the market with value added products they must face long established products, like new greater amberjack in front of established tuna markets, or pikeperch for carp.

The main challenge on production development for the new species is to choose appropriate buyers and the selection of market segments in which these species and their products can have a competitive advantage.

The full description of the work and results has 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

The objective of this task was to identify and map the main trends that are relevant for the European seafood market in general, and also more specifically for markets in the selected countries within DIVERSIFY. Besides the seafood market, relevant trends for meat products were also identified in this deliverable. Trends in these other markets are used for comparison and for cross-sector analysis. The general and specific trends in the DIVERSIFY project can support aquaculture producers in understanding the global and European context of the market for animal proteins, and the implications of the trends for their business activities. With these insights, aquaculture producers can seek for market opportunities to improve their business. The results of this deliverable could be used for the development of business models for the small and medium enterprises participating in DIVERSIFY, but also for other enterprises in the EU.

The identification of relevant trends was conducted by literature research and consultation of position papers and news articles. Point of departure was the identification of a number of general European trends that are relevant for the market for seafood, but when relevant, also for meat products and meat substitutes. Relevant trends can be on the macro, meso and micro level. Trends on macro level already have been described in the submitted ***Deliverable D27.1 Report on external environmental factors that affect or will affect the production chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.*** Therefore, this sub-task has focused on trends on meso (sector) and micro (household) level. Also within the trend-mapping, a distinction between long-term trends (more than five years) and short-term trends (less than five years) has been made. First, relevant trends for animal proteins at EU level have been identified. For each trend a description has been given, including how this trend can affect the market for animal proteins in the EU. Then, for each of the five countries some more detailed trends are described.

The main trends in the European animal protein market are:

Long term:
- Increasing demand for animal proteins
- Increasing production costs
- Substitution between fish and meat
- Growing importance of sustainability certification
- Growing interest in information about origin and production of animal protein

	(traceability)
	- Increasing attention for animal welfare
	- Growing interest in local and regional products
	- EU ambition to become more self-sufficient for seafood
Short term	- Other protein sources will increase in market share
	- Increasing demand for convenience products
	- Dominance of retailers can lead to conversion of consumption patterns
	- Growing market share in private labels of retailers
	- Flexitarism increases in north western EU countries

The main trends in the different European countries are:

UK trends:
- purchases of fish products have been declining since 2006, however future growth is expected
- The 'big five' dominate the market, but there are opportunities for diversification
- Personal well-being (health) is the primary driver for increasing fish consumption
- Sustainability issues can influence consumption trends
- UK consumers are more freshness, price and taste oriented than average EU consumers
- UK consumers eat meat relatively less often compared to EU average

Spanish trends:
- Sustainability is gaining importance, but is halted due to the economic crisis
- Spanish consumers are increasingly health conscious
- Shorter supply chains in the foodservice sector
- Immigration stimulates growing diversity in the foodservice sector
- Freshness, hygienic display, taste and local produce are the main priorities in buying meat

German trends:
- German fish consumption has grown, but is declining recently
- German consumers require knowledge on origin (traceability) and sustainability of food
- Taste, no GM, freshness and traceability are the most important consumer priorities in buying meat
- Country of origin is relatively important in Germany
- Increasing sales via the internet

Italian trends:
- Sales of fish products are consolidating
- Decreasing self-sufficiency in fish products
- Retail buying is concentrated, but consumer formulas in the Italian retail remain
- Only 20% of the Italians thinks about decreasing meat consumption
- Italians prefer locally produced products, no GM feed, traceability and hygienic display for meat

French tends:
- Preference for fresh (chilled) fish products for consumption at home
- Independent restaurant outlets are most popular for consumption out of home
- Overall fish consumption increases, but dependency on imports grows
- Consumer preferences differ by age
- Increasing importance of ethnic food
- Freshness, acceptable and affordable pricing, traceability and taste are most important buying motives
- Country of origin is very important in France

The objective of this sub-task has been to identify and map the main trends that are relevant for the European seafood market in general, and also more specifically for aquaculture markets in the selected countries within DIVERSIFY. The identified trends and developments reveal an increasing competition for animal proteins on the long term, which will increase fish prices, and the prices of other proteins. Prices of protein sources will also increase, because of growing production cost. For captured fish, fuel prices are an important cost driver while for aquaculture and other proteins feed prices will have an important effect. At the same time the EU market for animal proteins is facing increasingly higher requirements with respect to quality,

traceability, sustainability and animal welfare. Although some of these developments show some differences per country, the main trends are comparable.

The implications of the main trends for the DIVERSIFY project is that SME's could take these trends into account in developing their marketing strategy. In some of the countries included in this study a large share of the consumers have the intention to decrease their meat consumption. This holds opportunities for fish products. Also fish products benefit from being marketed as healthy products, especially compared with meat products. Demographic developments such as the ageing population in the EU will make health issues much more important. In the marketing of the six species of DIVERSIFY, the health aspects should therefore be taken into account. Although aspects such as sustainability, traceability and animal welfare can be seen as requirements that one must meet, they can also be interpreted as an opportunity to give the species within DIVERSIFY an identity. This identity can be used in the positioning the fish products in the market. In some countries, groups of consumers prefer to eat fish that was produced in their own country or region, rather than being imported. This holds both for native consumers and for immigrants. This preference can also contribute to the identity of the fish species.

Overall, key aspects that should be taken into account when marketing the species considered in DIVERSIFY are healthiness, an alternative for meat products, sustainable and local or regional. These characteristics make the species suitable for being marketed as a fresh product for fishmongers and local restaurants. For SMEs it is difficult to sell to large retailers with strict requirements and who demand stable quantities of fish. Fishmongers and restaurants are more interested in fresh fish products that are marketed as local or regional. Selling to fishmongers and restaurants, however, requires short supply chains in order to maintain the quality.

The full description of the work and results has been provided in ***Deliverable 27.4 Report on trend mapping for the European aquaculture and fisheries sector, and protein market in the (near) future***.

Task 27.3 Opportunities and barriers for growth (led by DLO)

Task 27.3.1 Success-failure study

Although essential for a firm's survival, new product launches are also associated with high risks and many new products fail to win over sufficient customers to become a commercial success. While estimates vary from 40% to 90%, depending on the product category, the criteria used to define success, and the stage at which products are included in the analysis, it is clear that new products fail at a significant rate. The objective of this deliverable was to identify critical success factors for market acceptance for new seafood products in the European market. A list of critical success factors for market acceptance is presented, based on what success factors for new products could be identified in the literature and a success-failure study of comparative cases. With these insights, aquaculture producers can seek market opportunities to improve their business. The results of this deliverable could be used for the development of business models for new fish products.

The identification of success factors was conducted by literature research. We mainly scanned relevant review or meta-analysis articles from the academic marketing literature to identify which factors are distinguished as drivers of new product success. In addition, to get an idea of the position of the European market for new fish products, the Global New Products Database of Mintel, Innova Database and different market research reports were consulted. Finally, for drafting the comparative cases with regard to success or failure of specific fish species in the European market (or one of the specific countries within Europe), desk research was conducted and an appeal was made to experts in the field (based on personal correspondence).

Based on these studies, we distinguish the following factors to play a role in the market acceptance of new products in **Table 27.3.1.1**.

Table 27.3.1.1 Key determinants of new product acceptance

Drivers of new product acceptance	Definition	Predicted effect on new product acceptance
Product advantage	Superiority and/or differentiation over competitive offerings	+
Product meets customer needs	Extent to which product is perceived as satisfying desires/needs of the customer	+
Product innovativeness	Perceived newness of the product	U
Brand/product category reputation	A brand/product category has a good reputation if consumers believe its products to be of consistent high quality	+
Market power	The power of the brands in the category and the power of the industry in general	+
Market competitiveness	The degree of competitive response to a new product	-
Market potential	Anticipated growth in customers/customer demand for a specific new product	+

Note: + means a positive effect, - is a negative effect and U is a U-shaped effect .

New fish product launches

The Mintel Global New Products Database contains new product launches in European countries. This database is used to check the number of new fish product launches in Europe. First of all, the number of new fish products on the European market has increased sharply over the last two decades. Europe has the highest share for fish and seafood launches (Innova Database, 2014). In the launched new products there is an increasing focus on local flavors, convenience and health benefits (Innova Database, 2014). The rise of new products appealing to these benefits meets consumers' demand for fish and seafood as part of a modern and healthy lifestyle. In the European market, 2482 new fish products were launched in 2014.

The top five countries with regard to launches of new fish products are France, Germany, Italy, Spain and UK. These top five countries correspond with the focal countries of the DIVERSIFY project. As can be deducted from **Figure 27.3.1.1** below, new product launches increased especially for France, Germany and Italy, while for Spain only the last 2 years showed a growth in number of fish product launches. The number of new fish product launches in the UK remained relatively stable over the past 5 years. This can be related to the fact that in the UK, in the same time period, fish purchases declined.

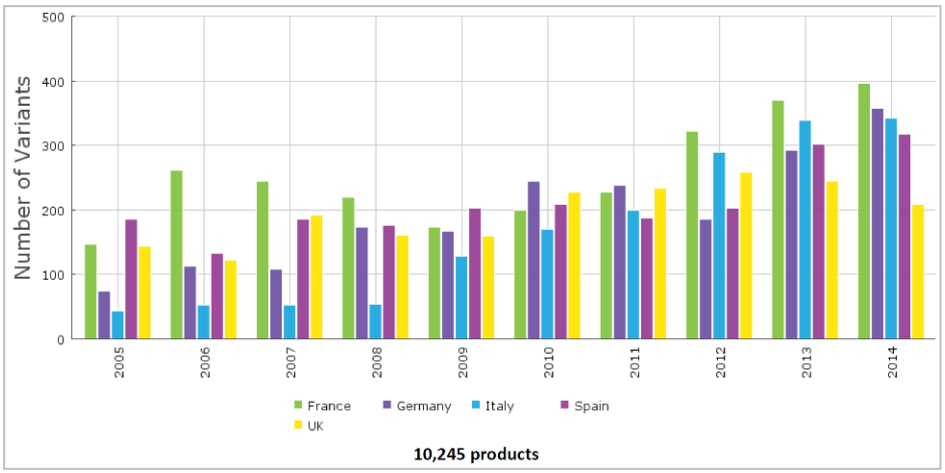


Figure 27.3.1.1. Fish product launches per year, for the top-5 EU countries (Mintel GNPD-database, 2014).

Most new fish product launches are new varieties, followed by new products or new packaging. The majority of new fish products are global brands (68%), but 32% of new fish products are private label products. It is expected that the share of private labels is going to increase over the next years. **Figure 27.3.1.2** below depicts the top 15 fish brands launched over the past 15 years. Notice that the 4 highest ranked brands in terms of product launches are all private labels, from which 2 brands from discount stores (*i.e.* Lidl and Aldi). This is underlined by the fact that most new fish products are launched through supermarkets or mass merchandise channels. Especially in countries such as Germany, hard discounters like Lidl and Aldi have a very strong position. Aldi carries no national brands and its market share has grown dramatically over the last decade. Consequently, its private label is often one of the largest in the category.

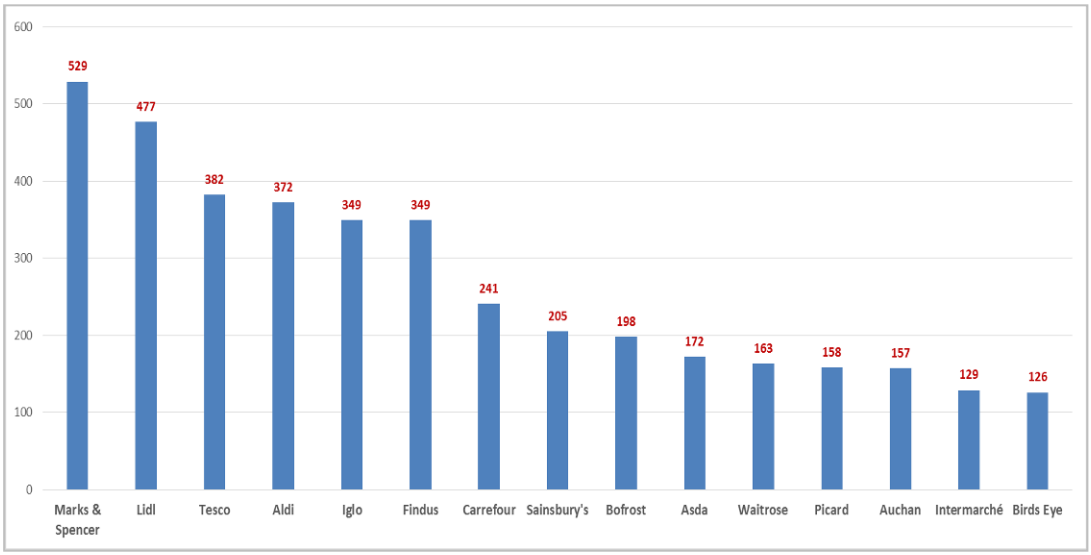


Figure 27.3.1.2. The top 15 fish brands launched over the last 15 years, with the number of products per brand (Mintel GNPD-database, 2014).

In addition, **Figure 27.3.1.2** reveals that the top 15 fish brands are mainly from northwest European companies (retailers). This corresponds with the aforementioned fact that most processed fish products are introduced in this part of Europe, whereas southern Europe mainly consumes whole fresh fish. Position claims of new fish products are related mainly to the environment and ethical aspects, especially in Germany and the UK. Convenience also plays an important role. France and Spain are dominant in this regard. Other product positionings are "natural" (*i.e.*, important in France and the UK), specific positionings like premium product (*i.e.*, in the UK), "minus" claims and "suitable for" claims. The "natural" claim refers to the use of natural ingredients and no artificial additives, the "minus" category represents products positioned with low/no/reduced formulations, whereas "suitable for" claims are related to specific diets such as reduced allergen and gluten-free.

In summary, what ultimately determines the success for new fish species is providing products that offer advantages that meet consumers' different needs, that can rely on a positive image and that have a strong market position based on cooperation between suppliers, the industry and retail.

The full description of the work and results has been provided in ***Deliverable 27.6 List of critical success factors for market acceptance***.

Deviations in WP 27 from Annex I and their impact:

All 3 Deliverable presented in this periodic report were delayed by few months. This was mainly due to the delay of Deliverable 27.1 and due to convergence of work around Christmas 2014. However all deliverables have been finalized and submitted before March 2015.

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 progress towards objectives and details for each task (13-30):

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)

The general objective of this activity was to explore and report new ideas through focus groups with consumers and experts with regard to the fish products resulting from the species under study in the focal markets (*i.e.*, UK, Germany, Spain, France and Italy). Two focus groups were conducted per country (n=10 focus groups in total). In every country and in each group, participants were mixed, coming from both the '*involved traditionals*' and the '*involved innovators*' psychographic segments (for more details about these segments see ***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)***. Each semi-structured focus groups were comprised of 6 participants, 3 from each of the two psychographic segments. Focus groups were internally heterogeneous (but externally comparable) in terms of their socio-demographic

profile, consisting of informants of different age, educational and general socio-economic background. Focus group interviews were recorded and transcribed for subsequent data analysis according to standard procedures.

The conducted focus groups were of a semi-structured nature, allowing the moderator of the discussion and the participants of the group to rise issues unknown or other than those already assigned in the agenda. The researcher/moderator was allowed to ask the participants about the comparisons between their views and experiences, facilitating a better understanding of why the participants agree or disagree.

The focus groups proceeded in three steps:

(1) Exploring consumers' personal experiences, preferences, attitudes, and perceptions regarding new food products in general (10 minutes);

(2) Exploring consumers' personal experiences, preferences, attitudes, and perceptions in relation to new fish products (with emphasis on the species under study) (20-30 minutes); and

(3) Exploring consumers' creative ideas for new farmed fish species by using creative techniques (*i.e.*, empathy map and brainstorming) (60-80 minutes) (**Figure 28.1.1**).

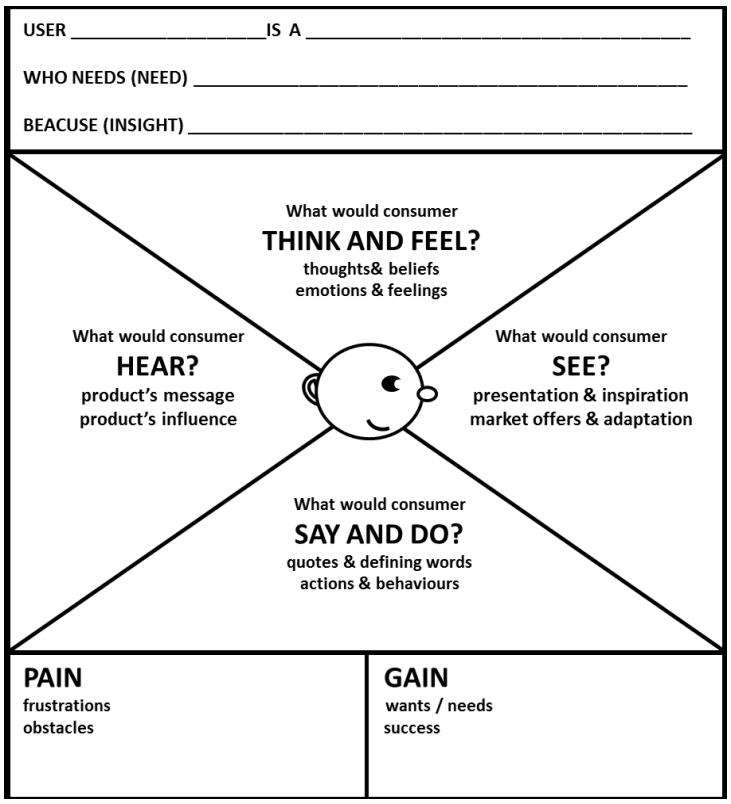


Figure 28.1.1. Empathy map.

By achieving these steps, this research provided a list of ideas for new product development that will be further tested in the experimental and quantitative research that touch upon the two main areas of the work, namely related to development and selection of new product concepts from selected fish species, and evaluation and optimization of newly developed fish products. Further, this research provided up to 3 new product ideas for each of the new fish species. Thus, in total fifteen feasible ideas were generated from consumer focus groups in selected countries (*i.e.* UK, Germany, Spain, France and Italy) from which 8 were chosen in order to move from the idea to the tangible product (**Table 28.1.1**).

Table 28.1.1. Final choice of creative ideas for new fish products.

Country	Idea
Germany	Frozen fish filet that is seasoned or marinated either traditional, Italian, Provence or Asian. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The product is in a sliding packaging, transparent vacuum packed bag made of recyclable material, with clear pictures of the unfrozen product on the cardboard sleeve.
UK	Fresh fish back fillet that looks like a roast in tray or bag that can be prepared in an oven or barbecue. This fish is accompanied with dips, sauces and dressings. The product is produced environmentally sustainable (containing ASC hallmark). It is labelled as a premium product, the country of origin is EU. The packaging is transparent bag or a tray where fish is laid and covered with transparent plastic.
Spain	Fresh ready to eat meal with fish fillet with different cheese and fine herbs. The fish is seasoned. This product is pre-cooked and can be prepared in the microwave in 5 minutes. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The packaging is individual with transparent window and a lid on the top under which you can smell the product.
Spain	Fish sausages and fish hamburgers. The main advantage of this product is that the product has no bones. The seasoning is very mild and therefore this product is therefore suitable for children. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The packaging is transparent vacuum packed or in a plastic tray with transparent plastic on the top.
Italy	Bread crusted crispy frozen fish product with a topping of vegetables and sauce made by the traditional recipe. This fish product is medium seasoned and easy to prepare in the oven or the microwave in the original packaging. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The packaging is a tray with transparent lid where image of the ready dish is presented.
France	Fresh fish Carpaccio that can be used as starter for a hot meal or as sandwich filling. This Carpaccio will be seasoned with ginger and chili and presented as scales of the fish. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The packaging is a plate that looks like a round box with the compartments and transparent wheel on the top that you can turn to rich different sections.
France	Botarga made of grey mullet and sliced like medallions. Botarga is a Mediterranean delicacy of salted, cured fish roe, typically from grey mullet or tuna. The product is similar to the softer cured mullet roe, karasumi from Japan and East Asia. The product is produced environmentally sustainable (containing ASC hallmark). It is labeled as a premium product; the country of origin is EU. The packaging is a tray with the transparent film on the top and product can be served in the same tray.
Spain	Liquid fish to make soups or drink. Liquid fish for soups is in mashed form. These products are without additives and thus highly suitable for diabetic and vegetarian people. The product is produced environmentally sustainable (containing ASC hallmark). It is labelled as a premium product; the country of origin is EU. The packaging for soups is tetra brik, while liquid fish for drinking is in the plastic bottle.

In a second step, interviews with experts were conducted by using a structured questionnaire in each of the five countries, UK, Germany, Spain, France and Italy. More specifically, four expert interviews were conducted in UK, one in Germany, two in Spain and three in France and Italy. This part provided the expert opinions with regard to feasibility of ideas for new fish products collected in the previous phase with consumer focus groups. The main objective of this phase was to explore possibility of creating new fish products from the ideas gathered from the focus groups from five focal fish markets (i.e. 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 considered that in overall these ideas could increase profits of fish industry due to the higher diversity of choice. On the other hand, experts had dividing opinions on increase of fish consumption based on the presented product concepts. Generally, they stated that these ideas had a possible prospective if they are developed with good coordination between the fish farmers and consumer itself. Experts consider that some of the product concepts should be revised in collaboration with consumers, that is that they require product testing and sampling.

In terms of general recommendations for new product development of selected fish species, **Figure 28.1.2** points to the most important drivers and barriers for the choice of the new product ideas most relevant for consumers.

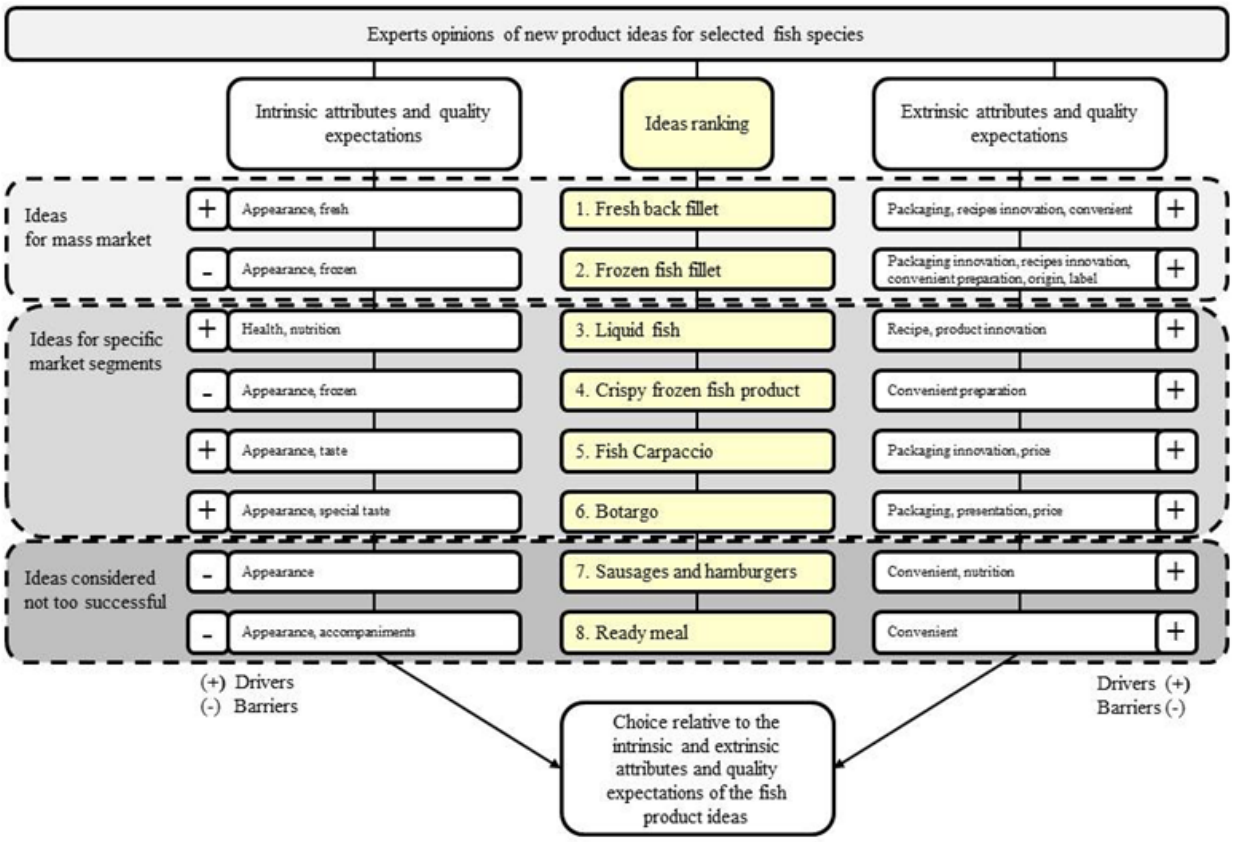


Figure 28.1.2. Drivers and barriers of new products ideas most relevant for consumers.

The full description of the work and results has 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)

The objective of was to generate a list of ideas for new product development. A combination of the market perceptions (***Deliverable 28.1 Report with results of focus groups with consumers and experts regarding ideas for new products***) and the technical limitations and the economical prospects efficiencies (*i.e.* within a socio-techno-economic study) was used to generate a pool of ideas about potential products. Within this report the perspectives of scientists from different scientific areas were included in order to justify feasibility for potential products.

A combining list of product ideas was generated: a) Those deriving from ***Deliverable 28.1***, and b) New product concepts, generated combining information of the market perceptions and the technical limitations and the economical prospect efficiencies, which were included in the socio-techno-economic study of WP27.

For the screening process of concepts a quantitative screening (rating on a scale of 1 to 7) was conducted in the frames of the present deliverable. In order to perform the quantitative screening of this deliverable, a list of screening criteria that consisted the basis for concepts rating, was formulated independently to the idea generation process (1. Nutritional benefit / value; 2. Healthiness; 3. Convenience in preparation (easy to cook); 4. Convenience for consumption (ready to eat); 5. Cost for consumer (price); 6. Technical feasibility (equipment & raw material); 7. Technical feasibility (know-how); 8. Specific consumer targeting; 9.

Familiarity; 10. Newness/innovativeness; 11. Existence of similar products/ Competitors; 12. Shares characteristics of successful products; 13. Perceived consumer freshness; 14. Safety; 15. Shelf life; 16. Packaging; 17. Added value; 18. Attractiveness (Appearance/ presentation); 19. Recipes (versatility / packaging)). To overcome the subjectivity, which lies in certain screening methods experts of different fields involved in the 28 WP of DOW rated all concepts in the criteria corresponding to their expertise. The results of the screening process facilitated the ranking of all potential solutions (concepts).

The fish technical characteristics were further analyzed and based on them each species technical compatibility for every product concept group was given. Furthermore, some product concepts which were found to be problematic in aspects of technical feasibility are presented. The possible technical difficulties new product development could face are presented in detail for each of the concepts. These product concepts were not suggested for further development irrespectively of their final ranking position:

Idea 16: frozen whole fish filled with spices & vegetables: problematic in aspects of freezing and defrost as well as preparation (cooking) due to differences in the nature and properties of its ingredients. *Idea 17: fresh whole fish filled with spices & vegetables:* This product may have issues in aspects of preservation and sensory properties due to coexistence (e.g. fluid exchanges) of fish and vegetables. Also may have a similar issue in preparation (cooking). *Idea 23: Varied meal with fish fillet, burgers, sausages*: This meal contains in the same package three different food types of different spoilage patterns and shelf life, and thus is technically incompatible. *Idea 26: Fresh ready to eat meal with fish fillet and cheese:* This is technically complicated dish since it has to combine cheese and fish and there are difficulties for both human sensory issues and aspects of preservation and shelf life. *Idea 32: bread crusted crispy frozen product with a topping of vegetables*: This product should not incorporate the topping with the fish, due to difficulties in preparation (different defrost - warming times for toping and fish). *Idea 41: Ready-made fish fillet / fish dices accompanied with cereals and vegetables:* This product should not incorporate cereals and vegetables to the main fish, because they will undergo alterations due to moisture excluded by the fish.

Moreover an analysis of selected ideas has been done. In order to divide the products into groups for the facilitation of the reader, their main characteristics are used as follows:

A. Mass market products: can be delivered through several distribution channels; are oriented to the widest variety of consumer segments. Similarities are that they can constitute a regular daily meal; price and versatile characteristics allow frequent use; are convenient, healthy, have high nutritional value; there are no bottlenecks in their production. The advantageous aforementioned characteristics make them competitive against fresh or frozen fish products that occur in mass market. These mass market products include:

1. Fresh fillet products without further processing including Idea 14: ***Fresh fish fillet with herbs and spices***, Idea 21: ***Fresh fish fillet with different 'healthy' seasoning and marinades***, Idea 40: ***Fresh fish fillet sliced presented in the shape imitating of fish scales***, Idea 29: ***Fresh fish fillet medallions with garnish and sauce***, Idea 20: ***Fresh back fish fillet*** and Idea 43: ***Fresh fish fillet that comes with 3-day plan***.

2. Ready to eat meals represented by Idea 4**: *Ready to eat meal, salad with fish***.

3. Fish steaks or large pieces or roasts represented by Idea 42: ***Fresh fish roast.***

4. Whole frozen fish products that include ideas 15 & 16, both included in the top 20 positions of the ranking.

5. Frozen fish fillets without further processing including: Idea 1: ***Frozen fish fillets with different recipes***, Idea 25: ***Frozen back fish fillet in transparent packaging and accompanying marinades***, Idea 19: ***Deep frozen white fish fillet in the transparent packaging with additional information***, Idea 13: ***Frozen fish filet that is seasoned or marinated***, and Idea 18: ***Frozen fish fillet with potatoes and vegetables***.

Regarding the suitability of the DIVERSIFY species, all species are suitable for the above ideas. However, Atlantic halibut, meagre, wreckfish and greater amberjack can offer a competitive technical advantage. Atlantic halibut products could be targeted to UK, Germany and France (already established market for these

species). Meagre products could be launched to the UK (already established market for this species) and Spain, France and Italy (countries with meagre production). There is demand for frozen products in German, as well as an established demand in the UK and Germany for pre-seasoned fish products.

B. Products targeted to specific market segments: delicacies/high end products, best if delivered to the consumers through specific distribution channels such as specialty stores or delicatessens. The price range is elevated due to their nature, and are oriented mainly to consumer prototypes such as *hedonic consumers* and *variety seeking consumers.* Competitive advantages include innovative nature and packaging; convenience; versatility in use. There are no obvious bottlenecks in their production.

1. Fish Carpaccios or tartars (including ideas 24, 30 & 38), were all included in the top 20 positions of the ranking. As an example is typically described ***Fish Carpaccio 2*** seasoned with ginger and chilli and presented in the form of fish scales-like cuts (Idea 38): The packaging is a plate that looks as a round box with compartments and a transparent wheel on the top that can be turned to reach different sections. Advantages include high nutritional value and freshness due to minimum processing, innovativeness and healthiness. The only identified weaknesses are higher price; limited shelf life; unfamiliarity with raw product concept. Still, major competitors have similar price range. A suggestion for possible changes in the product is the inclusion of a milder seasoning.

2. Bottarga sliced like medallions (Idea 39). Bottarga is a Mediterranean delicacy of salted, cured fish roe, typically from grey mullet or tuna. The product is similar to the softer cured mullet roe, "karasumi" from Japan and East Asia. The packaging is a tray with the transparent film on the top and the product can be served in the same tray. Due to high levels of salt it has a prolonged shelf life. Product packaging and presentation give added-value to the product, and the medallion-shaped roe cuts are convenient, innovative and attractive. The main identified weaknesses, are its price and the existing competition.

3. Fresh thin smoked fillets (Idea 2): the packaging is a plastic tray where the fillets are laid covered with a transparent plastic that allows visibility of the fillets. Ideas concerning the different uses of the fillets are included on the product's sleeve. This product can be targeted to wider market segments than product 4 or 5 due to more affordable price and higher familiarity in sensory characteristics (smoking). Among the advantages are versatility in uses; convenience; freshness; nutritional value; reasonable price/quality; familiarity in sensory characteristics. Thin smoked fillets are competitive due to price, high nutritional value and desirable appearance and because they are appropriate for several uses. Main identified weakness includes the low added value due to significant filleting losses.

All species are suitable for development of product 4 to 6:, however grey mullet and greater amberjack are advantageous due to the combination of higher fat contents (good sensory result in smoking) with good yields. Bottarga can be produced by the roe of any fish species that have adequate roe yield and specific sensory properties, but possible bottlenecks in the production could arise if other species than mullet are used (traditional). Since these are delicacies aimed at specific consumer segments, no specific countries are targeted. The UK, Germany and Italy show interest for greater amberjack products, Italy finds grey mullet products interesting and Spain asks for different species and diversification in the market.

C. Added-value products: can be targeted to mass market and delivered through several distribution routes. Common characteristics of these products include convenience; versatility; high nutritional value; affordable price; and high added value. Added value products may not by themselves increase significantly fish consumption, still they should be incorporated in the production parallel to other products. Such a practice could generate significant profits by using industries' low value by-products or discards.

1. Fish burgers, balls or sausages represented by Idea 6 ***Frozen fish burgers in the shape of fish, targeted to children*.** The burgers are ready to cook and prepared with a mild seasoning. The product is included in a transparent vacuum packed bag or in a plastic tray with transparent plastic on the top. Information on fish for educative purposes (children) and playful gifts (*e.g.*, sticker) are included in the packaging. This is intended to increase fish consumption in children. The advantages of this product includes absence of bones; attractive shape for children; reasonable price; versatility; familiar sensory characteristics; convenience;

healthiness and nutritional value. Main identified weaknesses include technical feasibility in equipment and low freshness (frozen).

2. Fish spreads, dried fish sticks, seasoning, sauces represented by Idea 19 ***fish pate***. Fish pate/ spreads prepared using different recipes. Can be used as starter or incorporated in a sandwich. The product is sustainably produced (containing ASC label). It is labelled as a premium product; the country of origin is EU. Its advantages are its convenient use, healthiness, affordable price, prolonged shelf life. The main identified weakness of this product is its reduced value of freshness.

For the creation of these products all fish species can be utilised as raw materials. Since product 9 is marketed frozen, low fat species, such as meagre are advantageous, followed by other low fat species such as Atlantic halibut and pikeperch. No specific country or market limitations occur for this group of products. Finally a convenient mass market product with characteristics depending on the choice of incorporated fish is:

3. **Fresh ready to eat salad that includes fish as well as an accompanying sauce** (Idea 4); fish and sauce are provided, and are separately packed and included within the original package. The fish included is either a smoked fillet (provided in slices), or vinegar-cooked, or alternatively bottarga; thus, the dish can be eaten cold. The packaging is composed of a bowl where the salad is placed; the fish pieces and the sauce are provided in separate transparent accompanying packages. A transparent lid exists on the top to allow product visibility and the packaging has the picture of the ready meal on it. Advantages include convenience; healthiness; freshness; nutritional value; and familiarity. The main disadvantage is short shelf life. Depending on the fish accompaniment, characteristics (such as price) can change.

The full description of the work and results has bee provided in ***Deliverable 28.2 List of ideas for new product development***.

Task 28.2 New Product Development (led by IRTA, Luis Guerrero)

Sub-task 28.2.1 (led by HCMR, Kriton Grigorakis)

This Task was partially reported during the previous Reporting Period.

One significant aspect of the market success potential of farmed fish is their quality as perceived by the consumer. Two important aspects in the multi-parameter set that constitute quality, are the nutritional and the technical characteristics of the fish. The former refers to the nutritional value of the food, while the latter to the processing technical losses and the edible yields. The technical quality expressed as somatic indexes and technical yields (dressing yield, filleting yield) on the other side, are mainly economic aspects and are of interest to both consumers and processors. Furthermore, the organoleptic quality of the fish, i.e. the human sensory impression, is among the capital factors for purchasing any fish. Accordingly, in this task the following parameteres were evaluated:

1. Somatometric indexes of the studied species at commercial sizes.
2. Correlation of commercial sizes with losses, edible proportions and filleting yields for each species in order to evaluate possible commercial sizes with best yields.
3. Analysis of the proximate composition of fillets of all species.
4. Sensory description of the appearance, aroma, flavour, taste and texture of the studied species.
5. A correlation of the sensory profile of the studied species with fillet composition and mechanical texture.

Specimens of meagre, greater amberjack, pikeperch and Atlantic halibut used in the present study were of aquaculture origin. On the contrary, wreckfish were obtained from two different locations from the wild, due to the absence of any farmed individuals, and grey mullet were also obtained from the wild due to absence of available farmed ones in commercial sizes. Fish characteristics, sampling and size information are all included in **Table 28.2.1**.

Table 28.2.1: Origin, season of sampling and fish size information of selected fish species.

Species	Season	N	Origin – farming conditions	Feed	Fish Size
Greater Amberjack (*Seriola dumerili*)	Feb. 2015	10	Farm (Corfu S.A.)-NW Greece -floating sea cages	Commercial extruded feed	1-1.5 kg
Greater Amberjack (*Seriola dumerili*)	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 –fresh water intensive farming	Commercial extruded feed	1-2 kg
Grey Mullet (*Mugil cephalus*)	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 (*Polyprion americanus*)	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

Apart from somatic measurements, one fillet from each fish was vacuum packed and stored in -20°C until sensory analysis, as it was necessary to gather all fish samples together to have the sensory analysis conducted. The other fillet was used for composition analysis. The following somatometric indexes were calculated individually: Condition index (CI) = [100× body weight (g) / body length^3 (cm^3)]; Dressing yield (DY) = [100×(gutted body weight /body weight)]; Filleting yield (FY) = [100 × (fillet weight /body weight)];

Hepatosomatic index (HSI) = [100 × (liver weight / body weight)]; Gonadosomatic index (GSI) = [100 × (gonad weight / body weight)] ;Viscerosomatic index (VSI) = [100 × (total viscera weight / body weight)] and Visceral fat index (VFI) = [100 × (visceral fat weight / body weight)].

Fish fillet proximate composition analysis (protein, fat, moisture and ash), was conducted by the custom AOAC (2005) methods.

Part of the sensory analysis work took place by choosing assessors and by doing significant preliminary work in sensory analysis. A list of sensory attributes was used to assess and score the fish samples. The list of descriptors was created starting from a list of 94 descriptors from a previous study. To narrow the list down, one session of Check-All-That-Apply (CATA) was performed to reduce the number of descriptors and use only those who were useful for describing and discriminating the five fish species under study. Afterwards, assessors scored the attributes previously selected with the CATA method in two sessions. Twenty-two descriptors were retained after applying a two way ANOVA (fish species and tasting session) keeping those able to discriminate among the samples. Once the final list was set up, panellists were trained to become familiar with the included descriptors, therefore references scales were developed to score the intensity of each attribute to assess (see ***Deliverable 28.3 Report on product and process solutions for each species based on technological, physical and sensory characteristics*** for further details). In all cases, samples were cooked in a convection oven at 115ºC for 20 min in individual transparent glass jars designed to make samples easy to visualize. Eight panellists with previous experience in sensory profiling of food products were recruited for this training before evaluating the samples. Sample analysis was performed in five sessions, testing all samples in each session. In each tasting session, the order of sample presentation

and the first order and carry-over effects were blocked. **Table 28.2.2** shows the mean values obtained for each species and sensory descriptor.

Three fish from each species (pikeperch, meagre, greater amberjack, grey mullet and wreckfish) were selected for the instrumental analysis of texture. Two different tests were carried: a non-destructive compression test (30%) with a spherical probe (18.4 mm diameter) and a Texture Profile Analysis (TPA, compression of 75%) (BOURNE, 1978) with a cylindrical probe (25 mm diameter). All the tests were performed with a TA-HD plus Texture Analyser (Stable Micro System, Surrey, England) at a constant speed of 1 mm/s. The non-destructive test (spherical probe) was performed both in the raw and cooked samples in three different locations of each filet whereas the TPA was only carried out in the cooked samples in two different locations of each filet.

Although, there are numerous inevitable uncertainties due to technical reasons (limited number of fish, limited dietary histories and limited seasons examined) some suggestions can be drawn from the obtained results.

1) Technical yields do not seem to be significantly influenced by fish size, with the exception of dressing yield that correlates positively with body weight in great amberjack and meagre. Thus for these two species it is technically more profitable to have fish of large commercial sizes, when commercialized as gutted.

2) Fish species reared in ponds or in freshwater, *i.e.* the grey mullet and the pikeperch, characterized by the presence of "earthy" odour and flavour, maybe be better used for the design of products that come with dressing spices or sauces that can cover these earthy characters that are mostly unwanted. Thus, ideas such as #14 and 21 (fresh products with spices or marinates) or 25 and 13 (frozen products with marinates) were presented in ***Deliverable 28.2 List of ideas for new product development can be ideal for these species***.

3) Greater amberjack may be advantageous for raw (*e.g.* Carpaccio or tartar, ideas 24, 30 and 38 of ***Deliverable 28.2***) and smoked products (idea 2 of ***Deliverable 28.2***) due to its high fillet fat contents and distinct sensory characteristics (high acid flavour and juiciness). On the other hand, it may be disadvantageous when referring to the frozen products, due to its vulnerability to fat oxidation. If frozen products are going to be designed for greater amberjack, they should be made with fish smaller fish of 1-2 kg, because they have relatively low fillet fats as opposed to larger fish (of 10-15 kg) that are more fatty.

4) Meagre and pikeperch may be ideal for fish burgers (*e.g.* fish burger in the shape of fish: idea 6 of ***Deliverable 28.2***) due to their low chewiness (as opposed to greater amberjack and wreckfish). This is justified by the fact that these new products refer to children, which would in their majority prefer less "chewy" fish.

The main results/conclusions that can be drawn from this task are:

1) The condition index of the fish is usually indicative of the nutritional status of the fish, meaningful only in intra species comparisons, and has been found to correlate positively with body weight in the cases of great amberjack and meagre;

2) The dressing yields for all species are quite similar, slightly exceeding 90%;

3) No other significant correlations of technical yields with fish size (body weight) were observed. This implies that body weight is not a crucial parameter in processing;

4) Great amberjack showed high fillet fat reaching 4% in 1-1.5 kg fish and exceeding 12% in 15-20kg fish. All other fish exhibited very low fillet fat, not exceeding 1%;

5) All fish species showed similar and typical fillet protein averaging 20%;

6) Average values of the sensory descriptors, as well as discriminant analysis, show significant differentiation in the sensory properties of the five fish species. Grey mullet and pikeperch are characterized by the presence of earthy odours and flavours. All marine origin species (greater amberjack, wreckfish and

meagre) are characterized by higher butter flavour. Wreckfish is characterized by acid flavour. Meagre and pikeperch are characterized by lower chewiness;

7) Texture profile analysis also showed differentiation between fish species and some relevance with the values received for the sensory textural descriptors;

8) Some processing recommendations – products best fitted for each species can be suggested.

Table 28.2.2. Mean values for each species and sensory descriptor.

Descriptor	Amberjack	Wreckfish	Meagre	Grey Mullet	Pikeperch	RMSE
O Butter	2.6^{b}	3.8^{a}	3.8^{a}	2.4^{b}	1.4^{b}	1.487
O Seafood	1.2	1.4	1.4	0.6	0.6	1.109
O Sardine	1.4^{ab}	2.3^{a}	1.7^{ab}	2.2^{a}	0.9^{b}	1.638
O Earthy	0.8^{b}	0.5^{b}	0.7^{b}	1.6^{ab}	2.7^{a}	1.509
Colour	3.7^{ab}	4.5^{a}	3.1^{bc}	3.9^{ab}	2.2^{c}	1.245
Colour homogeneity	8.6^{a}	7.4^{ab}	6.8^{b}	6.7^{b}	7.7^{ab}	1.602
Exudates	7.0^{b}	8.2^{a}	5.5^{c}	2.6^{d}	5.0^{c}	1.503
Turbidity	1.1^{c}	6.3^{b}	5.7^{b}	2.2^{c}	8.0^{a}	1.947
Fat droplets	5.9^{a}	6.1^{a}	5.7^{a}	0.8^{b}	0.8^{b}	2.000
Laminar structure	6.3^{a}	3.9^{b}	4.5^{b}	4.7^{b}	4.7^{b}	1.904
Acid	4.5^{a}	0.7^{b}	0.6^{b}	0.7^{b}	0.6^{b}	1.210
Bitter	2.0^{b}	1.6^{b}	1.8^{b}	3.2^{a}	1.5^{b}	1.485
Butter	2.5^{a}	2.1^{a}	2.3^{a}	1.0^{b}	0.8^{b}	1.369
Seafood	1.0^{ab}	1.1^{ab}	1.5^{a}	1.1^{ab}	0.4^{b}	1.057
Boiled vegetables	1.6^{b}	2.9^{a}	2.9^{a}	2.3^{ab}	2.6^{ab}	1.379
Earthy	0.7^{bc}	0.4^{c}	0.2^{c}	1.5^{b}	2.8^{a}	1.401
Firmness	5.8^{ab}	6.6^{a}	4.8^{bc}	5.8^{ab}	4.1^{c}	1.596
Crumbliness	5.5^{b}	4.0^{c}	6.4^{ab}	5.8^{b}	7.3^{a}	1.506
Juiciness	5.8^{a}	4.9^{ab}	5.8^{a}	4.3^{b}	5.2^{ab}	1.248
Chewiness	6.1^{ab}	6.5^{a}	4.0^{c}	5.2^{b}	3.8^{c}	1.291
Pastiness	3.9^{ab}	3.1^{bc}	4.2^{a}	2.7^{c}	4.3^{a}	1.342
Teeth adherence	6.8^{a}	3.3^{b}	3.7^{b}	3.5^{b}	2.8^{b}	1.593

a-c: mean values in the same row with different superscripts differ significantly ($p \leq 0.05$).

The full description of the work and results has 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 focused on the development of new products physical prototypes from four selected species: meagre, greater amberjack, pikeperch and grey mullet. Partner 1. IRTA was responsible of developing products of varying degree of processing from meagre and pikeperch, whereas P18. CTAQUA was responsible for those obtained from greater amberjack and grey mullet. Provided that wreckfish is not available as farmed fish, the possibility to create prototypes out of this species and conduct further consumer test was not possible.

The selection of fish products was made after considering the different product concept positions in the ranking shown in **Deliverable 28.2** and on the technical feasibility of the different fish species. The selected concept ideas for product development made with meagre were "frozen fish fillets with different recipes", ready to eat meal: salad with fish" and "fish burgers shaped as fish". The selected ideas made with pikeperch were "fish spreads /pate", "fresh fillet with different 'healthy' seasoning and marinades" and "ready-made fish tartar with additional soy sauce". The selected ideas made with greater amberjack were "frozen fish fillet that is seasoned or marinated", "ready-made fish tartar with additional soy sauce" and "fresh fish steak for grilling in the pan". The selected ideas made with grey mullet were "thin smoked fillets", "fresh fillet with different 'healthy' seasoning and marinades" and "ready-made fish fillets in olive oil".

In relation to the "frozen fish fillets with different recipes" idea, the low fat content of meagre was considered as crucial for this product success. Fish fillets with skin are frozen and vacuum packed in three or four 2-person portions. This process is relatively simple and thus entails minor difficulties. According to the literature, the shelf life of this product is expected to be of 9 months or higher. Provided that this concept idea includes different portions each one having different recipe from the others, different recipes were created based on kitchen books, Internet and own recipes. The different recipes and their overall acceptability scores were as follows: "*Fish fillet citrus sauce"*: 8.0; "*Baked fish":* 7.0; "*Fish with tomato (microwave)":* 7.5*; "Fish fillet in green sauce":* 8.0*; "Fish fillet with orange":* 8.0*; "Fish fillet with garlic and parsley":* 8.5*; "Fish fillet with vegetables":* 8.5*; "Three cheeses fish fillets":* 8.0. Based on their scores and product suitability the selected recipes were the following: "*Three cheeses fish fillets", "fish fillet with yogurt sauce and mushrooms", "fish fillet with vegetables", "baked fish with tomato sauce"* and *"fish fillet with orange".*

With respect to the "thin smoked fillets" idea, the high fat content of grey mullet represents an interesting opportunity for this idea product development. In this project, hot smoking with dry salting and addition of sugar was the procedure followed for the preparation of grey mullet smoked fillets. After being cooked (60ºC for 40 minutes), they are vacuum packed per three, two or individual portions (**Figure 28.2.1**). The elaboration of this product is relatively simple and, in consequence, presents minor difficulties. This product is stored at refrigeration temperatures and under such conditions the shelf life of this product is estimated to be less than 31 days of storage.

Figure 28.2.1. Prototype of smoked grey mullet fillets, vacuum-packed ready for consumption.

As for the "ready to eat meal: salad with fish" idea, several fish species, including meagre, have been reported to be of technical compatibility. Because of meagre low fat content it was considered to better marinate it with vinegar than smoking. Vinegar from apple cider was chosen for this product development as it provides smoother flavour. A mustard vinaigrette containing honey was also designed as accompanying sauce. These latter ingredients are separately packed. This salad also contains croutons, tomatoes and green

vegetables that are packaged in modified atmospheres (MAP). This product is stored under refrigeration and its shelf life is limited by the salad component.

In relation to the "fish burgers shaped as fish" idea, meagre fish is of particular interest for this product idea as it has a higher proportion of muscle trimmings. In addition, it is particularly indicated for frozen products due to its low fat content. Cheese was added to the formulation to make it more appealing to children's taste. As a processing aid, microbial transglutaminase was added to improve the texture of this product. This product is frozen and skin packaged (**Figure 28.2.2**). Therefore, the production of this product is relatively complex and its shelf life has to be similar to other related products that have been processed and under the same storage conditions and, in consequence, expected to be of 9 months or higher.

Figure 28.2.2. Physical prototype of the "fish burgers shaped as fish" product concept.

The "fish spreads/pate" idea was reported to be compatible with pikeperch in ***Deliverable 28.2***. On the other hand, this fish species is characterized by the presence of "earthy" odor and flavor ***(Deliverable 28.3***), which can be masked with the use of appropriate ingredients. Related with that, a thermally stable oil-in-water emulsion was added in the product formulation. With the addition of this ingredient it is possible to obtain a fish pate with the desired mouth feel and rheological characteristics. The ideal processing conditions include the use of special equipment to fill this product in a tube under aseptic conditions. Alternatively, the product can be filled into the tube manually, close and pasteurize it and store at refrigeration temperatures. Therefore, this product is highly processed and complex. According to microbial and sensory evaluations the shelf life of this product is expected to be of 30 days approximately.

In relation to the "frozen fish filet that is seasoned or marinated" idea, the greater amberjack was the selected fish species because it has good yields and when it is 1-2 kg weight has a relatively low fat content compared to larger fish (***Deliverables 28.2 and 28.3***). Soya sauce and honey were selected to prepare the marinade (Asian marinade). Once the product was elaborated, it was immediately vacuum packed and frozen. This product presents minor difficulties and the marinade can be tailor made in function of the target consumer. In order to assess the shelf life, this product was stored at -18ºC for one third of the corresponding estimated period and the remaining period stored at an abused temperature of 4ºC (day 7). At the end of this storage period the product was considered as not acceptable from a microbiological point of view.

Two different "fresh fish fillet with different 'healthy' seasoning and marinades" have been developed in ***Deliverable 28.4***. The selected fish species were pikeperch and grey mullet. The seasonings and marinades not only were healthy but also helped to mask the "earthy" odour and flavour of these fish species (***Deliverable 28.3***). Two seasonings are based on the utilization of olive oil whereas another one included yoghurt. Both products are stored under refrigeration. However, the product with grey mullet was vacuum

packaged whereas that with pikeperch involved the use of modified atmospheres. Therefore, the main limitation is the use of appropriate equipment for packaging in modified atmospheres. Both products are expected to have a shelf life of about 6 days.

There are also two different versions of the "ready-made fish tartar with additional soy sauce" idea. The selected fish species for this idea were pikeperch and greater amberjack. Greater amberjack was selected because of its high content in fat and distinctive sensory characteristics (***Deliverables 28.2 and 28.3***). However, other fish species such as pikeperch have also been considered as suitable for this concept idea. The prototypes presented in the deliverable combined different strategies including: fish gelatine, packaging and processing conditions. Consequently, these products are relatively complex. Greater amberjack was found to have a limited shelf life under the reported conditions whereas that of the tartar with pikeperch which involves the use of high hydrostatic pressure processing can be at least of 1 week.

Grey mullet was selected for the "ready-made fish fillets in olive oil" idea due to its technical compatibility and sensory characteristics (***Deliverables 28.2 and 28.3***). This idea is based on a traditional method of fish preservation and packaged in glass containers (**Figure 28.2.3**). Overall, the elaboration of this product is relatively simple and, provided that it is submitted to a sterilization process, its stability is expected to be relatively high.

Figure 28.2.3. Prototype of ready-made grey mullet fillet in olive oil.

Finally, greater amberjack was used in the development of the "fresh fish steak for grilling in the pan" idea. Fresh greater amberjack were descaled, eviscerated and the obtained clean fillets were cut in portions (**Figure 28.2.4**) and vacuum packed. This product presents minor difficulties. The shelf life of this product is of at least 5 days of refrigerated storage.

Figure 28.2.4. Fresh greater amberjack fillet ready to be cooked on the pan.

The full description of the work and results has 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)

No work done during this period.

Sub-task 28.3.2 (led by ULL, Covadonga Rodriguez)

No work done during this period.

Sub-task 28.3.3 (led by IRTA, Luis Guerrero)

No work done during this period.

Deviations from Annex I and their impact:

We have faced some delays mainly due to the difficulties to find the raw material needed for the different activities. **Deliverable 28.1** was the only one delivered on time in month 14. **Deliverable 28.2** had a delay of four months and was delivered in month 20 thus affecting **Deliverables 28.3** and D**28.4** that were submitted with a delay of five and three months, respectively. These delays have also affected some the related activities described in WP 29 (see later), although an important part of these delays has been absorbed and reduced by WP 29, thus minimising their impact in the correct development of the socio-economic tasks.

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 (P11)			
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 progress towards objectives and details for each task (11-30):

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)

The general objective of this task was to explore consumer attitudes towards (farmed) fish, as well as define consumer value perceptions in the form of trade-offs between perceived gains (i.e., benefits or 'values') and perceived losses (*i.e.* sacrifices or 'costs') from the consumption of the fish products resulting from the species under study in the focal markets (*i.e.* France, Italy, Germany, Spain and the UK. In order to achieve the objective of this sub task, an on-line consumer survey with a minimum of N = 500 consumers per each country (nationally representative samples) has been conducted in the five focal markets (i.e. France, Italy, Germany, Spain and the UK). Data has been operationalized and collected based on the proposed Customer Value (CV) model (**Figure 29.1.1**).

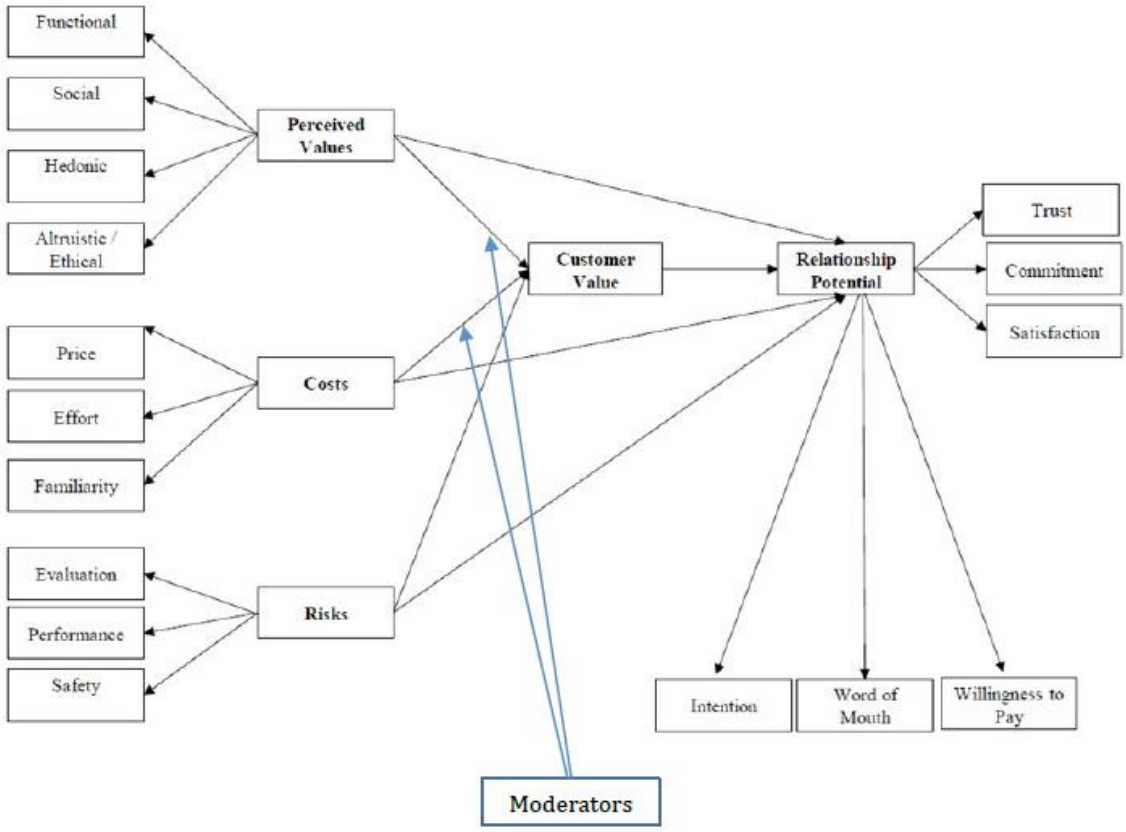


Figure 29.1.1. The Customer Value (CV) model.

The obtained data provided information on consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the examined hypothetical new (farmed fish) products from selected fish species. Convergence of the multi-item constructs has been

achieved across investigated countries (**Table 29.1.1**). These results have further allowed for the subsequent value-based segmentation in task 29.1.2.

Table 29.1.1. Results of factorial analysis for multi-item constructs (summary)

Construct	Countries (Cronbach's alpha of relevant dimensions)				
	France	**Germany**	**Italy**	**Spain**	**UK**
Functional value	0.949	0.906	0.957	0.961	0.952
Social value	0.888	0.878	0.883	0.869	0.894
Hedonic value	0.910	0.889	0.900	0.879	0.904
Ethical value	0.898	0.794	0.903	0.911	0.891
Emotional value	0.922	0.884	0.907	0.922	0.929
Price	0.799	0.740	0.862	0.820	0.797
Effort	0.839	0.873	0.868	0.880	0.886
Unfamiliarity	0.788	0.766	0.811	0.789	0.840
Evaluation cost	0.729	0.827	0.796	0.825	0.835
Performance risk	0.812	0.813	0.840	0.790	0.838
Safety risk	0.707	0.781	0.833	0.746	0.841
Customer value	0.862	0.895	0.898	0.911	0.918
Satisfaction	0.944	0.939	0.938	0.931	0.938
Trust	0.941	0.942	0.943	0.948	0.913
WOM	0.912	0.898	0.918	0.857	0.910
Intention to buy	0.861	0.857	0.817	0.821	0.878
Customer involvement	0.942	0.878	0.959	0.950	0.941
Domain specific innovativeness	0.860	0.884	0.858	0.860	0.899
Subjective knowledge	0.946	0.927	0.939	0.926	0.944
Optimistic bias	0.895	0.808	0.845	0.856	0.882
Social representations	0.739	0.731	0.789	0.741	0.756

The full description of this work and results has 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))

The general objective of the sub-task 29.1.2 has been the identification of consumer segments in the five focal European countries (i.e. France, Germany, Italy, Spain and the UK) based on the dataset collected in the previous sub-task 29.1.1. This value-based segmentation task gives insights into consumer sub-markets

(i.e. segments) across and within the five countries examined (i.e. national and international segments) with the highest potential for maximized consumer value perceptions, thus relevant for exploitation in subsequent activities of WP 29.

Based on the dataset collected in the previous sub-task 29.1.1 and by using Agglomerative Hierarchical Cluster Analysis (AHCA) (Ward method and Euclidian distance), three distinct consumer segments have emerged across the study countries: the *involved innovators*, *involved traditional*, and *ambiguous indifferent* (**Table 29.1.2** and **Table 29.1.3**).

Table 29.1.2. Segmentation analysis - psychographic profiles of the segments, mean scores

Construct	Involved innovators C1 (N=728)	Involved traditional C2 (N=911)	Ambiguous indifferent C3 (N=872)	Sig.*
Involvement	1.95 [b]	1.77 [a]	3.77 [c]	***.000***
Domain-specific innovativeness[d]	2.81 [a]	5.37 [c]	3.94 [b]	***.000***
Subjective knowledge	4.19 [a]	1.63 [a]	3.06 [b]	***.000***
Optimistic bias	3.89 [c]	2.62 [a]	3.57 [b]	***.000***
Social representation of food	3.00 [a]	4.43 [c]	3.87 [b]	***.000***

1 = Strongly agree, 7 = Strongly disagree*Results from the ANOVAs test
** All values in italic significant at $p > .001$
[a,b,c]Tukey HSD post hoc test, superscripts indicate post-hoc paired comparisons
[d]DS Innovativeness has negative polarity, meaning that agreement scores close to unit actually indicate lack of innovativeness and vice versa

Table 29.1.3. Country membership (%) in different consumer segments

Country	Involved innovators C1 (N=728)	Involved traditional C2 (N=911)	Ambiguous indifferent C3 (N=872)	Total per country
Germany (n=506)	**30.6*** **21.3**	**47.4** **26.3**	21.9 12.7	100.0
Spain (n=500)	26.4 18.1	**35.2** **26.3**	**38.4** **22.0**	100.0
France (n=500)	28.4 19.5	29.2 16.0	**42.4** **24.3**	100.0
Italy (n=500)	**33.8** **23.2**	**35.6** 19.5	30.6 17.5	100.0
UK (n=505)	25.7 17.9	33.9 18.8	**40.4** **23.4**	100.0
Total per cluster	100.0	100.0	100.0	

*Numbers shifted to the left represent percentage of membership of single country in three clusters, while numbers shifted to the right represent the membership of all countries per single cluster.

The fact that the segments have been found to be uniform across all countries shows a more homogeneous or converging fish-related culture and provides opportunity to fashion new product concepts through the careful use, novel combination, and conscious innovation of existing fish products at a cross-border European level.

This segmentation analysis of the European fish market further allowed for selection of the most important potential market segments to enter with new fish products from new fish species. More specifically, the most interesting consumer segments that could be a target of marketing positioning strategies for new fish products and new fish species are the *involved innovator and involved traditional.* These two market segments have been profiled on the basis of the different geographic, demographic, psychographic and

behavioural characteristics that allowed for better understanding of the needs and preferences of the each segment across and within the five countries examined.

Finally, characterization of each market segment has allowed for further prioritization and development of the more personalized communication of the key distinctive benefit(s) of the new fish products' market offering that will come in the subsequent steps of DIVERSIFY project. Thus, this particular sub-task allowed for identifying and targeting of specific market segments, as well as for setting the stage for the development of the new fish products to be specially tailored and relevant for the consumers from the established market segments.

The full description of this work and results is 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, Luis Guerrero and prepared by Luis Guerrero (IRTA)).

The objective of this task 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). In this task, the different physical product prototypes developed and tested in Tasks 28.1 through 28.3, related to new product development and monitoring technical quality of the products, were manufactured according to the amount needed and following strict hygienic conditions. These product samples are the basis for the acceptability test to be done later in Task 29.2. **Table 29.2.1** shows the new products developed for each species.

Table 29.2.1. New products developed for each species (in bold the products selected for tasting with consumers).

Fish species	**Product ideas**
Meagre	Idea 1*: Frozen fish fillets with different recipes **Idea 6: Fish burgers shaped as fish (H)** **Idea 4: Ready to eat meal: salad with fish (L)**
Pickeperch	Idea 21: Fresh fish fillet with different 'healthy' seasoning and marinades Idea 30: Ready-made fish tartar with additional soy sauce **Idea 9: Fish spreads/pate (H)**
Grey mullet	**Idea 2: Thin smoked fillets (M)** **Idea 33: Ready-made fish fillets in olive oil (M)** Idea 21: Fresh fish fillet with different "healthy" seasoning and marinades
Greater amberjack	Idea 13: Frozen fish fillet that is seasoned or marinated Idea 30: Ready-made fish tartar with additional soy sauce **Idea 34: Fresh fish steak for grilling in the pan (L)**

L: low processing; M: mid processing; H: high processing.

*See deliverables **Deliverables 28.2** and **D28.4** for a detailed description of each product idea.

The main aim of this task is to provide all the information needed to handle, store and prepare the different samples, the statistical design to follow in each location (order of presentation, sample distribution among participants, etc.) as well as some practical recommendations to carry out the test and recruit the participants properly. More specifically, participants will be 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***). Further all the sensory tests will be performed under controlled conditions in a central location per country. All the product samples will be shipped in advance to each location in the right conditions and guaranteeing the cold chain. Samples will be sent with detailed instructions about the right procedure to store them until analysis. Finally, ten tasting sessions will be held in each location in two consecutive days. In each tasting session, consumer will assess overall expectation with the product (see the design for the different presentation orders in **Table 29.2.2**) followed by blind tasting (as presented in Table 29.2.2) and overall expectation in informed condition (*i.e.* picture with full description of the product from **Deliverable 28.2**, see example in **Figure 29.2.1**).

Table 29.2.2. Order of presentation of the six new products in each session for all the countries.

Products to assess	**Tasting session**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Idea 6: Fish burgers shaped as fish (meagre)	1st	2nd	4th	6th	5th	3rd	2nd	1st	3rd	6th
Idea 33: Ready-made fish fillets in olive oil (grey mullet)	3rd	1st	2nd	4th	6th	5th	4th	2nd	1st	5th
Idea 2: Thin smoked fillets (grey mullet)	5th	3rd	1st	2nd	4th	6th	6th	4th	2nd	3rd
Idea 34: Fresh fish steak for grilling in the pan (greater amberjack)	6th	5th	3rd	1st	2nd	4th	5th	6th	4th	1st
Idea 4: Ready to eat meal: salad with fish (meagre)	4th	6th	5th	3rd	1st	2nd	3rd	5th	6th	2nd
Idea 9: Fish spreads / pate (pickeperch)	2nd	4th	6th	5th	3rd	1st	1st	3rd	5th	4th

Figure 29.2.1. Example of product to be displayed on the computer screen according to the presentation orders shown in Table 29.2.2. (Idea 6: Fish burgers shaped as a fish).

The full description of this work and results has 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***. ***Deliverable 29.4 Report on the actual product's sensory profiling in the five countries*** investigated was under final revision and will be submitted shortly.

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)

The main objective of this sub-task 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 (**Deliverable 29.4**). The task contains the following information: (i) the methodology used for development of mock-ups, and (ii) development of mock-ups for the experiments that include (a) images of the developed product ideas; (b) review of the consumer studies; (c) secondary analysis of the data on newly launched fish products, and (d) the experimental design.

According to this sub-task's objectives, in order to develop product mock-ups, select most important combination of intrinsic-extrinsic product attributes for targeted consumer segments established in Sub-task 29.1.1 (see Deliverable 29.2), and prepare experimental set-ups for subsequent Sub-task 29.3.2, the work done in Task 29.3.1 followed four-steps:

1. First, pictures have been taken of the developed physical product prototypes in the proper packaging and without any labelling information, so resemble as much as possible to final products.
2. Subsequently, a literature review of previous consumer studies that involved experimental set-ups with fish products (i.e. Conjoint or Discrete Choice experiments) has been undertaken to cease the most important product attributes and their levels.
3. Following, a crosscheck of the findings from the previous studies with existing secondary data on new fish product launches has been undertaken based on datasets from the Mintel[3] Global New Products Database (GNPD).
4. Finally, based on the above, an experimental design with product attributes and attribute levels has been proposed across developed product ideas.

Pictures of new products developed

The images of the six physical product prototypes developed (see Deliverable 29.3) have been taken by a professional with high resolution camera with each image having at least 5″ x 4″ (3750 x 3000 pixels) at 300 dpi on the white background. This is done in order to obtain a sharp reproduction of fish product images for our experiments. Images are further manipulated in GIMP Image Manipulation Program and set on a black background in a 1280 x 1024 pixels resolution where the image was centred and set within the square of 1080 x 700 pixels. The finally manipulated images of the each product idea will constitute the stimuli for the experiments in the subsequent sub-task 29.3.2.

Review of relevant consumer studies

Review of previous literature with regard to fish product experiments assisted in selecting the most important extrinsic product attributes (*i.e.* product labeling elements). Key references in relation to fish products and different experimental set-ups have been identified through a systematic literature search. These references were mainly related to studies that have applied conjoint or discrete choice experimental designs in a fish context, as subsequent sub-task 29.3.2 will apply similar designs, to ascertain final combination of attributes with highest consumer value perceptions per product. In summary, previous research reported that most important attributes in relation to fish products are:

[3] The Mintel Global New Products Database (GNPD) (http://www.gnpd.com/) monitors product innovation and retail success in consumer packaged goods markets worldwide. GNPD offers unrivalled coverage of new product activity for competitor monitoring, category awareness, opportunity identification and inspiration for new product development.

- Country of origin (e.g. domestic *vs* imported);
- Price (attribute levels vary depending on species and countries);
- Storage conditions (e.g. fresh *vs* frozen);
- Production method (e.g. wild caught *vs* farmed);
- Organic and natural claims (e.g. organic salmon: yes *vs* no);
- Certification labels (e.g. certified for sustainability *vs* certified for quality *vs* uncertified);
- Ethical animal claims (e.g. fewer salmon suffer from external injuries: yes *vs* no), and
- Brand (e.g. manufacturer *vs* retail brand).

Analysis of secondary data on newly launched fish products

Analysis of existing secondary data on newly launched products has additionally assisted in the selection and fine-tuning of the most important extrinsic product attributes for fish products, as it provided a clear picture of the existing products in the marketplace and what works for consumers. Therefore, an extensive search has been undertaken in Mintel GNDP Database. The search of newly launched fish products has been carried out during June 2016. Moreover, it has been expanded to include all new products launched from 1st of January 2011 to 31st of May 2016. Furthermore, the search has been restricted to the products belonging to the "Fish product category" (existing in Mintel GNDP Database), as well as to the product ideas developed in DIVERSIFY project (**Deliverable 29.3**). Therefore, the information on the number of new products launches, prices, certification logos and the claims has been accessed for the last five years and for five countries investigated (*i.e.* France, Germany, Italy, Spain and the United Kingdom).

Finally, prices, claims, and logos of the selected products were listed and then summarized to demonstrate overall similarities and differences across countries. Additionally, cross-comparison of the real-life data provided from Mintel GNDP Database with primary data obtained from the Diversify project (see **Deliverable 28.1**) allowed for a more effective generation of product mock-ups that can subsequently be tested in sub-task 29.3.2 with consumers from the identified market segments (D29.2). The secondary data analysis has been undertaken separately for each of the six new product ideas.

Experimental design

In order to prepare mock-ups for the experiments in sub-task 29.3.2 (*i.e.* Discrete choice experiments) in the five countries investigated, the product attributes and attribute levels in the experimental design must be carefully chosen in order to reflect key product characteristics or dimensions which consumers may use to assess the newly developed fish products. In particular, the attributes should embrace the most relevant attributes to potential consumer segments (**Deliverable 29.2**). Attribute levels correspond to the points along chosen attribute dimensions and should likewise cover most representative levels. As only two out of six product ideas will be tested across the investigated countries, the same attributes and their levels have to be assumed. This being said, and based on the previous literature review and secondary data analysis, the suggested attribute levels for product mock-ups, are as follows:

1. **Country of Origin** - (produced in EU/own country/produced outside of EU);
2. **Price** - 1, (50%, 100%, +200% of average price[4]);
3. **Nutritional claim** – Omega 3 (yes/no);
4. **Nutritional claim** – High in protein (yes/no); No gluten (yes/no);
5. **Nutritional claim** – No gluten (yes/no);
6. **Health claim** – Improves brain function (yes/no);
7. **Health claim** – Improves cardiovascular function (yes/no);
8. **Environmental claim** – ASC logo, responsibly sourced (yes/no), and
9. **Ethical claim** – Fish welfare logo (yes/no)

[4]Average price for each product idea based on the secondary data analysis of new product launches from Mintel GNDP Database, see section 3.3.

The above attributes and their levels were varied according a 3^2x2^7 orthogonal design in SPSS statistical software, which produced 30 experimental sets of product mock-ups. The design was further partitioned into ten versions of choice set size of three that will be randomly assigned to consumers.

Product mock-up stimuli will be presented in a visual shelf simulation mimicking realistic purchase decision and in order to capture subliminal effects of different labelling information. Thus, to conduct the choice experiment, consumers will be first asked to imagine that they want to buy a fish product from aquaculture production to eat with their family and/or friends. In each screen, they will be asked to indicate the product they are most likely to choose and if that would be their realistically purchased product.

The full description of this work and results has 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 main objective of this sub-task is to develop a number of experimental set-ups through an on-line survey to test the mock-ups developed in the previous sub-task 29.3.1. The experiments will be run with consumer from selected segment from Sub-task 29.1.2 (and Deliverable 29.2). Thus, participants will belong to the segments (national and international) with the highest/best value perceptions per product in order to achieve a best match between ideal extrinsic/intrinsic attribute combinations and high-potential segments.

This Sub-task is planned to start at the end of 2016 (Mo 36).

Task 29.4 Communication effectiveness in behavioural change (led by AU, Athanasios Krystallis, prepared by Marija Banovic).

The main objective of this task is to explore communication effectiveness in relation to developed product prototypes (Task 29.2 and **Deliverable 29.4**) and optimal [product attributes x segments] combinations (Sub-tasks 29.1.1 to 29.3.2). Thus, the effects of different forms of label communication will be tested for their effectiveness on consumer buying intentions and willingness to pay. More specifically, this task examines if the communication parameters (*i.e.* message type, process, and source) have the ability to influence consumer value perceptions and cause attitudinal change, as well as purchasing intentions, willingness to pay and actual behaviour, thus causing behavioural change.

This task will include those communication parameters' combinations as experimental stimulus that will be used in the communication experiments in the five countries investigated (i.e. France, Germany, Italy, Spain, and the UK), resulting in Deliverable D29.7. Following, a second round of experimental set-ups with product samples similar to those from Sub-task 29.3.2 will be designed and implemented in an on-line survey, in order to test for communication effects. This will result in Deliverable D29.8 and report on the results of the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers' attitudes and behaviour.

Deviations from Annex I and their impact:

There were no deviations from Annex I in this WP.

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	P24. ITTICAL	P25. DOR	P28. CANEXMAR
P29. ASIALOR				
Lead Scientist preparing the Report (WP leader):	Edwin Nijssen			
Other Scientists participating:	Michel van der Borgh (P10), Lluis Guerrero (P3), Gemma Tacken (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 progress towards objectives and details for each task (13-30):

Task 30.1 Business models (led by TU/e, Edwin Nijssen; Michel van der Borgh).

Sub-task 30.1.1

According to the DOW, the value proposition for the producers and partners (involved SMEs) will be described and specified for specific customer segments targeted in close cooperation with the SMEs. The information gathered in Task 27.3, will be used as a basis. This will include quantitative elements of price and efficiency, and qualitative matters of overall customer experience and outcome (P10. TU/e, P6. DLO P12. APROMAR). This Sub-task will result in ***Deliverable D30.1 Report on value propositions for the producers and Partners*** and will be delivered in Mo 46 (September 2017).

Preparations (*e.g.* contacting partners and producers; detail data collection protocol; determining final products) for this sub-task start in Mo 34 (September 2016) and the data itself will be collected during a workshop with producers and partners (involved SMEs) in January 2017.

Sub-task 30.1.2

According to the DOW, the resources necessary to create value for the customer will be described. It identifies the partner network and determines the actual resources that are required in order to optimize operations and reduce risks of a business model. Guidelines for the organizations to cultivate buyer-supplier relationships will be developed. Complementary business alliances will be explored, including options for joint ventures to expand globally (P10. TU/e, P12. APROMAR). This will be finished in Deliverable D30.2 and will be delivered in month 46 (September 2017). Report on indications of resources for creating customer value for the specific products.

Preparations (*e.g.* contacting partners and producers; detail data collection protocol; determining final products) for this sub-task start in month 34 (September 2016) and the data itself will be collected during a workshop with producers and partners (involved SMEs) in January 2017.

Sub-task 30.1.3

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 (P10. TU/e, P12. 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 Revenue (pricing & costs structures) model per species*** will present the results of this Sub-task and will be delivered in Mo 48 (November 2017).

Preparations (e.g., contacting partners and producers; detail data collection protocol; determining final products) for this sub-task start in month 34 (September 2016) and the data itself will be collected during a workshop with producers and partners (involved SMEs) in January 2017.

Task 30.2 New product marketing strategy development (led by TU/e, Edwin Nijssen; Michel van der Borgh).

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).

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).

Preparations (e.g., identifying viable product solutions from WP28 & WP29; checking availability of product for actual market test; designing market test) for this sub-task start in month 34 (September 2016). Critical for this sub-task is the availability of fish product developed in WP28 and further tested in WP29. Discussion with partners and researchers from the Diversify project during the annual meeting in Nancy indicated that the availability of products is a potential bottleneck. As a consequence we need to consider alternative ways to conduct the envisioned market tests (e.g., simulated store tests). Discussions on alternative scenarios are currently on the way with DLO (P.6).

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). This task starts as soon as market projections are ready and financial insights can be given of production of the species. This is dependent on the technical tasks.

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).

Deviations from Annex I and their impact:

There were no deviations from Annex I in this WP.

WP31 Dissemination

WP No:	31	**WP Lead beneficiary:**		P18. CTAQUA
WP Title (from DOW):	Dissemination			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P7. IMR	P8. IEO
P9. UL	P12. APROMAR	P13. UNIBA	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), Javier Ojeda (P.12), Aldo Corriero (P.13), Laslo Varadi (P.35), Mathias Keller (P.34)			

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 previous 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 progress towards objectives and details for each task (13-30 Mo):

Task 31.1 Project website and brochure (led by CTAQUA, Rocio Robles).

WEBSITE

Although the Deliverables corresponding to this Task have been already reported in the previous reporting period (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***, the web page of the project has continued to be updated continually, providing essential information on project activities.

The structure of the website maintained its initial pages, although a new page named "Scientific articles" (http://www.diversifyfish.eu/scientific-articles.html) has been included, in order to adapt the web to the availability of scientific publications from the results of the research done in the different WPs of the project. This change of structure complies with the first objective of WP31, to Disseminate the knowledge acquired to the scientific community, in order to promote further research.

The website structure is as follows:

1. News: This page is updated often (monthly), 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. The most relevant results of the undergoing experiments from the different WPs are highlighted, as well as providing information and links to dissemination activities (conferences, presentations, news releases, videos, etc.).

2. Summary: The page remains as it was designed, including the full title of the project, a brief description of its objectives and contact details of the Project Coordinator (PC), geographical distribution of the partners, general structure of the work to be implemented (Pert diagram) and a list of the organization of the consortium and its governing bodies and research area groups (Management structure).

3. Partners: These page also remains as designed originally, providing the contact information of each partner and the Principal Investigator (PI), including the logo of the partner. During the second reporting period, one of the partners has exited the consortium of DIVERSIFY (Culmarex) and has been removed from the web site.

4. Species: The presentations given by the species leader at the DIVESIFY Special Session during the Aquaculture Europe conference in 2015 (AE 2015), are included in the sections of this page (**Figure 31.1.1.**). Each species leader presented a summary of the work carried out on each species: Dr. Alicia Estévez for meagre *Argyrosomus regius;* Dr. Nikos Papandroulakis for greater amberjack *Seriola dumerili*, Dr. Pascal Fontaine for pikeperch *Sander lucioperca,* Dr. Birgitta Norberg for Atlantic halibut *Hippoglossus hippoglossus*, Dr. Tito Peleteiro for wreckfish *Polyprion americanus* and Dr. William (Bill) Koven for grey mullet *Mugil cephalus*.

5. Research area: These pages are maintained with all the information on how the research tasks are designed to address the identified bottlenecks in each selected species including the six (6) disciplines of the project: Reproduction and genetics, Nutrition, Larval husbandry, Grow out husbandry, Fish health and Socioeconomics (which includes final product and quality).

6. Intranet: 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 31.1.2.**).

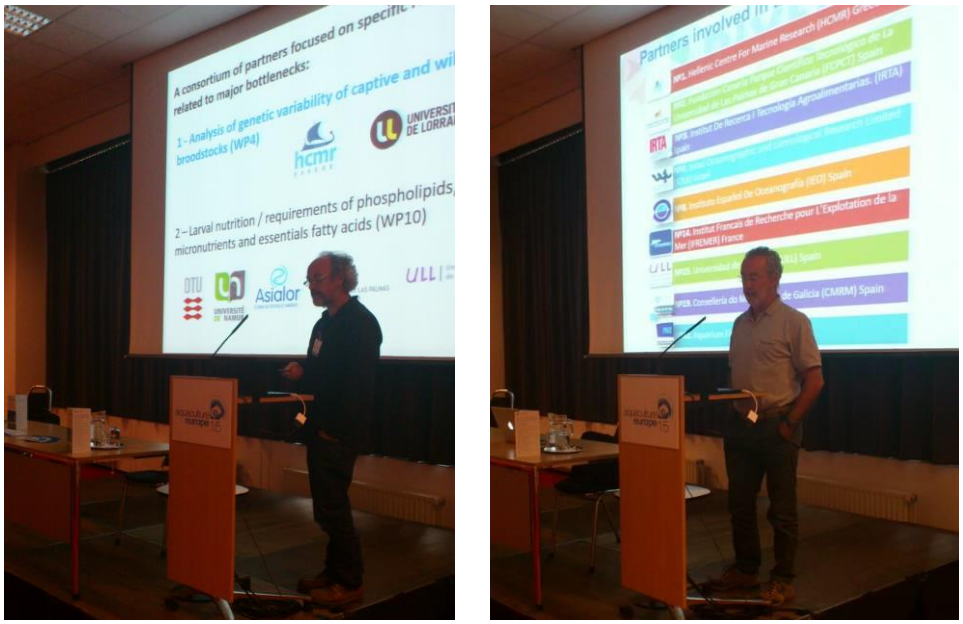


Figure 31.1.1. Pike perch leader (Dr. P. Fontaine) and wreckfish leader (Dr. T. Peleteiro) presenting the advances of the experiments during the DIVERSIFY Special Session at AE 2015, Rotterdam, Netherlands. These presentations available at http://www.diversifyfish.eu/pikeperch-sander-lucioperca.html and http://www.diversifyfish.eu/wreckfish-polyprion-americanus.html

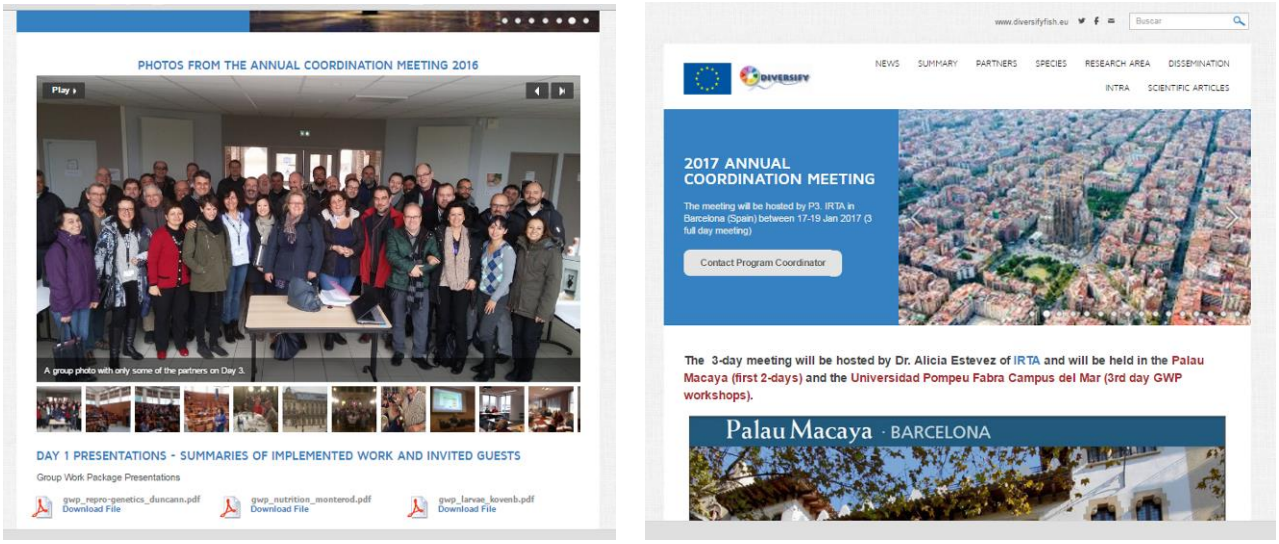


Figure 31.1.2. Desktop captures of the DIVERSIFY page "Intranet- Meetings &Activities", including the information of the past ACM 2016 held in Nancy, France, with the summary documents of the different sessions; besides the announcement of the next ACM 2017 to be held in Barcelona, Spain.

7. Dissemination: These pages keep being updated with all the dissemination activities done in the project. It includes the following sections:

- Newsletter: already four issues (**Figure 31.1.3**) have been published and are available at the website www.diversifyfish.eu/newsletter.html; the fifth one is in progress and will be uploaded in month 31.

- Articles in magazines and the internet with project information in other languages of the consortium; latest update includes the publication of an article about the project in the publication "Sustainable Aquaculture Magazine", The Fish Site, UK (http://www.thefishsite.com/focus/5m/264/sustainable-aquaculture-magazine)(**Figure 31.1.4**). Our P37. 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/).
- Scientific publications: in this section scientific publications related with the species and research areas of DIVERSIFY are included. A dedicated page to scientific articles produced within the frame of the project, has been included as separate page.
- Presentations and posters: all the presentations presented at the Special Diversify Session during AE 2015 in Rotterdam, Netherlands, have been included in this section (**Figures 31.3.5 to 31.3.7**).
- Photo gallery.

8. Scientific articles: this is a new page specially incorporated to present the scientific articles that are being produced in the frame of the project tasks. Five scientific articles have been published so far (**Figure 31.1.6**) and are included in the page. These publications are available online or by direct contact with the authors. At the time of the preparation of this report (July 2016), a total of 7 (seven) scientific articles in ISI-Index journals have been published and 1 (one) has been admitted, and these articles have been uploaded in the appropriate site of the ECAS site of the EU. More scientific results are expected to be published in the coming months, since several manuscripts are under preparation and two are under review (see later).

Figure 31.1.3. Slide presenting the four issues of the DIVERSIFY Newsletter, available at http://www.diversifyfish.eu/newsletter.html

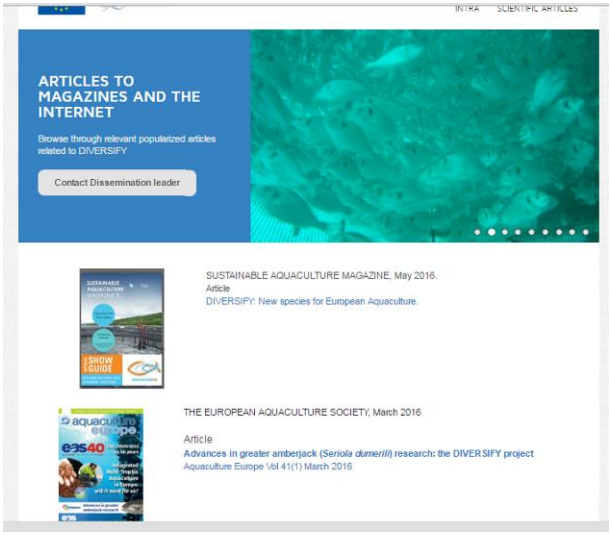


Figure 31.1.4. Desktop capture of the web page including the latest magazine articles published about DIVERSIFY.

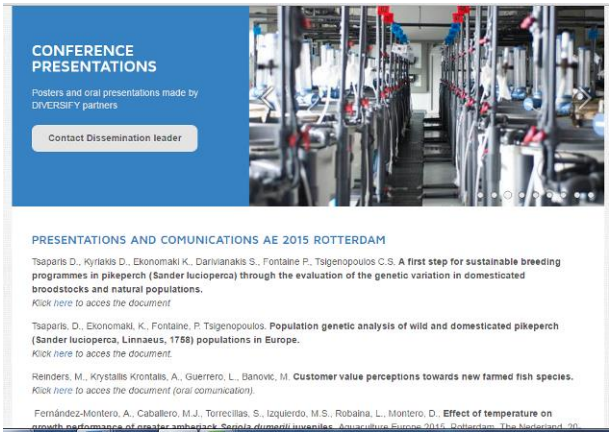


Figure 31.1.5. Desktop capture of the web page with the presentations at the aquaculture Europe 2015 conference in Rotterdam.

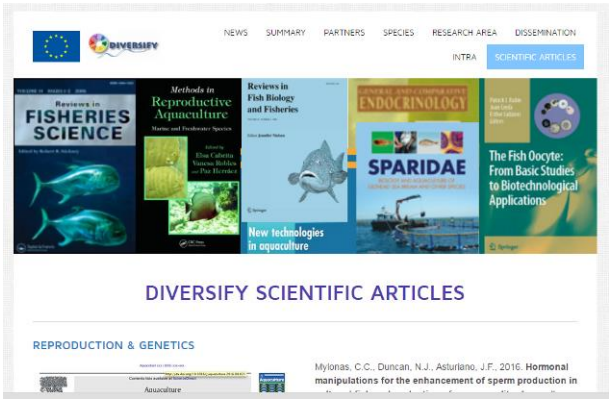


Figure 31.1.6. New page included in the web of DIVERSIFY, were all the scientific articles resulting from the project are being listed, with a copy of the first pages of the published article and a link to the corresponding author.

DIVERSIFY LOGO

The logo keeps being used in all the pages and subpages of the website, and is included in all the dissemination material produced within the project, in the profiles of the social networks, Facebook, twitter, in all dissemination material and as part of the signature of the partners. During the ACM 2016 in, 2016, an updated version of the logo has been presented (**Figure 31.1.6**), and was included in all the dissemination material of the project, such as the merchandising material (T-shirts). The updated logo includes a sentence summarizing the objective of the project: "New species for EU aquaculture".

Figure 31.1.6: The updated logo design of DIVERSIFY.

DIVERSIFY BROCHURE AND BOOKMARK

The project brochure and bookmark have been distributed with the occasion of several aquaculture conferences and events such as:

- EATIP (European Aquaculture Technological and Innovation Platform) Annual meeting, Brussels, May 2015.
- Aquaculture Round Table, Bonn, Germany, July 2015. Presentation of DIVERSIFY by P32 at the Aquaculture Round Table.
- Lecture event "Fisch-Forum" 2015, Hamburg, Germany.
- Sectorial conference ANFACO, Vigo Spain, September 201.
- AQUACULTURE EUROPE (AE 2015) conference (Rotterdam, Netherlands, October 2015), Special Diversify Session during the conference.
- ACM in Nancy, France 2016.
- TRAFOON project training workshops "Food quality and safety in aquaculture sector", Poland, April 2016.
- Offshore Mariculture Conference (Barcelona, Spain, April, 2016)
- Seafood Expo Global and Seafood Processing Global, (Brussels, Belgium, April).

PRODUCTION AND RELEASE OF DOWNLOADABLE AUDIOVISUAL MATERIAL

Several videos have been produced and released during 2015 and 2016. All the videos are included following a chronological order in the "News" page of the project website.

During February 2015, two videos have been uploaded in the web and are available at Youtube. 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 uploaded in February 2015, shows a sampling done by researchers from the P15. University of La Laguna (Tenerife), with two specimens of wreckfish at the facilities of Makro in La Laguna (Tenerife, Spain) (**Figure 31.1.7)**.

Figure 31.1.7. Desktop captures of the wreckfish sampling done by the researchers of University of La Laguna (P15. ULL).

In May 2015, a short video was recorded 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) (**Figure 31.1.8**). The video is also available in the project Youtube channel https://www.youtube.com/watch?v=W1G9hAk4x3E

Figure 31.1.8: Desktop captures of the evaluation of reproductive condition of greater amberjack broodstock specimens.

Also in July, another short video was released with an interview of the PC commenting on the progress and results of the project after the first 18 months. This video is also available in Youtube https://www.youtube.com/watch?v=SUba5STbFz8 .
In January 2016, a video compiling the presentations given during the Special Diversify Session at Aquaculture Europe 2015, was released (**Figure 31.1.9. to Figure 31.1.16.**). Major achievement within each Research Area and species were summarized in this production. The video is also available in Youtube https://www.youtube.com/watch?v=I_JTnaPskK8 .

Figure 31.1.9. Desktop capture of the first sequence of the Special DIVERSIFY Session.

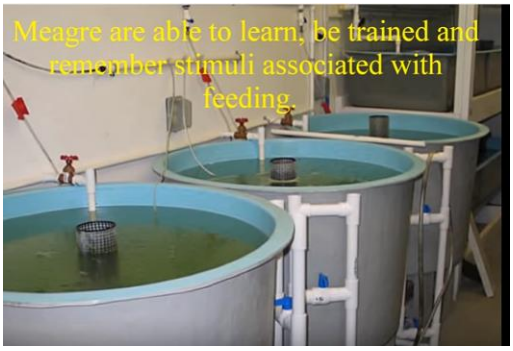


Figure 31.1.10. Desktop capture of a sequence with results from meagre.

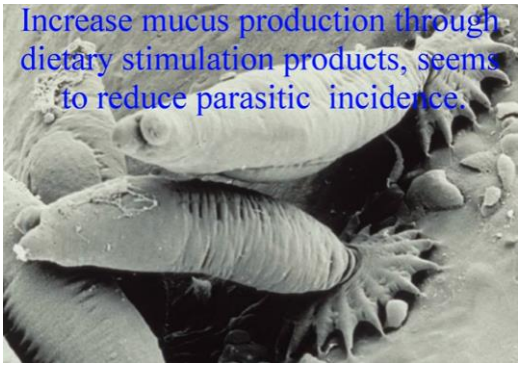


Figure 31.1.11. Desktop capture of a sequence showing greater amberjack findings.

Figure 31.1.12. Desktop capture of a sequence with results from pikeperch.

Figure 31.1.13. Desktop capture of a sequence with results from halibut.

Figure 31.1.14. Desktop capture of a sequence with results from Reproduction & Genetics of wreckfish.

Figure 31.1.15. Desktop capture of a sequence with Nutritional findings in grey mullet.

Figure 31.1.16. Desktop capture of a sequence with results from Socioeconomic Area with regard to New Product Development.

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. The video has been uploaded to the web and it is also available in Youtube https://www.youtube.com/watch?v=taHt2_dYYbQ (**Figure 31.1.17**).

Figure 31.1.17. Desktop capture of the initial sequence of the video presenting the standardization of sperm analysis.

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 **(Figure 31.1.18).**

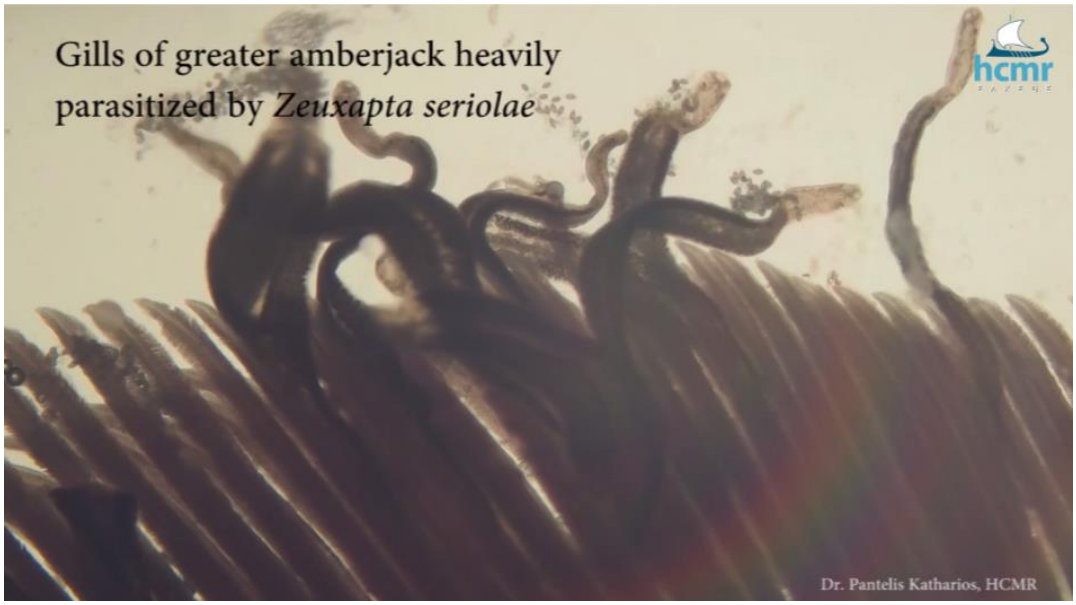


Figure 31.1.18. Desktop capture showing the gills of greater amberjack heavily infected by *Zeuxapta* seriolae gill parasite.

ADDITIONAL DISSEMINATION ACTIVITIES

During 2015 and the first six months of 2016, various dissemination activities were 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 2015-2016 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
58	09/01/2015	All	All	2 nd DIVERSIFY Newsletter	Web	English
59	10/01/2015	All	All	DIVERSIFY 2nd VIDEO	Web	English
60	16/01/2015	All	All	International Green Week 2015 in Berlin. Dr. Keller informed interested consumers about the project and distributed the flyer and the bookmarks. Berlín, Germany	Exhibition and distribution of dissemination material	German
61	26/01/2015	All	All	VIDEO: "Meagre work IRTA 2014"	Web	English
62	10/02/2015	All	All	Los primeros meros de acuicultura podrían llegar a los mercados en 10 años	Web	Spanish
63	10/02/2015	All	All	Los primeros meros de acuicultura podrían llegar a los mercados en 10 años	Web	Spanish
64	10/02/2015	All	All	Los primeros meros de acuicultura podrían llegar a los mercados en 10 años	Web	Spanish
65	10/02/2015	All	All	Los primeros meros de acuicultura podrían llegar a los mercados en 10 años	Web	Spanish
66	10/02/2015	All	All	Científicos viguenses creen que el mero de acuicultura puede llegar al mercado en una década	Web	Spanish
67	10/02/2015	All	All	Científicos de Vigo apuran la salida comercial del mero de acuicultura	Web	Spanish
68	10/02/2015	All	All	Científicos de Vigo apuran la salida comercial del mero de acuicultura	Web	Spanish
69	10/02/2015	All	All	El Aquarium Finisterrae participa en el proyecto "Diversify para impulsar la industria de la acuicultura en Europa"	Web	Spanish
70	10/02/2015	All	All	Las chernas del acuario coruñés promueve su inclusión en un proyecto europeo	Web	Spanish
71	10/02/2015	All	All	"Visita del equipo de investigadores del proyecto europeo Diversify, en el que participa Aquarium Finisterrae"	Web	Spanish
72	11/02/2015	All	All	"Farmed Atlantic wreckfish could reach the market in 10 years"	Web	English
73	11/02/2015	All	All	"Los primeros meros de acuicultura llegarán a los mercados en 10 años"	Web	Spanish
74	11/02/2015	All	All	"El Aquarium Finisterrae participa en el proyecto Diversify para impulsar la industria de la acuicultura europea"	Web	Spanish
75	20/02/2015	All	All	Wild wreckfish sampling	Web, video	English
76	01/03/2015	All	All	Advances in Meagre (Argyrosomus regius) research during the first year of the project Diversify	Magazine	English
77	26/03/2015	All	All	Perceived Consumer Value towards New Farmed Fish Species: A Psychographic Segmentation in top five EU Markets	Oral Presentation	English
78	28/04/2015	All	All	Presentation of Diversify and its 1st year progress EATIP	Oral Presentation	English
79	13/05/2015	All	All	Assessing genetic diversify in domesticated pikeperch (Sander lucioperca) broodstocks	Poster	English
80	14/05/2015	All	All	Lateral line ontogeny and chronic ulcerative dematopathy of meagre (A. regius)	Oral Presentation	English

81	14/05/2015	All	All	Fish reproduction dysfunctions. Manipulation of maturation in aquaculture fish. Dr. C. Mylonas	Conference	English
82	30/05/2015	All	All	DIVERSIFY. The fisrt year completed with success	Magazine	English
83	12/06/2015	All	All	Presentation of Diversify activities during the annual general assembly of the German federation for fish processirs and wholesalers	Oral Presentation	English
84	15/05/2015	All	All	Fish innovators: European consumers with an interest in farmed fish	Magazine	English
85	25/06/2015	All	All	Farmed fish a healthy and sustainable choice?	Web	English
86	15/07/2015	All	All	Presentation of Diversify at "Runder Tisch Aquakultur"	Oral Presentation	English
87	22/07/2015	All	All	Trasncriptome Characterization and Market Discovery in Meagre (A. regius)	Poster	English
88	26/07/2015	All	All	A first step for Sustainable Breeding Programmes In Pikeperch (Sander lucioperca) through the evaluation of the genetic variation in domesticated broodstocks and natural populations	Oral Presentation	English
89	23/08/2015	All	All	Comparison of CATA vs FCP in generating descriptive attributes with trained assessors	Poster	English
90	23/08/2015	All	All	Consumer beliefs regarding farmed versus wild fish: a cross- cultural perspective	Poster	English
91	31/08/2015	All	All	Interview with the Project Coordinator	Interview	English
92	08/09/2015	All	All	Advances in pikeperch (Sander lucioperca) research during the last 18 months of the project	Magazine	English
93	09/09/2015	All	All	Optimized spawning induction protocol for meagre (A. regius) using weekly GnRHa injections	Oral Presentation	English
94	08/10/2015	All	All	Presentación do proxecto da UE: Diversificación e potencial de cultivo de especies emerxentes na acuicultura europea: caso de cherna. Diversify.	Oral Presentation	Gallego
95	09/10/2015	All	All	Population genetic analysis of wild and domesticated pikeperch (Sander lucioperca) , Linneaeus, 1758) populations in Europe	Poster	English
96	12/10/2015	All	All	The DIVERSIFY project, Interview with Mrs Anneke Meyer, freelance journalist for German National Radio	Interview	English
97	13/10/2015	All	All	Monogeneos en el cultivo de Seriola dumerili en la región atlántica canaria	Poster	English
98	13/10/2015	All	All	Maduración sexual de tres stocks de cherna (Polyprion americanus) en Galicia	Poster	Spanish
99	21/10/2015	All	All	Progess in understanding the ontogeny of the immune system in meagre (A. regius) Results of the EU diversify Project in 2014 and 2015	Poster	English
100	21/10/2015	All	All	Biochemical composition of wild wreckfish (Polyprion americanus)	Poster	English
101	21/10/2015	All	All	Fish reproduction dysfunctions. Manipulation of maturation in aquaculture fish. Dr. C. Mylonas	Poster	English
102	21/10/2015	All	All	Evaluation of different feeding frequences in Seriola dumerili juveniles: effects on hematological and biochemical parameters	Poster	English
103	21/10/2015	All	All	Growth performanceof seriola dumerili juveniles fed on differente feeding frequences	Poster	English
104	21/10/2015	All	All	Solving bottlenecks in commercial production of Atlantic halibut (Hippoglossus hipoglossus L.)	Oral Presentation	English
105	21/10/2015	All	All	Biometric parameters of wild wreckfish (Polyprion americanus)	Poster	English
106	21/10/2015	All	All	Diversify brochures and bookmarks	Flyers	English
107	22/10/2015	All	All	Special Session: New/emerging finfish species	Conference	English
108	22/10/2015	All	All	Update on the first reporting period of the project diversify	Oral Presentation	English
109	22/10/2015	All	All	New advances in meagre (A. regius) culture. Results of the EU Diversify Project in 2014 and 2015	Oral Presentation	English
110	22/10/2015	All	All	The time is right for first product innovation: an exploration of European consumers attitudes towards sustainable new fish product ideas	Oral Presentation	English
111	22/10/2015	All	All	Customer value perceptions towards new farmed fish: A European consumer segmentation	Oral Presentation	English
112	22/10/2015	All	All	Greater Amberjack (Seriola dumerili) Aquaculture Advances in the frame of the diversify project	Oral Presentation	English
113	22/10/2015	All	All	Preliminary studies on rotifer enrichment of greater amberjack larviculture	Poster	English
114	22/10/2015	All	All	Fatty acid profile of wild greater amberjack female gonads from Mediterranean and Atlantic areas	Poster	English
115	22/10/2015	All	All	Induced spawning of paired meagre (A. regius) with male rotation: an approach to produce multiple full and half-sib families for genetic breeding programs	Oral Presentation	English
116	22/10/2015	All	All	Results for the first year of wreckfish (Polyprion americanus) culture	Oral Presentation	English
117	22/10/2015	All	All	FSH agonist: a missing therapeutic agent facilitating breeding for captive grey mullet (mugil cephalus) broodstocks	Oral Presentation	English
118	30/12/2015	All	All	DIVERSIFY AFTER 18 MONTHS	VIDEO	English

119	15/01/2016	All	All	DIVERSIFY 4th Newsletter	Newsletter	English
120	25/01/2016	All	All	Flyers sent to Trafoon Project Coordinator	distribution of dissemination material	English
121	25/01/2016	All	All	Flyers sent to Trafoon Project Fish WP leader	distribution of dissemination material	English
122	23/03/2016	All	All	Advances in greater amberjack (Seriola dumerili) research: the DIVERSIFY project	Magazine	English
123	06/04/2016	All	All	DIVERSIFY presentation at Offshore Mariculture Conference 2016	Presentation	English
124	06/04/2016	All	All	Flyers distributed at Offshore Mariculture Conference 2016	distribution of dissemination material	English
125	25/04/2016	All	All	Flyers distributed at the Seafood Expo Brussels organized in Brussels (May 2016).	distribution of dissemination material	English
126	27/04/2016	All	All	FISHBOOST-An EU 7FP project on breeding programs for EU aquaculture species. An invited presentation on DIVERSIFY and its status was given during the coordination meeting of FISHBOOST	Oral presentation	English
127	27/04/2016	All	All	Flyers distributed at FISHBOOST-An EU 7FP project on breeding programs for EU aquaculture species	distribution of dissemination material	English
128	09/05/2016	All	All	EMBO Practical Course on ''Computational Molecular Evolution''	distribution of dissemination material	English
129	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

PRESS RELEASES

Most of the press releases done during the second period were related to the research work performed with wreckfish in the Area of Reproduction & Genetics by the Insitituo Español de Oceanografia (P8. IEO). The press releases were made in Spanish (Fundación OESA, Faro de Vigo, La voz de Galicia, Noticias de Galicia; La opinion) and one of them in English for the internet site Fish Information and Services, www.fis.com (**Figure 31.1.19 to Figure 31.1.21**).

Figure 31.1.19: Desktop capture press release about DIVERSIFY project at Faro de Vigo www.farodevigo.es/mar/2015/02/10/cientificos-vigo-apuran-salida-comercial/1181724.html

Figure 31.1.20: Desktop capture press release about DIVERSIFY project at Fundacion OESA website www.fundacionoesa.es/noticias/los-primeros-meros-de-acuicultura-podrian-llegar-a-los-mercados-en-10-anos

Figure 31.1.21: Desktop capture press release about DIVERSIFY project at FiS http://fis.com/fis/worldnews/worldnews.asp?monthyear=&day=11&id=74532&l=e&special=&ndb=1%20target=

DIVERSIFY is also present in the social media, via a twitter account and facebook account. Twitter account counts with 206 followers (May 2016).

ARTICLES

Three feature articles have been published by the European Aquaculture Society during 2015 and 2016. 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. Following this plan, in the 1st Semester issue of 2015, the research done with meagre was published, describing the progress during the first year of the project in all the Research Areas of the species **(Figure 31.1.22).** In the second semester, the feature article was devoted to pike perch (**Figure 31.1.23)** and in the first semester publication of 2016, greater amberjack has been the species addressed (**Figure 31.1.24).**

The latest article about DIVERSIFY has been published in the Sustainable Aquaculture Magazine, digital magazine from The FishSite (**Figure 31.1.25**).

Figure 31.1.22: Cover page of the article published about meagre in the European Aquaculture Society magazine. Aquaculture Europe vol. 40 (1) March 2015.

Figure 31.1.23: Cover page of the article published about pike perch in the European Aquaculture Society magazine. Aquaculture Europe vol. 40 (2) September 2015.

Figure 31.1.24: Cover page of the article published about pike perch in the European Aquaculture Society magazine. Aquaculture Europe vol. 41 (1) March 2016.

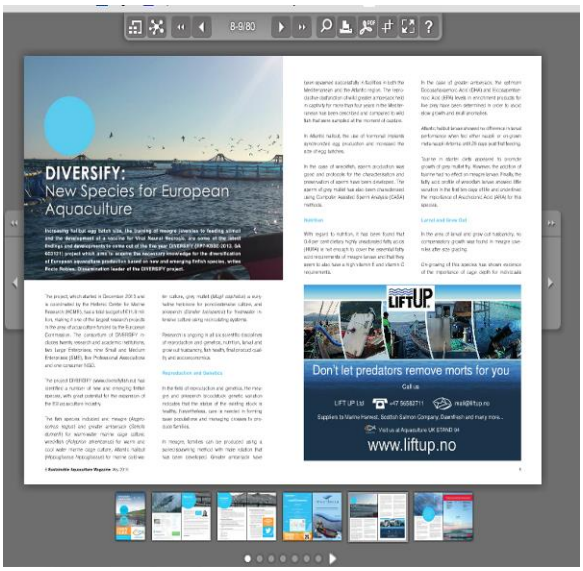


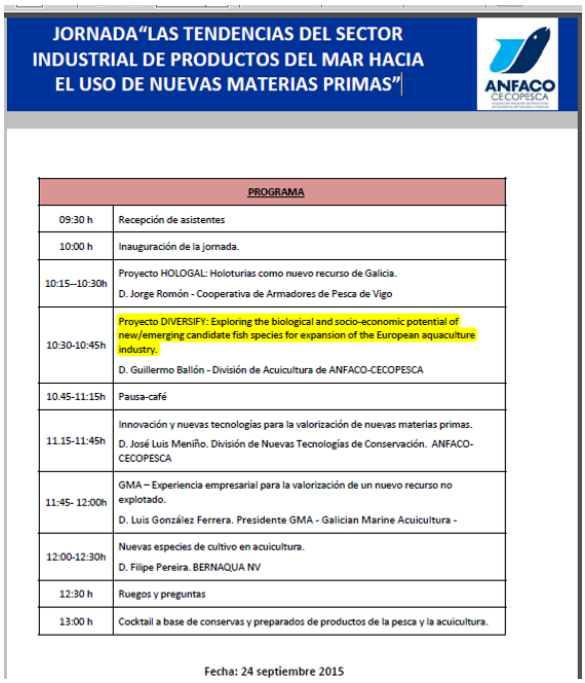
Figure 31.1.25: Desktop capture of the article about DIVERSIFY published in "Sustainble Aquaculture Magazine", The FishSite www.thefishsite.com/focus/5m/264/sustainable-aquaculture-magazine

WORKSHOPS

On April 28, 2015, the PC and Dissemination leader attended the Annual meeting of the European Aquaculture Technology & Innovation Platform (EATIP) held in Brussels, to present the project to the members of this platform. A summary of the progress and achievements of the project during its first year was presented.

In June, 2015, Dr. Matthias Keller from P34. BVFi (Bundesverband der Deutschen Fischindustrie und des Fischgrosshandels E.V.), presented DIVERSIFY to the members of the German federation of fish processors and wholesalers (Hamburg, Germany), during their annual general assembly. Later on, in July 2015, the same partner introduced DIVERSIFY during the celebration of the Aquaculture Round Table, Bonn, Germany. Dr. Keller (**Figure 31.1.26**) presented the most relevant results of the project after one year, with special emphasis on the work packages related to the socio-economic dimension of DIVERSIFY. Results obtained from WP28 related to consumer perception of aquaculture products were presented at the meeting.

Figure 31.1.26. Presentation of DIVERSIFY by P32 at the Aquaculture Round Table.



JORNADA "LAS TENDENCIAS DEL SECTOR INDUSTRIAL DE PRODUCTOS DEL MAR HACIA EL USO DE NUEVAS MATERIAS PRIMAS"

ANFACO CECOPESCA

PROGRAMA	
09:30 h	Recepción de asistentes
10:00 h	Inauguración de la jornada.
10:15--10:30h	Proyecto HOLOGAL: Holoturias como nuevo recurso de Galicia. D. Jorge Romón - Cooperativa de Armadores de Pesca de Vigo
10:30-10:45h	Proyecto DIVERSIFY: Exploring the biological and socio-economic potential of new/emerging candidate fish species for expansion of the European aquaculture industry. D. Guillermo Ballón - División de Acuicultura de ANFACO-CECOPESCA
10.45-11:15h	Pausa-café
11.15-11:45h	Innovación y nuevas tecnologías para la valorización de nuevas materias primas. D. José Luis Meniño. División de Nuevas Tecnologías de Conservación. ANFACO-CECOPESCA
11:45- 12:00h	GMA – Experiencia empresarial para la valorización de un nuevo recurso no explotado. D. Luis González Ferrera. Presidente GMA - Galician Marine Acuicultura -
12:00-12:30h	Nuevas especies de cultivo en acuicultura. D. Filipe Pereira. BERNAQUA NV
12:30 h	Ruegos y preguntas
13:00 h	Cocktail a base de conservas y preparados de productos de la pesca y la acuicultura.

Fecha: 24 septiembre 2015

In September 2015, P36. ANFACO presented DIVERSIFY during a Sectorial Workshop organized in Spain, entitled "Towards the use of new raw materials in the industrial sector of products of the sea". The DIVERSIFY project was also presented to the sector during this event (**Figure 31.1.27**).

Figure 31.1.27. Program of the sectorial workshop organized by ANFACO (P36) in September 2015; DIVERSIFY project was presented to the sector during the conference.

The DIVERSIFY project has been presented also during the 6th Offshore Mariculture Conference, held in Barcelona on 6-8 of April 2016. The PC Dr. CC Mylonas (**Figure 31.1.28.**) gave a talk introducing the project and presenting a summary of the latest achievements.

Figure 31.1.28. The Project Coordinator presenting the DIVERSIFY project at the 6th Offshore Mariculture Conference, held in Barcelona on 6-8 of April 2016.

During May 2016, Mr Javier Ojeda from P12. APROMAR, participated in the Seafood Expo Global held in Brussels, Belgium (**Figure 31.1.29.**), with a stand in which DIVERSIFY brochures and bookmarks were displayed and distributed to the attendees of the exposition and providing information about the project status.

Figure 31.1.29. The APROMAR stand displaying the brochure and bookmark of DIVERSIFY at the Seafood Expo Brussels organized in Brussels (May 26-28, 2016) and Javier Ojeda providing explanations about the project to an attendant.

Task 31.2 Annual Coordination Meetings (led by HCMR, Constantinos Mylonas).

The full description of the work and results of the ACM 2014 has been submitted as ***Deliverable D1.3 Annual Coordination Meeting for Y2***.

The full description of the work and results of the ACM 2016 has been submitted as ***Deliverable D1.6 Annual Coordination Meeting for Y3***. A brief presentation is also provided in the following pages.

The ACM 2016 was hosted by Dr. Pascal Fontaine from the University of Lorraine (P9. UL) and was held at the Museum-Aquarium of Nancy (Day 1) and the Brabois Campus of the University of Lorraine (Day 2 & 3) on 2-4 February 2016 (Mo 27). The 3-day meeting was attended by 87 persons: 78 coming from the DIVERSIFY consortium and 9 invited guests from outside the consortium. No representative attended from three Beneficiaries (P26. GEI, P28. CANEXMAR and P37. EUFIC). Beneficiary P10. TU/e was unable to attend the first day of the meeting, but attended the second and third day.

As for the previous meetings, information regarding the meeting was uploaded continually on the project's web site (www.diversifyfish.eu/INTRA/Meetings & Activities/2016 Annual Coordination Meeting) to ensure that all participants had access to the most updated information. The Agenda (**Table 31.2.1**) was developed with assistance from GWP leaders and consisted of:

(a) DAY 1: a common session for all participants during DAY 1 (including invited guests) presenting summaries of the work implemented in all six Scientific Disciplines, specific presentations from various WPs or tasks, and presentations from invited guests,
(b) DAY 2: Six (6) Scientific Discipline-specific workshops running in three parallel sessions during DAY 2, and
(c) DAY 3: a common session dealing with Dissemination, Scientific and Financial Reporting, and Management. A meeting of the Steering Committee was also held at the end of the ACM. In addition, a special 2-hour meeting was held with all the Partners being involved in work with greater amberjack (*Seriola dumerili*) under any Scientific Discipline, in order to address some issues related to the grow-out experiments.

The morning session started with a welcoming presentation by the PC, 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. The invited guests included Dr. Pierrick Haffray and Mrs Anastasia Bestin from the Syndicat des Sélectionneurs Avicoles et Aquacoles Français (SYSAF, an animal breeding company), Dr. Joao Correia and Mr. Mauricio Francisco from Flying Sharks (a fish capture and transport company), the secretary of the European Aquaculture Society Dr. Alistair Lane, Prof. Jonna Tomkiewicz from the Danish Technical University and members of the technical staff of four aquaculture production companies (**Andromeda SA** from Greece/Spain, **Le Poisson du Soleill** from France, **Isidro de la Cal** from

Spain and **Galaxidi SA** from Greece). As for the previous ACM 2014, the attendance of a number of researchers and producers from outside the consortium allowed the prompt and effective dissemination of DIVERSFY results to relevant stakeholders.

The presentations from the invited guests, 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. Pierrick Haffray (SYSAF, France) on the development of breeding programmes in aquaculture fish and of Prof. Jonna Tomkiewicz (DTU, Denmark) on the breeding and larval rearing research of Atlantic eel (*Anguilla anguilla*). Both presentations are extremely relevant to work undertaken in DIVERSIFY and we hope that we will establish further contacts with these researchers. The participation of four 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. All presenting partners and invited guests agreed to have the presentations of the ACM 2016 available for the wider public, and they have already been uploaded on the website of the project, and are available to all interested stakeholders.

Table 31.2.1. Agenda of DAY 1 of the Annual Coordination Meeting 2016, which took place on the 2-4 February 2016 (Mo 27), at the Museum-Aquarium of Nancy, Nancy, France.

DAY 1		2 Feb	Tuesday (Open Day & Summary presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration	U of Lorraine Staff	Register, receive badge, submit presentations
9.00	9.30		Welcoming	Constantinos Mylonas (HCMR), Pascal Fontaine (UL)	Meeting logistics, agenda, welcoming from UL
9.30	10.00		GWP presentation – Repro & Genetics	Neil Duncan (IRTA)	
10.00	10.15		Dysfunctional reproductive maturation in captive greater amberjack	Aldo Corriero (UNIBA)	Reproduction & Genetics
10.15	10.30		Population genetic analysis of wild and domesticated pikeperch populations and their application to future breeding programs	Costas Tsigenopoulos (HCMR)	Reproduction & Genetics
10.30	11.00		GWP presentation – Nutrition	Daniel Montero (FCPCT)	
11.00	11.30	coffee			
11.30	12.00		GWP presentation – Larval husbandry	Bill Koven (IOLR)	
12.00	12.15		The nutrient profile of Artemia is greatly improved by ongrowing nauplii for 3 days on Dri-Green	Kristin Hamre (NIFES)	Larval rearing
12.15	12.30		First larval rearing efforts with wreckfish	Tito Peleteiro-Nikos Papandroulakis-Antonio Vilar	Larval rearing
12.30	13.00		GWP presentation – Grow out husbandry	Nikos Papandroulakis (HCMR)	
13.00	14.00	Lunch	Lunch at "Restaurant Cesar" at Place Stanislas		
14.00	14.15		The effect of different stimuli on meagre feeding behaviour	Ioannis Papadakis (HCMR)	Grow out husbandry
14.15	14.30		Multifactorial approach to identify rearing conditions optimising growth, physiological status and immune defense in pikeperch	Patrick Kestemont (FUNDP)	Grow out husbandry
14.30	15.00		GWP presentation – Fish Health	Chris Secombes (UNIABDN)	
15.00	15.15		Pathologies of fish not included in the DIVERSIFY DOW	Pentelis Katharios	Fish health
15.15	15.45		GWP presentation – Socio economics	Gemma Tacken (LEI/DLO)	
15.45	16.00		Consumer value perceptions and attitudes towards farmed fish products in top-five EU markets	Marija Banovic and Thanassis Krystallis (AU)	Socieconomics
16.00	16.30	coffee			
16.30	16.45		Selection of new products and product development	Kriton Grigorakis (HCMR)	Socieconomics
16.45	17.00		Breeding selection in aquaculture fishes, with emphasis on the meagre	Pierrick Hafray, SYSAFF	Invited guest
17.00	17.15		Capture of wild fish for aquaria and research	Joao Coreia (Flying Sharks)	Invited guest
17.15	17.30		European eel breeding, larval culture and first-feeding attempts	Jonna Tomkiewicz (DTU)	Invited guest
17.30	17.45		Greeting from the EU Officer of DIVERSIFY	Marta Iglesias (EU DG RTD)	EU Scientific Officer
17.45	18.00		Wrap up	Constantinos Mylonas (HCMR)	Agenda for next day
18.00	19.00		Visit the Aquarium	Pascal Fontaine	
20.00			Dinner at "Grande Salons Hotel De Ville, Stanislas Place"		

Task 31.3 Presentation of DIVERSIFY at the AQUA EUROPE meetings (led by HCMR, Constantinos Mylonas).

A presentation (20 min) was given by the PC in the Diversification Session, at the AQUACULTURE EUROPE 2014 conference held between 14-17 October 2014 at Donostia-San Sebastian, Spain. Coincidentally, Drs. Tito Peleteiro from P8 IEO and Rocio Robles from P18 --the WP31 Dissemination leader-- chaired the session. Since this was the first of such presentations and a limited number of results was available at the time, it was decided to make an introductory presentation of DIVERSIFY, familiarizing the aquaculture community with the project. An estimated 120 persons attended the presentation.

The objective of the project were presented, together with the justification of the project's species selection, which includes 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 euryhaline herbivore for pond/extensive culture, and pikeperch (*Sander lucioperca*) for freshwater intensive culture using recirculating systems (**Fig. 31.3.1**).

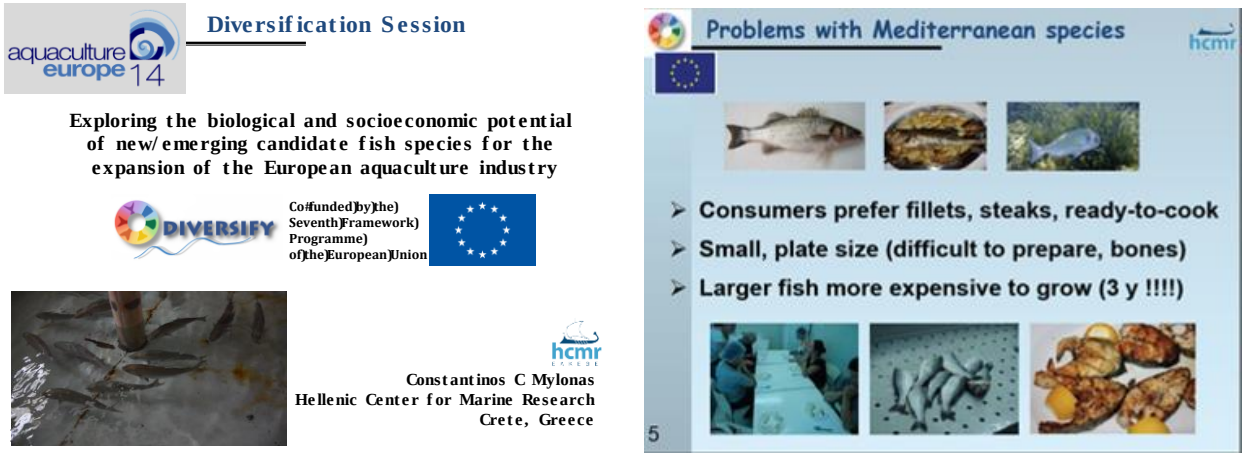


Figure 31.3.1. Representative slides of the DIVERSIFY presentation at AQUACULTURE EUROPE 2014, with the opening slide (left) and the justification of the species selection of the project (right).

Then there was a brief description of the identified bottlenecks of each of the selected species, and a brief outline of the work planned in the six scientific disciplines, which are Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health and Socioeconomics, including final product quality. Afterwards, a presentation of the website (www.diversifyfish.eu) and its organization was provided, encouraging people to seek information on the project, either according to species of interest or of scientific discipline (**Fig. 31.3.2**). At the end of the presentation, the PC informed the audience about the next Annual Coordination Meeting and the possibility to attend the open day of the meeting, for interested researchers and industry managers. The attendees were also handed out the DIVERSIFY brochure and bookmark produced recently to introduce the project (**Fig. 31.3.2**). Brochures were also made available to all conference participants at specific literature distribution tables and relevant sessions. Also, the PC and the WP31 Leader handed out DIVERSIFY brochures to researchers and industry managers with whom they discussed the project and the potential for dissemination and collaboration with other National initiatives.

Figure 31.3.2. Representative slides of the DIVERSIFY presentation at AQUACULTURE EUROPE 2014, with the project's webpage (left) and the brochure produced and distributed to the conference at large (right).

The full description of the DIVERSIFY activities at the AQUACULTURE EUROPE 2014 conference has been submitted as ***Deliverable D31.6 Annual presentation of DIVERSIFY (Y1) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator***.

A Special Session was organized at the AQUACULTURE EUROPE 2015 conference held between 20-23 October 2015 at Rotterdam, The Netherlands (**Fig. 31.4.1**), focusing on the work carried out in the last 2 years in the DIVERSIFY project. The session was chaired by the PC of DIVERSIFY and the WP31 Dissemination leader. As committed in the DOW, summary presentations (20 min) were given by each of the six Species Leaders (SL) of DIVERSIFY including the 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 euryhaline herbivore for pond/extensive culture, and pikeperch (*Sander lucioperca*) for freshwater intensive culture using recirculating systems.

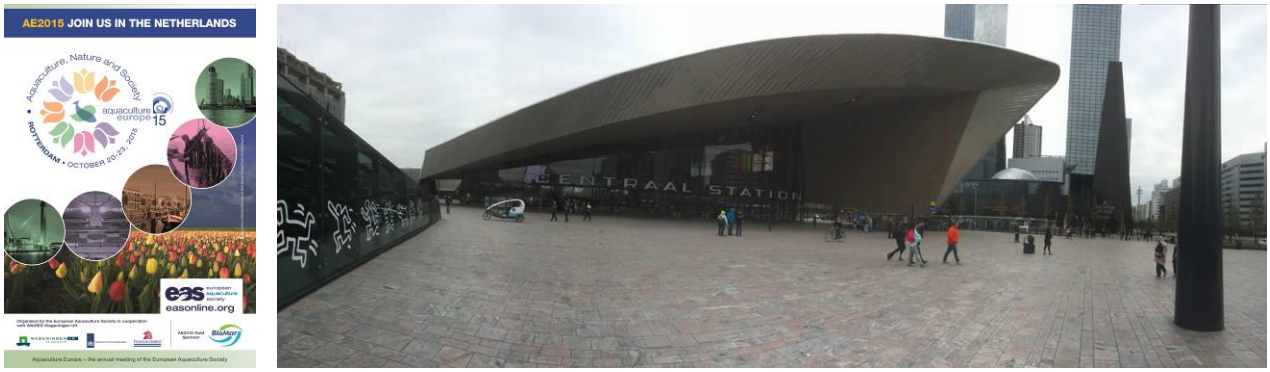


Figure 31.4.1. The announcement of the AQUACULTURE EUROPE 2015 conference that is organized every year by the European Aquaculture Society, and a panoramic view of Rotterdam, The Netherlands.

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 of DIVERSIFY, given by the PC of the project **-see *Deliverable 31.9 Annual presentation of DIVERSIFY (Y2) at a relevant conference***. Following each of the six Species Leaders summary presentations,

presentations were also given by DIVERSIFY researchers on specific Tasks of the DOW. The Special Session lasted for the whole day (10:30 to 17:00) and an estimated of 30-120 persons were present at the different presentations in the designated room. Overall, this Special session demonstrated that significant progress has been achieved in the study of new/emerging species for the EU aquaculture industry. The six Species Leaders from the DIVERSIFY project (**Fig. 31.4.2, right**) have contributed immensely to the success of this Special Session, by presenting all the work relevant to the diversification of the European aquaculture industry.

16.50 **Shawn Robinson**, K. P. Ang, A. Buschmann, T. B. R. Chopin, B. A. MacDonald, G. R. Reid
GAZING INTO THE CRYSTAL BALL FOR AQUACULTURE DEVELOPMENT: WHAT IS IN THE GLOBAL FUTURE FOR INTEGRATED MULTI-TROPHIC AQUACULTURE (IMTA)?

17.10 **Thierry Chopin**, Stacy Murray, Hamid Khoda Bakhsh, Ellen Belyea, Adrian Hamer, Miko Leavitt, Jake Ettlott, Cory Taylor, Mitchell Dickie
FIMTA + MIMTA = going ETP all the way with IMTA!

THURSDAY

NEW/EMERGING FINFISH SPECIES (EU DIVERSIFY PROJECT)
Thursday, October 22 10.30 - 17.30 Weelde Room
Chairs: Constantinos Mylonas, Rocio Robles

10.30 **Constantinos C. Mylonas**, Rocio Robles
UPDATE ON THE FIRST REPORTING PERIOD OF THE PROJECT "DIVERSIFY" (7FP-KBBE-GA 603121): EXPLORING THE BIOLOGICAL AND SOCIO-ECONOMIC POTENTIAL OF NEW/EMERGING CANDIDATE SPECIES FOR THE EXPANSION OF THE EUROPEAN AQUACULTURE INDUSTRY

10.50 **Estevez Alicia**, Neil Duncan, Cindy Campoverde, Juan Manuel Afonso, Costas Tsigenopoulos, Contastinos Mylonas, Lidia Robaina, Marisol Izquierdo, Nikos Papandroulakis, Karl Andree, Ana Roque, Pantelis Katharios, Stavros Chatzifotis, Maria Ioanna Tsertou
NEW ADVANCES IN MEAGRE *Argyrosomus regius* CULTURE: RESULTS OF THE EU DIVERSIFY PROJECT IN 2014 AND 2015

11.10 **Neil Duncan**, Constantinos C. Mylonas, Ioannis Fakriades, Edwards Milton Sutton, Zohar Ibarra-Zatarain, Marco Chiumento, Olinser Aviles Carrillo
INDUCED SPAWNING OF PAIRED MEAGRE *(Argyrosomus regius)* WITH MALE ROTATION: AN APPROACH TO PRODUCE MULTIPLE FULL AND HALF-SIB FAMILIES FOR GENETIC BREEDING PROGRAMS

11.30 **Nikos Papandroulakis**, Marisol Izquierdo, Maria Carmen Hernández-Cruz, Hippolito Fernández-Palacios, Aldo Corriero, Salvador Jerez, Covadonga Rodriguez, Chris Secombes, Pantelis Katharios, Constantinos C. Mylonas
GREATER AMBERJACK *Seriola dumerili* AQUACULTURE ADVANCES IN THE FRAME OF THE DIVERSIFY PROJECT

11.50 **Alvaro Fernández-Montero**, Maria Jose Caballero, Silvia Torrecillas, Marisol Izquierdo, Lidia Robaina, Daniel Montero
EFFECT OF TEMPERATURE ON GROWTH PERFORMANCE OF GREATER AMBERJACK *Seriola dumerili* JUVENILES

12.10 **Fontaine Pascal**, Ivar Lund, Robert Mandiki, Costas Tsigenopoulos, Enric Gisbert, Marirol Izquierdo, Fabrice Teletchea, Patrick Kestemont
REDUCTION OF MAJOR BOTTLENECK IMPACTS TO SUSTAIN THE INTENSIVE CULTURE OF PIKEPERCH *Sander lucioperca*

35

Figure 31.4.2. The Aquaculture Europe 2015 program page showing the Special Session (left) and the six Species Leaders (right). From left to right, Papandroulakis, N. (greater amberjack, P1. HCMR), Estevez, A. (meagre, P3. IRTA), Norberg, B. (Atlantic halibut, P7. IMR), Koven, W. (grey mullet, P4. IOLR), Fontaine, P. (pikeperch, P9. UL) and Peleteiro, J.B. (wreckfish, P8. IEO).

The full description of the DIVERSIFY activities at the Aquaculture Europe 2015 conference has been submitted as ***Deliverable D31.10 Annual presentation of DIVERSIFY (Y2) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator***.

Task 31.4 Scientific presentations and submission of manuscripts (led by HCMR, Constantinos Mylonas).

A total of 44 **scientific presentations** have been made during the 2nd Reporting Period (**Table 31.4.1**). Most of the presentations are related to the results and advances of the work done in the Research areas of Reproduction & Genetics, Nutrition and Socio-economics. It is expected that in the coming months more presentations will be done as more experimental results will become available, especially from the discipline areas of Larval husbandry, Grow out husbandry and Fish Health.

Table 31.4.1. List of scientific presentations done during the second reporting period of DIVERSIFY project.

#	Main leader	Title	Date	Place
1	P3. IRTA	Advances in Meagre (*Argyrosomus regius*) Research during the first year of the project DIVERSIFY	01/03/2015	"Aquaculture Europe" magazine of the European Aquaculture Society, Vol 40, March 2015
2	P11. AU	Perceived Consumer Value towards New Farmed Fish Species: A Psychographic Segmentation in Top-five EU Markets.	26/03/2015	143rd European Agricultural Economics Association (EAAE) Seminar, Napoli, Italy
3	P1. HCMR	Presentation of DIVERSIFY and its 1st year progress.	28/04/2015	EATiP Annual General Meeting, Brussels, Belgium
4	P1. HCMR	Assessing genetic diversity in domesticated pikeperch (*Sander lucioperca*) broodstocks.	13/05/2015	11th Panhellenic Symposium of Oceanography & Fisheries,Mytilene, Greece - http://www.symposia.gr/en/
5	P1. HCMR	Lateral line ontogeny and chronic ulcerative dermatopathy of meagre (*Argyrosomus regius* (Asso, 1801)).	14/05/2015	11th Oceanography & Fisheries Conference, Mitiline, Greece
6	P15. ULL	Fish reproduction dysfunctions. Manipulation of maturation in aquaculture fish.	14/05/2015	Faculty of Sciences (La Laguna University)
7	P3. IRTA	Fish meal substitution in diets for flathead grey mullet (*Mugil cephalus*) fry.	12/06/2015	Avenços en Recerca en Aqüicultura, Institut de Estudis Catalans, Barcelona, Spain
8	P1. HCMR	Transcriptome Characterization And Marker Discovery In Meagre *Argyrosomus regius*.	22/07/2015	ISGA XII - The International Symposium On Genetics In Aquaculture, Santiago De Compostela, Spain
9	P1. HCMR	A First Step For Sustainable Breeding Programmes In Pikeperch (Sander lucioperca) Through The Evaluation Of The Genetic Variation In Domesticated Broodstock And Natural Populations.	26/07/2015	ISGA XII - The International Symposium On Genetics In Aquaculture, Santiago De Compostela, Spain
10	P3. IRTA	Comparison of CATA vs FCP in generating descriptive attributes with trained assessors.	23/08/2015	11th Pangborn Sensory Science Symposium, Gothenburg, Sweden
11	P3. IRTA	Consumer beliefs regarding farmed versus wild fish: a cross-cultural perspective.	23/08/2015	11th Pangborn Sensory Science Symposium, Gothenburg, Sweden

12	P9. UL	Advances in pikeperch (*Sander lucioperca*) research during the last 18 months of the project	08/09/2015	"Aquaculture Europe" magazine of the European Aquaculture Society, Vol 40 (2), September 2015
13	P1. HCMR	Optimized spawning induction protocol for meagre (*Argyrosomus regius*) using weekly GnRHa injections.	09/09/2015	5th International Workshop on the Biology of Aquatic Gametes, Ancona, Italy
14	P19. CMRM	"Presentación do proxecto da UE: Diversificación e potencial de cultivo de especies emerxentes na acuicultura europea: caso da cherna. Diversify".	08/10/2015	Foro Recursos Mariños, O Grove (Pontevedra) Spain
15	P1. HCMR	Population genetic analysis of wild and domesticated pikeperch (*Sander lucioperca*, Linnaeus, 1758) populations in Europe.	09/10/2015	13th International Congress on the Zoogeography and Ecology of Greece and Adjacent Regions, Irakleio
16	P8. IEO	Monogeneos en el cultivo de *Seriola dumerili* en la región atlántica canaria.	13/10/2015	XV Congreso Nacional y I Congreso ibérico de Acuicultura. Huelva (Spain)
17	P8. IEO	Maduración sexual de tres stocks de cherna (*Polyprion americanus*) en Galicia.	13/10/2015	XV Congreso Nacional y I Congreso ibérico de Acuicultura. Huelva (Spain)
18	P3. IRTA	Progress in understanding the ontogeny of the immune system in meagre (*Argyrosomus regius*). Results of the EU diversify Project in 2014 and 2015.	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
19	P19. CMRM	Biochemical composition of wild wreckfish (*Polyprion americanus*).	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
20	P3. IRTA	Fish meal substitution in diets for flathead grey mullet *Mugil cephalus* Fry	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
21	P8. IEO	Evaluationof different feeding frquencies in *Seriola dumerili* juveniles: effects on hematological and biochemical parameters.	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
22	P8. IEO	Growht performance of *Seriola dumerili* juveniles fed on different feeding frequencies.	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
23	P7. IMR	Solving bottlenecks in commercial production of Atlantic halibut (*Hippoglossus hippoglossus* L.): The Diversify Project.	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)

24	P8. IEO	Biometric parameters of wild wreckfish (*Polyprion americanus*).	21/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
25	P1. HCMR	Special Session: New/emerging finfish species (the EU DIVERSIFY project)	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
26	P1. HCMR	Update on the first reporting period of the project DIVERSIFY: Exploring the biological and socio-economic potential of new/emerging candidate species for the expansion of the European aquaculture industry.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
27	P3. IRTA	New advances in meagre (*Argyrosomus regius*) culture. Results of the EU Diverisfy Project in 2014 and 2015.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
28	P11. AU	The time is right for fish product innovation: an exploration of European consumers' attitudes towards sustainable new fish product ideas.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
29	P6. DLO	Customer value perceptions towards new farmed fish: A European consumer segmentation.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
30	P1. HCMR	Greater amberjack (*Seriola dumerili*) aquaculture advances in the frame of the DIVERSIFY project.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
31	P15. ULL	Preliminary studies on rotifer enrichment for the improvement of greater amberjack larviculture.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
32	P15. ULL	Fatty acid profile of wild greater amberjack female gonads from Mediterranean and Atlantic areas.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
33	P3. IRTA	Induced spawning of paired meagre (*Argyrosomus regius*) with male rotation: an approach to produce multiple full and half-sib families for genetic breeding programs.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
34	P8. IEO	DIVERSIFY: Results for the first year of wreckfish (*Polyprion americanus*) culture.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
35	P4. IOLR	FSH agonist: a missing therapeutic agent facilitating breeding for captive grey mullet (*Mugil cephalus*) broodstock.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)

36	P1. HCMR	Evaluatio of the genetic variation in domesticated broodstocks and wild populations of pikeperch (*Sander lucioperca*) in Europe as a tool for future breeding programs.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
37	P16. FUNDP	Impact of dietary L-trytophan on neurophysiologicla and immune responses of pikeperch to emersion stress.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
38	P9. UL	Reduction of major bottlenecks impacts to sustain the intensive culture of pikeperch (*Sander luciperca*).	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
39	P9. UL	Effects of four environmental factors on the survival and development of the larvae of pike perch (*Sander lucioperca*).	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
40	P4. IOLR	First summary of studies carried our on the grey mullet (*Mugil cephalus*) in the EU project: DIVERSIFY.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
41	P3. IRTA	P aired induced spawning of meagre (*Argyrosomus regius*) with male rotation: an approach to produce families for genetic breeding programs.	22/10/2015	Aquaculture Europe 2015 (EAS conference), Rotterdam (Netherlands)
42	P1. HCMR	Advances in greater amberjack (*Seriola dumerili*) research: the DIVERSIFY project	23/03/2016	"Aquaculture Europe" magazine of the European Aquaculture Society, Vol 41(1), March 2016
43	P1. HCMR	DIVERSIFY: an EU project exploring the biological and socio-economic potential of new/emerging candidate finfish species for the expansion of the European aquaculture industry.	06/04/2016	Offshore Mariculture Conference 2016, 76-7 April, Barcelona, Spain
44	P1. HCMR	DIVERSIFY research status. Coordination meeting of FISHBOOST (EU 7FP project on breeding programs for EU aquaculture species).	27/04/2016	Heraklion, Crete, Greece

In terms of **scientific articles**, the following 7 papers have been published during the 2nd Reporting Period:

Andree, K.B., Roque, A., Duncan, N., Gisbert, E., Estevez, A., Tsertou, M.I., Katharios, P., 2016. 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, 1-5 (online).

El Kertaoui, N., Hernández-Cruz, C.M., Montero, D., Caballero, M.J., Saleh, R., Afonso, J.M., Izquierdo, M., 2015. The importance of dietary HUFA for meagre larvae (*Argyrosomus regius*; Asso, 1801) and its relation with antioxidant vitamins E and C. Aquaculture Research, 1-15 (online).

Lazo, O., Claret, A., Guerrero, L., 2016. A comparison of two methods for generating descripting attributes with trained assesors: check-all-that-apply (CATA) vs. free choice. Journal of Sensory Studies 31, 163-176.

Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2016. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture, 1-26 (online).

Grigorakis, K., 2015. Fillet proximate composition, lipid quality, yields and organoleptic quality of Mediterranean farmed marine fish: A review with emphasis on new species. Critical Reviews in Food Science and Nutrition. DOI: 10.1080/10408398.2015.1081145.

Gisbert, E., Mozanzadeh, M.T., Kotzamanis, Y., Estévez, A., 2016. Weaning wild flathead grey mullet (*Mugil cephalus*) fry with diets with different levels of fish meal substitution. Aquaculture 462, 92-100.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016. Optimized spawning induction protocol for meagre *Argyrosomus regius* using weekly injections of GnRHa. Aquaculture, 1-9 (on line)

All the above articles have been uploaded in the appropriate site of the ECAS site of the EU. In addition, the following 2 manuscripts are currently under review:

Banovic, M., Krystallis, A., Guerrero, L., and Reinders, M.J. Consumers as co-creators of new product ideas: An application of projective and creative research techniques. *Food Research International* (under review).

Reinders, M.J., Banovic, M., Guerrero, L., and Krystallis, A. Consumer perceptions of farmed fish: A cross-national segmentation in five European countries. *British Food Journal* (under review).

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)

No work done during this period

Task 31.6 Promotional workshops (led by CTAQUA, Rocio Robles).

No work done during this period

Task 31.7 Dissemination to the food industry and consumers (led by APROMAR and EUFIC, Javier Ojeda and Laura Fernández).

In order to complete the objective of stablishing collaboration agreements with representative associations of consumer and supermarkets, P12. APROMAR, has established a collaboration agreement with the Association of International Seafood Professionals, AISP (seafoodprofessionals.org/). The AISP network is a tool to disseminate knowledge about fish, seafood and associated products, which is in line with the objectives of DIVERSIFY. A document explaining the link and collaboration between the project and the association was created and included in the blog of the AISP in November 2015

To transfer the research advances and achievements of the different Research Areas of DIVERSIFY to the industry, Dr. Matthias Keller, from P34. Fischindustrie und des Fischgrosshandels E.V.), has presented project results to the members of the German Association of Fish Processorsm and wholesalers during their annual general assembly in Hamburg (Germany) (see Workshops section). The same partner the celebration of the Aquaculture Round Table, Bonn, Germany; most relevant results of the project after one year, with special emphasis on the work packages related to the socioeconomic dimension of DIVERSIFY were presented.

Figure 31.7.1. Desktop image of the ASIP blog where the linkage between AISP and DIVERSIFY was launched and addressed.

Following the objective to transfer DIVERSIFY developments to the industry, the Dissemination leader has established a link of collaboration with the Coordinator of the TRAFOON project Dr. F. Javier Casado.

(www.trafoon.eu), and to Dr. Tomás Policar (WP leader "Traditional Products of fish"), from the same project. The project has the objective to establish a knowledge transfer network with a focus on food products, to support traditional food producing SMEs. One of the focus products is fish (work package "Traditional Products of fish"). To support traditional food SMEs, TRAFOON organizes Training Workshops for SMEs. DIVERSIFY dissemination information has been sent and distributed during the workshop held in Czech Republic last 1-2 February 2016 (" Improving fish feed and feeding techniques, fish processing, labelling and marketing with fish products") (**Figure 31.7.2).**

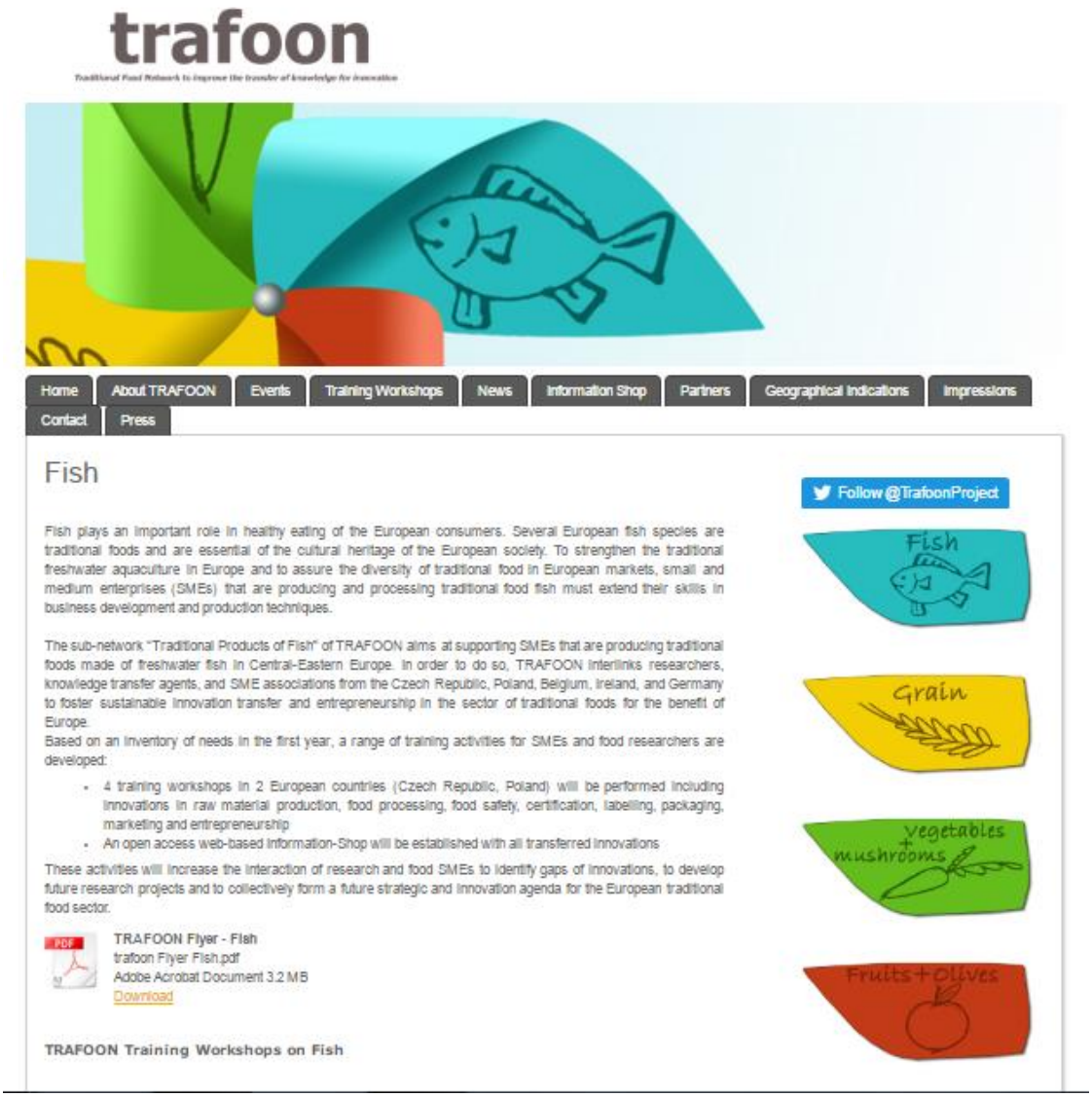


Figure 31.7.2. Desktop capture of the fish section within the TRAFOON project website.

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

This task has been completed and reported during the 1st Reporting Period. Some modifications have been made in the composition of the management bodies, which are explained below.

Group Work package Leaders (GWPL): These are the researchers leading the groups of related RTD WPs in the areas of Reproduction & Genetics, Nutrition, Larval husbandry, Grow out husbandry, Fish health and Socioeconomics were selected from the stage of the proposal preparation, again given their expertise and excellence in research in the scientific discipline. The original GWPLs were:

- Dr. Neil Duncan (P3. IRTA) for all Reproduction & Genetics WPs,
- Dr. Marisol Izquierdo (P2. FCPCT) for all Nutrition WPs,
- Dr. Bill Koven (P4. IOLR) for all Larval husbandry WPs,
- Dr. Nikos Papandroulakis (P1. HCMR) for all Grow out husbandry WPs,
- Dr. Chris Secombes (P5. UNIABDN) for all Fish health WPs, and
- Drs. Gemma Tacken (P6. LEI/DLO) for all Socioeconomics WPs.

Because of problems with the communication and availability of Dr. Marisol Izquierd, the GWP leader for Nutrition (See Deliverable ***D1.3 Annual Coordination Meeting Y2, 2014***), the PC proposed to the Steering Committee (SC) to change the leader. The SC and PC agreed with the proposition by Dr. Izquierdo, to appoint Dr. Hipolito Fernandez-Palacios (P2. FCPCT) as the new GWPL for this scientific discipline.

Unfortunately, Dr. Hipolito Fernandez-Palacios retired in the late 2015 and an extention of his stay was not approved by his institution, so it became necessary to appoint yet another researcher as the GWPL for Nutrition. The new researcher in charge of this GWP is Dr. Daniel Montero (P2. FCPCT), who was approved by the PC and SC during the ACM 2016.

Work package Lead Beneficiaries (LB): These are the researhers leading each WPs, and have the responsibilities for (a) the timely execution of all planned research activities in their specific WP, following the projects time schedule (Table 1.3ii Gantt chart), (b) the compilation of all results and preparation of the periodic reports and (c) liaising with the PC, WP31 Dissemination leader and the Species Leaders. No modification in the LBs has been done during the 2nd Periodic Period.

Species Leaders (SL): The SLs were selected from among the consortium based on their involvement, expertise and excellence in research with the selected species (one per species). During the implementation of the program, the SLs have been involved in overseeing, and in the following years with compiling and disseminating the work done in the various RTD WPs of the project. The SLs have been:

- Dr. Alicia Estevez (P3. IRTA) for meagre,
- Dr. Nikos Papandroulakis (P1. HCMR) for greater amberjack,
- Dr. Pascal Fontaine (P9. UL) for pikeperch,
- Dr. Birgitta Norberg (P7. IMR) for Atlantic halibut,
- Dr. Jose Benito (Tito) Peleteiro Alonso (P8. IEO) for wreckfish, and
- Dr. Bill Koven (P4. IOLR) for grey mullet.

Due to the retirement of Dr. Jose Benito (Tito) Peleteiro Alonso (P8. IEO), it has become necessary to appoint a new member of the DIVERSIFY consortium to take his place as the SL for wreckfish. The researchers involved in the work with wreckfish, together with Dr. Peleteiro decided to appoint Dr. Blanca Alvarez from the same partner (8.IEO), as the new SL for wreckfish.

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. Now, Mrs Tu-Linh Ly will also have to leave her position in the SC, as P29. ASIALOR will exit the consortium due to financial troubles (see later for details). A new member will be appointed to the SC from one of the partners.

Coordination meetings

The ACM 2016 was hosted by Dr. Pascal Fontaine of the University of Lorraine (P9. UL) and was held at the Museum-Aquarium of Nancy (Day 1) and the Brabois Campus of the University of Lorraine (Day 2 & 3) on 2-4 February 2016. The 3-day meeting was attended by 87 persons: 78 coming from the DIVERSIFY consortium and 9 invited guests from outside the consortium. No representative attended from three Beneficiaries (P26. GEI, P28. CANEXMAR and P37. EUFIC). Beneficiary P10. TU/e was unable to attend the first day of the meeting, but attended the second and third day.

As for the kickoff meeting, information regarding the meeting was uploaded continually on the project's web site (www.diversifyfish.eu/INTRA/Meetings & Activities/2016 Annual Coordination Meeting) 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: a common session for all participants during DAY 1 (including invited guests) presenting summaries of the work implemented in all six Scientific Disciplines, specific presentations from various WPs or tasks, and presentations from invited guests,
(b) DAY 2: Six (6) Scientific Discipline-specific workshops running in three parallel sessions during the day, and

(c) DAY 3: a common session dealing with Dissemination, Scientific and Financial Reporting, and Management. A meeting of the Steering Committee was also held at the end of the ACM. In addition, a special 2-hour meeting was held with all the Partners being involved in work with greater amberjack (*Seriola dumerili*) under any Scientific Discipline, in order to address some issues related to the grow-out experiments.

Table 2.3.1. Agenda of DAY 1 of the Annual Coordination Meeting 2016, which took place on the 2-4 February 2016, at the Museum-Aquarium of Nancy, Nancy, France.

DAY 1		2 Feb	Tuesday (Open Day & Summary presentations)		
Start	End		Title	Presenter	Details
8.00	9.00		Registration	U of Lorraine Staff	Register, receive badge, submit presentations
9.00	9.30		Welcoming	Constantinos Mylonas (HCMR), Pascal Fontaine (UL)	Meeting logistics, agenda, welcoming from UL
9.30	10.00		GWP presentation – Repro & Genetics	Neil Duncan (IRTA)	
10.00	10.15		Dysfunctional reproductive maturation in captive greater amberjack	Aldo Corriero (UNIBA)	Reproduction & Genetics
10.15	10.30		Population genetic analysis of wild and domesticated pikeperch populations and their application to future breeding programs	Costas Tsigenopoulos (HCMR)	Reproduction & Genetics
10.30	11.00		GWP presentation – Nutrition	Daniel Montero (FCPCT)	
11.00	11.30	coffee			
11.30	12.00		GWP presentation – Larval husbandry	Bill Koven (IOLR)	
12.00	12.15		The nutrient profile of Artemia is greatly improved by ongrowing nauplii for 3 days on Ori-Green	Kristin Hamre (NIFES)	Larval rearing
12.15	12.30		First larval rearing efforts with wreckfish	Tito Peleteiro-Nikos Papandroulakis-Antonio Vilar	Larval rearing
12.30	13.00		GWP presentation – Grow out husbandry	Nikos Papandroulakis (HCMR)	
13.00	14.00	Lunch	Lunch at the Restaurant "Cesar" at Place Stanislas		
14.00	14.15		The effect of different stimuli on meagre feeding behaviour	Ioannis Papadakis (HCMR)	Grow out husbandry
14.15	14.30		Multifactorial approach to identify rearing conditions optimising growth, physiological status and immune defense in pikeperch	Patrick Kestemont (FUNDP)	Grow out husbandry
14.30	15.00		GWP presentation – Fish Health	Chris Secombes (UNIABDN)	
15.00	15.15		Pathologies of fish not included in the DIVERSIFY DOW	Pentelis Katharios	Fish health
15.15	15.45		GWP presentation – Socio economics	Gemma Tacken (LEI/DLO)	
15.45	16.00		Consumer value perceptions and attitudes towards farmed fish products in top-five EU markets	Marija Banovic and Thanassis Krystallis (AU)	Socieconomics
16.00	16.30	coffee			
16.30	16.45		Selection of new products and product development	Kriton Grigorakis (HCMR)	Socieconomics
16.45	17.00		Breeding selection in aquaculture fishes, with emphasis on the meagre	Pierric Hafray, SYSAFF	Invited guest
17.00	17.15		Capture of wild fish for aquaria and research	Joao Coreia (Flying Sharks)	Invited guest
17.15	17.30		European eel breeding, larval culture and first-feeding attempts	Jonna Tomkiewicz (DTU)	Invited guest
17.30	17.45		Greeting from the EU Officer of DIVERSIFY	Marta Iglesias (EU DG RTD)	EU Scientific Officer
17.45	18.00		Wrap up	Constantinos Mylonas (HCMR)	Agenda for next day
18.00	19.00		Visit the Aquarium	Pascal Fontaine	
20.00			Dinner at "Grande Salons Hotel De Ville, Stanislas Place"		

DAY 1 – Summary presentations of implemented work and invited guests

The morning session started with a welcoming presentation by the PC, 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. The invited guests included Dr. Pierrick Haffray and Mrs Anastasia Bestin from the Syndicat des Sélectionneurs Avicoles et Aquacoles Français (SYSAF, an animal breeding company), Dr. Joao Correia and Mr. Mauricio Francisco from Flying Sharks (a fish capture and transport company), the secretary of the European Aquaculture Society Dr. Alistair Lane, Prof. Jonna Tomkiewicz from the Danish Technical University and members of the technical staff of four aquaculture production companies (**Andromeda SA** from Greece/Spain, **Le Poisson du Soleill** from France, **Isidro de la Cal** from Spain and **Galaxidi SA** from Greece).

The presentations from the invited guests, 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. Pierrick Haffray (SYSAF, France) on the development of breeding programmes in aquaculture fish and of Prof. Jonna Tomkiewicz (DTU, Denmark) on the breeding and larval rearing research of Atlantic eel (*Anguilla anguilla*). Both presentations are extremely relevant to work undertaken in DIVERSIFY and we hope that we will establish further contacts with these researchers. The participation of four 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 and Galaxidi SA, 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.

DAY 2 – Scientific Discipline-specific workshops

During the second day 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 third year (2016) of the project (**Table 2**).

The workshops of DAY 2 were running in parallel (3 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 Socioeconomics requested a full-day Workshop, 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 (**Table 2)**. 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.

DAY 3

Management (Amendment, Deliverables and Scientific Reporting)

The Partners were informed of the submitted **2nd Amendment to the Annex I**. The reasons for the amendment were explained (loss of greater amberjack broodstock in P.24 ITTICAL and transfer of activities to P23. ARGO; exit of P30. CULMAREX and modification of work carried out in WP20, etc.). As in the previous ACM 2014, a presentation was given by the PC to explain how the scientific reporting needs to be done for the project. This included both Deliverables and Periodic Reports. The presentation had begun with a reiteration of the roles of the PC, the SLs the GWPLs and the WP leaders (LBs), in an effort to clarify their responsibilities and to remind the information that must be provided by the rest of partners to the WP leaders and GWP leaders.

Table 2.3.2. Agenda of DAY 2 & 3 of the Annual Coordination Meeting 2016, which took place on the 2-4 February 2016, at the Museum-Aquarium of Nancy, Nancy, France.

DAY 2		3 Feb	Wednesday (GWP Worskshhops)		
Start	End		ROOM 1 (Gruber)	ROOM 2 (Galle)	ROOM 3 (Daum)
[illegible]	9,30		GWP 3 Nutrition (mullet)	GWP 2 Repro & Gen (meagre)	GWP 7 Socioeco
9,30	10,00		GWP 3 Nutrition (wreckfish)	GWP 2 Repro & Gen (pikeperch)	GWP 7 Socioeco
10,00	10,30		GWP 3 Nutrition (halibut)	GWP 2 Repro & Gen (amberjack)	GWP 7 Socioeco
10,30	11,00		GWP 3 Nutrition (pikeperch)	GWP 2 Repro & Gen (amberjack)	GWP 7 Socioeco
11,00	11,30	coffee			
11,30	12,00		GWP 3 Nutrition (amberjack)	GWP 2 Repro & Gen (halibut)	GWP 7 Socioeco
12,00	12,30		GWP 3 Nutrition (meagre)	GWP 2 Repro & Gen (wreckfish)	GWP 7 Socioeco
12,30	13,00		GWP 5 Grow out (mullet)	GWP 2 Repro & Gen (wreckfish)	GWP 7 Socioeco
13,00	13,30		GWP 5 Grow out (meagre)	GWP 2 Repro & Gen (mullet)	GWP 7 Socioeco
13,30	14,00	Lunch	Lunch at the University Restaurant		
14,00	14,30				
14,30	15,00				
15,00	15,30		GWP 5 Grow out (pikeperch)	GWP 4 Larval (meagre)	GWP 7 Socioeco
15,30	16,00		GWP 5 Grow out (amberjack)	GWP 4 Larval (halibut)	GWP 7 Socioeco
16,00	16,30		GWP 6 Fish health (amberjack)	GWP 4 Larval (pikeperch)	GWP 7 Socioeco
16,30	17,00		GWP 6 Fish health (meagre)	GWP 4 Larval (mullet)	GWP 7 Socioeco
17,00	17,30		GWP 6 Fish health (meagre)	GWP 4 Larval (wreckfish)	GWP 7 Socioeco
17,30	18,00		GWP 6 Fish health (halibut)	GWP 4 Larval (amberjack)	GWP 7 Socioeco
To be arranged			Guided tour of the historic center of the city		

DAY 3		4 Feb	Thursday (dissemination-reporting-administration)		
Start	End		Title	Presenter	Details
[illegible]	9,30		WP31 Dissemination	Rocio Robles	
9,30	10,00				
10,00	10,30		Amendments (2nd), Reporting	Constantinos Mylonas	
10,30	11,00				
11,00	11,30	coffee			
11,30	12,00		Deliverables, Participants Portal	Constantinos Mylonas	
12,00	12,30				
12,30	13,00		Steering Committee meeting	Coordinator, GWP leaders, SME representatives (ARGO, ASIALOR) APROMAR	
13,00	14,00	Lunch	Lunch at the University Restaurant		
14,00	14,30		Greater amberjack meeting (Room Cuenot)	Species Leader (Nikos Papandroulakis) & greater amberjack partners	Address issues related to the implementation of the large scale grow-out experiments
14,30	15,00				
15,00	15,30				
15,30	16,00				
18,00	22,00		Social event (to be arranged)	Pascal Fontaine	

Regarding the Deliverables, it was emphasized again to the Partners that they must be uploaded **on time** electronically in the Participants Portal. To ensure that all Deliverables are uniform throughout the consortium –in terms of appearance, format, and content quality and extent--, the PC has prepared a specific format file (**Fig. 2.3.1**), as well as explicit instructions on the preparation of the Deliverables, which are included in the website (http://www.diversifyfish.eu/2016-annual-coordination-meeting-feb.html). The Participants were also reminded of the Participants Portal and its functions that are relevant to the uploading of the Deliverables.

Then the PC discussed the status of the Deliverable submission, making a summary of the number of Deliverables that have been submitted so far and the ones that have requested a delay (**Fig. 2.3.2**). So far only 80% of the expected Deliverables have been submitted, but it is expected that as time goes on less delays will be faced and by the time of the Mid Term evaluation we will have almost all due Deliverables submitted. To ensure that the all Deliverables are not only uniform throughout the consortium in terms of

appearance, but also that they are of high scientific quality, the PC has explained and emphasized the procedure that has been employed so far for the preparation and submission of the Deliverables.

Figure 2.3.1. Instructions on the format file created by the PC for the preparation of the Deliverables of the project (available at www.diversifyfish.eu/INTRA/Forms & Protocols) and presentation of the Participants Portal section where Deliverables are listed and can be uploaded and downloaded by consortium members.

Figure 2.3.2. A summary of the Deliverables due and submitted so far and a presentation of the DIVERSIFY web page where all the submitted Deliverables are available to the consortium members, in the INTRA section of the website (http://www.diversifyfish.eu/deliverables.html).

The session continued with a brief discussion dealing with the upcoming Periodic Report (Period 13-30 months, due July 2016). As for the Deliverables, special format files have been produced by the PC for each Work Package and will be sent to the Lead Beneficiaries of each Work Package (in April 2016) to help them compile the results and data from each Task. It was stressed that the Periodic Report must include the work carried out during the reporting period with enough detail, but without excessive and unnecessary information, as it has been done for the 1st Periodic Report. This will allow the Consortium members to follow the major achievements as well as problems encountered during the 2nd period, and will enable both the EU Scientific Officer and the Mid-Term Evaluation committee to evaluate the work in relation to the

DOW, and be able to make any necessary recommendations. A question must be made to the EU Scientific Officer regarding the inclusion in the report of the data obtained in the 1st Reporting Period (1-12 months).

As before, to speed up the process of preparing the report, while at the same time ensuring that a uniform and high quality document is presented (both in terms of format and content), the work has been delegated to all Consortium members as follows:

a. The LBs for each Work Package (WP leaders) will request the text/figures/tables for each Task from the Task leaders, who are responsible to coordinate their writing with all scientists participating in their task. This process has already begun to some extent, in preparation for the ACM 2016, but must be updated with work that will take place in the next 3 months and completed by **20 May 2016**,
b. The LBs then will compile all the information into a single document for each Work Package, review it for content/format/editorial errors and submit it to the GWP leader (**30 May 2016**),
c. The GWP leaders will then compile all the Work Packages into a single document for each GWP, review it for content/format/editorial errors and submit it to the PC (**10 June 2016**),
d. The PC will then compile all the GWPs into a single document to prepare the 2nd Periodic Report and review it for content/format/editorial errors (**30 June 2016**),
e. The GWP leaders will also have to prepare following information (**20 June 2016**):
    i. 3.1 Publishable Summary (0.5 page per GWP),
    ii. 3.2.1 Project objectives for the period (0.5 page per GWP),
    iii. 3.2.2 Project progress and achievements for the period (1 page per GWP).

This information will then be submitted to the PC for incorporation in the Periodic Report. The PC will prepare the remaining sections required (e.g., 3.2.3 Project management for the Period, Deliverables and Milestones, etc.) and will complete the 2nd Periodic Report by the end of June 2016 and upload it in the Participants Portal. **All Partners agreed to the procedure and time schedule, and will do their utmost to complete the documents as requested and within the proposed deadlines.**

The PC then discussed the issue of preparing the work done in DIVERSIFY for submission to scientific magazines. Already 4 scientific articles have been published (See WP31. Dissemination) and many more researchers expressed their intention to start submitting their work. A change was agreed on the project's website, by moving the "Scientific Publications" page to the main menu bar, so that visitors will have a more rapid and direct access to the scientific work of the Consortium.

Financial Reporting

Then, the PC discussed briefly some Financial Issues, regarding the payments received and the need for all Partners to **abide by the budget allocation as described and agreed in the DOW**, as much as possible (**Fig. 2.3.3**). Partners were reminded that each Partner has requested a specific budget, and allocated the EU support to clearly defined and described types of expenses, such as Personnel, Subcontracting, Travel, Consumables, Durable Equipment and Other types of expenses. The objective is to avoid unnecessary modifications that would require transfer of budget between types of expenses, or spending of the money in items not indicated in the DOW. Of course, it is recognized that there is always the possibility of unforeseen costs, but the Partners were encouraged to keep this to the absolute minimum. The PC mentioned that so far a number of minor modifications have been made, always after the agreement of the EU Scientific Officer, who has so far always accepted such requests, provided a reasonable explanation and justification has been provided.

It was agreed that **Form C would be submitted to the PC by all Partners before 15 June 2016**, so that the PC would have time to review and ask for corrections (if necessary) to complete the process by the end of the year. It was emphasized that in the previous reporting, all Form Cs were submitted to the PC except from one Partner, thus delaying the submission of the Financial Report by almost 1 month (still it was submitted before the official deadline!).

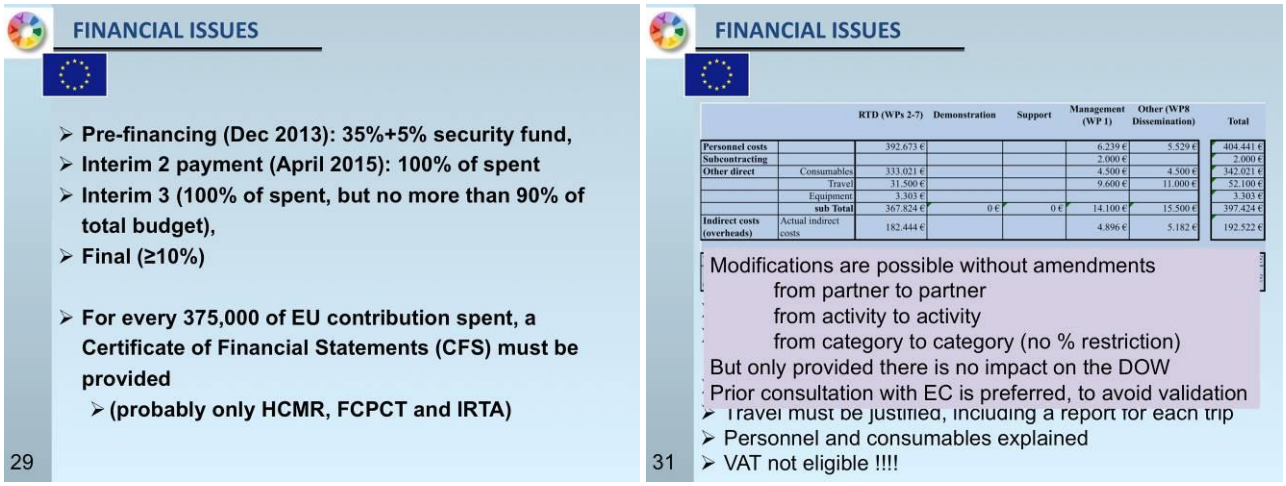


Figure 2.3.3. Representative slides from the discussion on Financial Issues, which included the payments received so far and an urge to the Partners to respect as much as possible their budgets and resource allocation (the whole presentation is available at http://www.diversifyfish.eu/2016-annual-coordination-meeting-feb.html).

At the end of the presentation, the date and location of the next ACM meeting was discussed. The next ACM has been agreed for Jan 2017 and will be hosted by IRTA (P3. UL) in Barcelona, Spain. As this time no Periodic Report is due, the PC suggested that the format of the meeting is modified slightly. It was suggested that we hold 2 Open Days, where instead of summary presentations of the GWP leaders, we allow as many Partners as possible to present their work in 20-30 min presentations. This will allow the consortium members to have a more detailed view of the work carried out and will encourage the Partners to prepare their work for presentation and then publication.

Finally, the Partners were presented with a slight modification of the project's logo that we used recently for the new t-shirts. The new logo provides some information as to the objective of the project and the PC thinks it is more informative (**Fig. 2.3.4**). Still, the decision was not to modify all the DIVERSIFY documents with this "new" logo, but to use it only in our new t-shirt and some of our new activities and presentations.

Figure 2.3.4. The modified logo, as it appears on the new t-shirts.

Steering Committee meeting

At the end of the third day, a meeting of the Steering Committee (SC) was held, as planned in the DOW. The SC members are the PC, the six GWP leaders, two representatives of SMEs (since P30. CULMAREX that was the third industry representative has exited the consortium) and the representative from a professional organization. The people attending this meeting were Mylonas, C.C. (PC, P1. HCMR), Duncan, N. (GWP leader, P3. IRTA), Montero, D. (GWP leader, P2. FCPCT), Koven, W. (GWP leader, P4. IOLR), Papandroulakis, N. (GWP Leader, P1. HCMR), Secombes, C. (GWP leader, P5. UNIABDN), Tacken, G. (GWP leader, P6. DLO), Daniil, M. (P23. ARGO), Deves, K. (P29. ASIALOR) and Ojeda, J. (P12. APROMAR).

No official agenda was prepared for the meeting, but the PC addressed the following issues:

1. 2nd Amendment – The PC explained in more detail the major aspects of the amendment, especially the budget reallocation amounts to the various Partners.
2. Some discussion was made on possible changes of Partners and a further Amendment to the Annex 1. Specifically, it was mentioned that the PI from one Partner may be moving to another organization and would like to continue his involvement in DIVERSIFY. The PC will contact the EU Scientific Officer and examine the procedure for this. Secondly, one organization may be changing its structure, which would involve a change in name but also of legal documents (e.g. VAT registration number). As above, they would still like to be in the consortium, so we need to address the procedure for their validation and then joining of the consortium.
3. Also, two of the commercial partners may also be changing legal status or stopping their activities, so the consortium must already start examining the potential of other partners joining the consortium to undertake the work planned for these Partners.
4. Management - Substitution of the leader for GWP Nutrition Dr. Hipolito Fernandez by Dr. Daniel Montero, due to the retirement of Dr. H. Fernandez has been requested by P2. FCPCT and has been reported and approved by the SC.
5. The PC mentioned that he is encouraging the participation/collaboration of more commercial operations outside the consortium, and introduced Isidro de la Cal as one company interested in having more collaborations with the consortium in the area of wreckfish reproduction and larval rearing. The company has a large stock of wreckfish breeders (more than 30!) and would be a very valuable collaborator.
6. Next ACM 2017 will be held in January in Spain, organized by P3. IRTA and hosted by Alicia Estevez - The location of the meeting will be in Barcelona, Spain. The GWP leaders were encouraged once again to invite relevant scientists from the international community to attend this meeting.

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 2016 has been submitted as Deliverable ***D1.6 Annual Coordination Meeting for Y3 (2016).***

Consideration of Gender Aspects in the Project

Although gender equality in the European work force has been advocated as a core policy since 1957 (Rome treaty), the reality is different. During FP5 and FP6 women were systematically under-represented in research projects. For FP7, the EU has set a target of at least 40% women participation at all levels of research in order to encourage equal opportunities under gender sensitive working conditions. It is worth mentioning that Aquaculture (especially land-based hatcheries) is among the industrial and scientific activities with a greater presence of women.

In DIVERSIFY, **several issues have been addressed regarding equal participation of women as RTD researchers and SME staff**, and a commitment to use gender-impartial language will be made. Particular attention has also been given to gender-sensitive issues when organising the project, such as scheduling annual meetings that require mobility. Work from men and women will be valued equally.

In the current project women participate as **Species Leaders** (2 of 6; IRTA and IMR), **Group Work package Leaders** (3 of 8; FCPCT, LEI and CTAQUA), **Work package Leaders** (12 of 31; from FCPCT, IRTA, DLO/LEI, IMR, NIFES, CTAQUA, CMRM) and the **Principle Investigators** in charge for the Partners (10 of 38; FCPCT, IRTA, DLO/LEI, IMR, ULL, NIFES, CTAQUA, CMRM, ANFACO, HRH). In terms of Researchers and Technicians, the overall participation of women in all the work packages is 42.5%. Several of the participating institutions (Partner No 1, 2, 3, 4, 5, 7, 13, 14, 15, 20, 21, 22, 25) have policies targeting gender equity at work. The hiring process for new personnel (technicians, graduate students, post-doctoral fellows) will be based on equal gender opportunity in order to contribute to the advancement of women in post-doctoral and top decision-making positions. The SMEs participating in this proposal employ women, mainly in land-based facilities in activities that include broodstock management, live food production, hatchery, nursery and pathology. Finally, when performing the consumer demand studies, results will be presented by gender and when required they will be analysed separately.

In an attempt to perform gender-sensitive research, the scheduling and organizing of meetings and other activities requiring mobility take into account gender issues and places for such activities are chosen taking into account not only practical logistics, but also requests from parents with limited mobility. As proposed during the proposal stage, DIVERSIFY has appointed Dr. Ana Roque from P3. IRTA (**Figure 2.3.5**) to deal with gender issues, should they arise. With that in mind, Dr. Ana Roque prepared a questionnaire and sent it to participating Partners to find out whether participating researchers, especially women have limited availability to travel and which time of the year and for how long is convenient to travel.

We have obtained 24 individual responses from **female researchers** from 20 partners. Below are the questions and the responses obtained, which I believe indicate that the consortium is making an effort to address gender aspects:

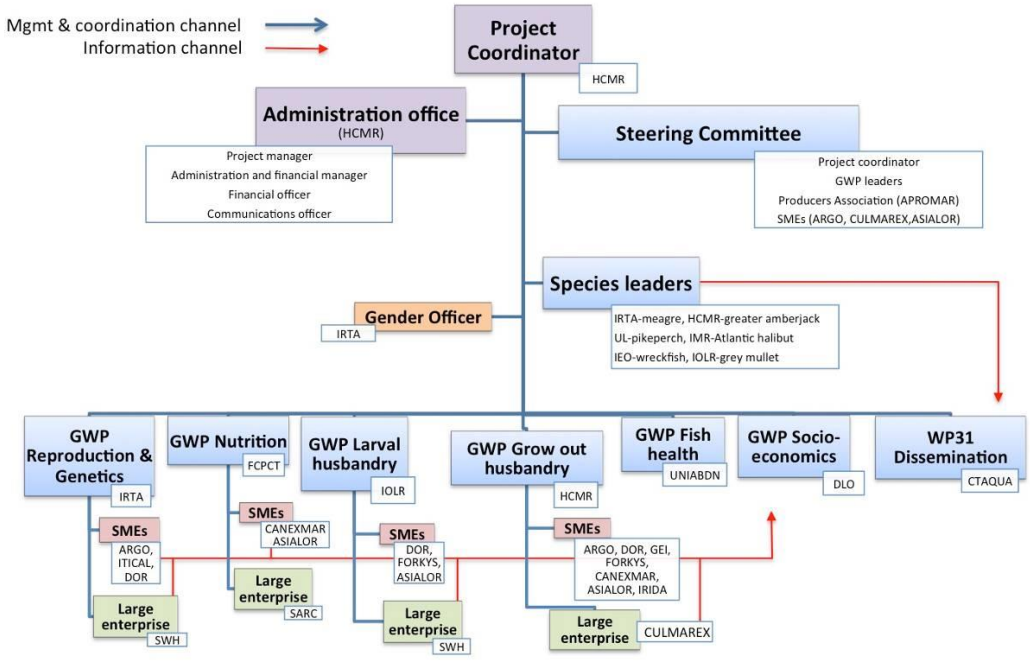


Figure 2.3.5. Management structure of the DIVERSIFY consortium.

Q1: Do you feel there are gender inequality issues that have not been addressed during the definition of the roles and tasks of each participant of the DIVERSIFY project?

All female participants replied "No"

Q2: Within the DIVERSIFY project we have as Species leaders 2 female participants out of 6, as Group Work Package leaders 3 female participants out of 8 and as Principle Investigators 11 female participants out of 38. Do you think this is because of :

- Interest - 4, Out of the 25 female participants who answered the questionnaire 4 think (opinion) the reason was female participants were not interested in undertaking these responsibilities
- Will – 9 female participants simply don't want to do it
- Feedom – 6 female participants don't have enough freedom to do it
- Confidence – 2 female participants are not confident enough to do it
- Qualities - 0, nobody doubts that female participants are well qualified to perform these roles

Q3: As a participant in the DIVERSIFY project, can you travel anytime of the year?

- Yes - 15 female participants can travel when they want
- No – 5 female participants cannot travel anytime of the year
- Some female participants did not answer this question

Q4: How easy is it for you to organize your trip?

- Entirely personal decision - 9 female participants stated they do not need to consult with anybody
- Need to arrange with Spouse/Partner - 12 female participants stated they need to consult with others
- Need to consider their dependents - No female participant needs to arrange directly with dependents

Q5: How long could you travel away for the needs of DIVERSIFY?

- 1 day - 2 female participants can go for a 1-day activity
- 3 days - 3 female participants can go for a 3-day activity
- 7 days - 9 female participants can go for a week-long activity
- 30 days - 6 female participants can go for more than 1-month activity

Q6: How many meetings of the project do you see yourself attending?

- 0 meetings - 3 female participants state they will not attend any meeting of the project
- 1 meeting - 2 female participants will attend one meeting
- 3 meetings- 3 female participants will attend meetings
- All-12 female participants will attend all meetings

Q7: If the answer above was not "all of them", why?

- Because of the dates of the meetings
- Because the female participant responding is not the Partner's Principle Investigator
- Because of budget limitations

Q8: If the answer to the above was "None", why?

Here 2 answers were due to the fact that the female participant responding is a technician and the other because the female participant has a small participation in the project compared to her colleagues.

Q9: Do you have any comments regarding Gender Issues in DIVERSIFY?

We obtained only the 6 comments below:

- In DIVERSIFY there are a lots of partners, so it is difficult for me to evaluate some aspects about Gender Issues.
- I think that this project have gender equity in general.
- Question Q2 should contain information about how many researchers are in DIVERSIFY and sex proportions. I have the impression that we are less women than men and this could be the reason why there are less leader women.
- Yes. I think the survey is discriminatory because is only for women, should be addressed to men as well.
- This questionnaire should be applied to all.
- In the project as such, I do not see that there are a lot of issues. There might be issues in some of the groups, but I am not sure what would be the best way to address this.

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 (Feb 2016). Deliverable ***D1.16 Annual Coordination Meeting for Y3 (2016)*** reporting on the meetings have also been uploaded on the Participants Portal.

The consortium, through 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. To this effect, a meeting was arranged at the headquarters of DG-RTD in Brussels, in January 2015, where the PC (Dr. C.C. Mylonas) and the WP31. Dissemination leader (Dr. R. Robles) met with the EU Scientific Officer (Dr. Marta Iglesias) and Financial Officer (Mrs Elisabeth Aichinger), as well as some other relevant EU officers invited by the Scientific Officer. During the meeting, the PC made a presentation of the project and the major achievements to that point, and also addressed some of the problems encountered. The meeting helped in developing a more personal and effective relationship between the DIVERSIFY consortium and the EU officers in charge, and we believe the importance of this developed relationship became more apparent in the following months, when a number of issues had to be addreseed, from approval of the Scientific and Financial Reports, to the submition of the 2nd Amendment and the approval of various modifications in the budget items.

We believe that an excellent communication channel exists between DIVERSIFY and the EU Scientific, Financial and Legal Officer (Mr. Pawel Suchon), who have been very responsive to all requests for information, in a prompt and very constructive and effective way.

Mid-term Evaluation of Progress

The mid-term evaluation will be scheduled by the EU Scientific Officer towards the end of 2016, and the PC has already started preparing for this evaluation.

Interactions with other projects

As planned in the DOW, in order to stimulate synergies and complementarities, links were established between the DIVERSIFY project and other relevant national and EU ongoing projects when the appropriate opportunities occurred. Fro example, links have been formed with the project "KRANIOS" coordinated by P1. HCMR, a Greek National project on the production of meagre. Also, links have been formed with "KRIPIS", also a Greek National project coordinated by P1. HCMR on the reproduction, larval rearing and cage grow of greater amberjack. There was also a link with the coordinator of Targetfish (EU 7FP) who confirmed there interest and willingness to collaborate with DIVERSIFY in the area of vaccine development of VNN (WP 26). Other projects with which DIVERSIFY has formed interactions include the COST Actions "AQUAGAMET" and "FITFISH", and PROAQUA a project is funded by the Committee for Strategic Research in Health, Food and Welfare, the Danish Research Council. More specific information has been provided in the submitted Deliverable ***D1.5 Interactions with other projects.*** There are also "passive" interactions with other, recently funded projects (PARAFISHCONTROL, REPROBOST,

CLIMEFISH, TAPAS) through the joined participation of some DIVERSIFY partners in the above mentioned projects.

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).

Deviations from the DOW

Some deviations from the DOW have been made, either as a necessity or a choice, in order to achieve the objectives of the project. The majority of deviations involve delays in the delivery time (in general of a few months) of some deliverables. A very small number of Deliverables needed to be delayed by a longer period of time (12 monts) in order to repeat the work proposed, due to technical problems or significant mortalities occurring in the cultured populations. Overall, we consider the deviations as minor, and with either minor effect or in many cases a positive effect on the quality of the work to be done, and further details –were necessary- are provide below on a WP-by-WP basis.

One notable exception to the minor nature of deviations from the DOW is the abolishion of a task from WP20 Grow out husbandry – meagre, which came about due to the exit of P30. CULMAREX from the consortium. This commercial partner (Large Enterprise) was in charge of studying the effet of an automatic feeding system on the performance of meagre in sea cages. However, due to problems with the sea cage sites of the company, the company could not implement the original experiments. An effort to identify another commercial partner, either in Spain or Greece was not successful. This forced the consortium to abolish this task, but in order to obtain as much relevant information towards the original objective of this WP and Task, a series of new experiments were planned by P3. IRTA, and will be implemented during 2016 and 2017 in tanks, in order to study the feeding response and growth performance of meagre under different feeding management regimes. Given the circumstances, we believe the solution is a satisfactory one, and will provide relevant and significant information to fulfil the objectives set out in the DOW.

Until the time this report was being prepared (Mo 32), a total of 64 Deliverables have been submitted, out of a total of 73 expected for the 1st and 2nd Reporting Periods (Mo 1-32) according to the DOW (2nd Amendment for 2016), and out of a total of 202 Deliverables for the whole project. We believe that all submitted deliverables are of good quality and fulfil their commitment as described in the DOW. In some situations where the results obtained were considered unsatisfactory –such as for eaxmple when high fish mortality or low quality of eggs was observed), new updated deliverables have been or will be submitted once the experiments were/are repeated.

WP1 Project management.

No deviations in the implementation of this WP were faced during the 2nd Reporting Period. The management of the project proceeds well, with timely and effective reporting procedures, submission of deliverables, reporting of dissemination activities and uploading of published scientific articles.

WP2 Reproduction & Genetics - meagre.

In addition to the paired-spawning induction trials that will be undertaken by P3. IRTA (as per DOW) under Task 2.1, P1. HCMR has carried out a similar experiment (2014) and will also carry out another one in 2015 in order to obtain more relevant information for this deliverable. The additional data will also facilitate the publication of the results, since it will contain a mirrored experiment undertaken in two different facilities, in different geographic locations, but under the exact same protocol. This work is without any extra cost to DIVERSIFY, and it is undertaken because spawning induction is carried out anyway at P1. HCMR in order to produce eggs for juvenile production for other WPs, in which the partner is involved. The work has already been submitted in ***Deliverable D2. Protocol for paired spontaneous tank spawning of meagr***e (Mo

22), which is currently under preparation to be submitted for publication. The previous work carried out in 2014 has been submitted and already published, and the citation has already been uploaded in the relevant section of the ECAS Participant Portal.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016b. Optimized spawning induction protocol for meagre *Argyrosomus regius* using weekly injections of GnRHa. **Aquaculture**, 464, 323-330

Also, 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, the resarcher responsible (P1. HCMR, Dr. Costas Tsigenopoulos) has proposed to modify Task 2.5, in terms of (a) the method to be used and (a) the time-schedule. Because it is the first time that these methods are going to be applied in meagre, it is believed that we will need a 6-month extension of the associated deliverables, from Mo 30 to 36. No change in budget allocation, staff effort or the number of deliverables will result form this modification. The main reasons for the requested modifications are the following:

1. Dominance of RAD-based methodologies over RNA-Seq in SNP discovery and QTL mapping.

The aim of task 2.5 is to identify SNPs that are associated with growth in meagre. Towards that end, we have suggested the use of RNA-Seq for detecting SNPs in the coding regions of the genome and afterwards the construction of a SNP-chip. Our original proposal was to design and then test the SNP chip to see whether any proportion of these loci might be associated with growth. In that testing phase, we planned to include ~800 individuals to increase the chance of identifying potential candidates for growth. However, the majority of those SNPs might be random polymorphic sites or associated with irrelevant traits with no apparent association with the trait of interest.

Nowadays, RAD (Restriction site Associated DNA) Sequencing is increasingly regarded as the dominant methodology in SNP discovery projects. Multiple studies have shown its pertinence for such tasks and several 'versions' of the 'classic' RAD-Seq protocol have emerged offering a variety of options that are appropriate based on the needs of each project. RAD-Seq leads to the discovery of polymorphic loci evenly distributed across the genome and is not limited to the small and functionally constrained protein-coding part of it. Depending on the sequencing platform used (MiSeq, HiSeq, etc.) and the level of multiplexing (how many individuals we use in the library preparation) which influence coverage rate, the methodology allows to study from a few hundred to tens of thousands of loci across the genome reducing greatly the required sequencing effort per individual and increasing the chance of sequencing the same loci in all samples.

Given the rapid development of RAD-based methodologies, we propose to change the planned strategy and conduct instead a mini-QTL project on targeted families of meagre using RAD-Seq on top of our already implemented RNA-Seq experiment. We propose to use at least two F1 families of meagre in a ddRAD Sequencing strategy, aiming at genotyping approximately 1,000 SNPs in the majority of progeny of the two families. This will lead to various beneficiary outcomes for meagre aquaculture such as:

i. information on the gene content of meagre and SNPs discovered in the protein-coding region of the genome through RNA-Seq,

ii. Linkage mapping through RAD-Seq

iii. Preliminary QTL mapping for growth through RAD-Seq.

Regarding the main aim of the task, we are confident that our new proposal will greatly increase the chance of discovering loci related to growth, exploring the cutting-edge methodology of genotyping-by-sequencing and setting the ground for future QTL studies and marker assisted selection on the species.

2. Drawbacks for the Open Array Platform that was originally suggested in the DOW

The Open Array technology we initially intended to apply, currently reaches a maximum of 256 SNPs for 960 samples (unfortunately SNP-arrays cannot be purchased for formats inferior than 960 fish which is why we intended to test so many fish at the beginning) but recent experience shows that a major characteristic for this approach in a non-model species like meagre, is that a non-negligible percentage of SNPs is going to be less polymorphic than expected and/or not-amplifiable. This was unfortunately evidenced in parentage assignment results obtained for the European sea bass and the gilthead sea bream through the EU funded REPROSEL project (pers. commun. with H. Chavanne and N. Duncan). The RAD-seq technique, and more specifically the ddRAD approach, may instead generate more than 1,000 SNPs markers in each library prepared with some 140 fish and run on a HiSeq2000 or HiSeq2500 Illumina platforms. Therefore, with the RADseq methodology we will make use of lower number of fish, study a much greater number of SNPs and possibly identify few that are linked to Quantitative Trait Loci (QTL) responsible for growth.

3. Maximize research and publication record output from a wide network of scientific and SME partners.

This intentional shift to more up-to-date technologies and methodologies will lead to at least three publications with high interest for the research community and SMEs participating in the project: one for the RNA-seq results and the transcriptome characterization of meagre, a second one with the linkage map of the species and a last one with the QTL study for growth. Moreover, interested SMEs will have the chance to directly essay their breeders in order to future select for enhanced growth.

The requested modification along with the justification has been presented to the EU Scientific Officer (Dr. Marta Iglesias), who approved of the change with an email message of 14 April, 2015.

WP3 Reproduction & Genetics – greater amberjack.

Task 3.2. Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean (led by HCMR) will probably 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 (outside the scope of this report) with great success, and is reported only briefly in this 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. In the following year (Y4), different doses will be tested, and the last year (Y5) the best time of administration will be evaluated if still needed, since with the many trials we have attempted in the first 3 years of the project in the various broodstock sites, we believe we have a pretty good idea as to the optimal time for GnRHa treatment, based on the time or the year and the ambient temperature.

Another issue that arose during this reporting period is that no new broodstock was acquired by P24. ITTICAL and no effort was made by the company to examine the maturation status of their fish and induce spawning in Y3 (Mo 30 or 31). So, it is the opinion of the WP leader and the PC that this partner is underperforming in the consortium and a motion will be made to the Steering Committee to discontinue their participation in the project and transfer their activities to another partner (either P23. ARGO which is already a partner in the consortium or GMF, which is not a member of the consortium, but which has dedicated their broodstock and facilities to the experiments of WP 3 for no charge to the Consortium. At the same time, the CEO of P24. ITTICAL expressed their intention to exit the consortium, through an email to the PC on 19 July 2016. This will be discussed with the EU Scientific Officer in the coming months. No negative impact

on the success of the project is foreseen from this deviation, and in fact we believe that there will be an improvement of the work implemented, due to the closer proximity of any of the proposed substitutes (P23. ARGO or GMF) to P1. HCMR, which is leading this Task, as well as the greater interest and commitment that that these SMEs have demonstrated so far, and their access to better facilities (sea cages and tanks).

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 broostock 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 present report.

Additional Task added. Our observations made in the first 3 years of the project, lead us to believe that there are some significant differences in the reproductive biology of greater amberjack from the Mediterranean Sea (Italy, Greece) vs the Atlantic Ocean (Canary Islands). Specifically, Atlantic Ocean broodstocks (1) adapt much more readily to tanks (even of small diameter) and undergo successful gametogenesis, (2) have a greater propensity to undergo spontaneous spawning without the use of any exogenous hormones, (3) respond better to GnRHa injections (*i.e.* pulsatile stimulation) than GnRHa implants (*i.e.* sustained stimulation) and (4) reproduce for a much longer period of time, from May to September. So, we hypothesize that the greater amberjack populations in the Mediterranean Sea and the Atlantic Ocean may not represent the same strain or stock, and there may be significant genetic differences between them, which have not been examined so far. So, we decided to undertake such a population genetics study for greater amberjack, taking advantage of the existence of a large number of broodstocks from different geographic locations (both captive and sampled from the wild as part of Task 3.1), as well as the access of the consortium to other broodstocks through professional contacts. This work will be undertaken at no extra charge to the project, and we already collected fin-clips from broodstocks of the various partners involved, such as FCPCT, IEO, UNIBA, HCMR, ARGO, FORKYS and other commercial hatcheries such as SAGRO Aquaculture (Cyprus) and KILIC FISHERIES (Turkey). This deviation will have a positive impact on the further contribution of the project to the knowledge of greater amberjack population structure and reproductive biology.

WP5 Reproduction & Genetics – Atlantic halibut.

Due to a prolonged sick leave for the WP leader Dr Birgitta Norberg from May to December 2015, some of the work in Task 5.1 was moved from 2015 to 2016. As a consequence, ***Deliverable 5.1*** will be delayed by a maximum of three months. All the data collection in Task 5.1 is done, as is reported here, but statistical analyses remain and will be completed shortly. Sterling White Halibut no longer keeps wild-caught broodstock and the collected data are from farmed females only. They were compared to the results obtained by IMR, and also used when Task 5.2 was planned. Due to the delay in Task 5.1, Task 5.3 and ***Deliverable 5.3 Fecundity regulation***, have been postponed by one year (starting in August 2016) and the Deliverable will be submitted in M48. We do not expect any impact on the overall work and achievements of the WP.

WP6 Reproduction & Genetics – wreckfish.

It has been so far proven more difficult to control ovulation (for *in* vitro fertilization) or spawning (for spontaneous tank fertilization) and production of fertilized eggs in response to hormonal therapy. The small number of breeders available by some partners (especially P1. HCMR) has been limiting the ability of a large number of trials during every annual reproductive season. Therefore, ***Milestones 34-36 Successful maturation and spawning of wreckfish to produce good quality eggs*** in Y1, Y2 and Y3, respectively, have been acomplisehed, but not to the complete satisfaction of the consortium. This has, in turn, limited the progress made in the WP 18 Larval husbandry – wreckfish. Nevertheless, slow but important progress is being made and we expect that by the end of the project we will succeed in producing a large number of

viable eggs, to enable the experiments with larval rearing to be implemented in time. Already, better spawning and egg quality has been obtained this summer (July 2016, Mo 31-32), and some larval rearing trials were underway when this report was being prepared.

WP7 Reproduction & Genetics – grey mullet.

Due to poor egg quality and fertilization rates in 2015, the egg shipment protocol that was submitted as ***Deliverable D7.4 Protocol for shipping grey mullet eggs***, was not thoroughly tested during the present reporting period and these studies should be repeated, and a new deliverable will be submitted. Nevertheless, the data collected so far does indicate that the SELFDOTT transport protocol can be readily adapted to shipping grey mullet eggs to the partners in DIVERSIFY. An updated version of the Deliverable will be submitted in Mo 36.

For P24. ITTICAL, it was planned to obtain a stock of wild grey mullet fingerlings (0+ year class) and maintain them until sexual maturity, to implement Task 7.4. In 5 December 2014, we were informed by the P.I. (Dr. Fulvio Cepollaro) that they acquired 5,000 fingerlings of 0.2 g in average size. However, upon examination of the stock maintained in the facilities of P24. ITTICAL in the Spring of 2016 by P13.UNIBA, it was found that the stock consisted of about 200 fish having a body weight ranging between 70 and 750 g in body weight, suggesting that this stock consisted of fish from different year classes (certainly no 1+ year class), and certainly not fish obtained as fingerlings in 2014 as reported by P24. ITTICAL. Therefore, this stock cannot be used for the purpose of Task 7.4 (as we do not know their real age) and contingency plans were made, by using a stock of wild fingerlings obtained by P26. GEI for the Grow out experiments (WP 23). These fish will be maintained for at least 2 yrs or until sexual maturity (as opposed to only 1 year to perform the WP 23 grow out study). In addition, a wild stock of fingeling has been acquired by P4. IOLR and will be also maintained for at least 2 years to be able to fulfil Task 7.4.

WP10 Nutrition – pikeperch.

There have been some delays within the period due mainly to some technical failures (1 experiment) or high cannibalism of larvae (2 experiments) meaning that 3 trials had to be repeated within the time frame of the 2nd Reporting Period. These trials have been repeated, so we have managed to keep on track in terms of planning and future deliverables. The use of resources and planned man months are higher than expected due to repetition of expensive trials, but within the framework of the budget.

WP12 Nutrition – grey mullet.

The effect of DHA/EPA ratio on larval and juvenile performance during rotifer and *Artemia* feeding was planned for the autumn of 2015 and was scheduled to conclude in June 2016. However, due to poor and very few grey mullet spawns, this sub-task is postponed to the autumn of 2016, as well as subtasks 13.3.2 and 13.3.3. However, about 400 F2 juvenile grey mullet were produced from 2015 season and these fish are presently being used to test the effect of dietary DHA/EPA ratio on older juveniles. Subtasks 13.2.1 and 13.2.2 were delayed due to the difficulties in finding student/technician to carry out the work on samples already collected from task 13.1. Having said this, these tasks are currently being carried out.

WP14 Larval husbandry – meagre.

The protocol for early weaning was scheduled for month 18 of the project, however taking into account the problems of cannibalism we decided to repeat the experiments and delay the submission of the ***Deliverable 14.1***, which included the protocol until month 30, in order to be sure that the protocol designed was feasible. There was no impact on other tasks related to meagre rearing.

WP15 Larval husbandry – greater amberjack.

In Task 15.1. a repetition is scheduled for later in 2016 and, therefore, the submission of ***Deliverable 15.2*** is delayed and this was requested earlier this year and was approved by the PC. In Task 15.3. and subtask 15.3.1 a trial will be repeated in 2016 due to total larval mortality in the previous attempt. The submission of ***Deliverable 15.3*** is also delayed as requested earlier this year and approved by the PC.

WP16 Larval husbandry – pikeperch.

The work for ***Deliverable 16.1 Determine effect of environmental factors on pikeperch larval rearing*** initially planned for the 1st Reporting Period, was delayed until month 30, because the experiment was repeated in 2015 due to high and abnormal mortality during the first trial done in 2014. As a result, the Deliverable was in its final revision by the PC at the time this report was prepared, and will be submitted promptly. The work for ***Deliverable D16.2*** initially planned on month 24 was postponed to month 30 because the experiment was delayed in early 2016, as a consequence of the delay of the work for Deliverable 16.1. We do not expect any impact on the project from these delays.

WP17 Larval husbandry – Atlantic halibut.

In Task 17.4, a deviation was necessary as it was not possible to produce enough on-grown *Artemia* beyond 28 days post first-feeding. Therefore, the experiment was terminated at 28 dpff instead of 45 dpff. There are no other deviations during the reporting period.

WP18 Larval husbandry – wreckfish.

The few spawns for poor quality obtained during 2015 was likely due to an unsuitable feeding protocol and the young age of the brood stocks (sexual maturity is achieved > 10 kg), as well as the variable and abnormal environmental conditions compared to previous years at the P32. MC2. Spawn quality at P32. MC2 has improved considerably while the brood stock at the P8. IEO has started to deliver good quality spawns, which suggests that 2016 will be a better year. Nevertheless, we will have to delay the submission of ***Deliverables D18.1***, ***D18.2*** and ***D18.3***, which depend on the availability of an adequate number of eggs of good quality. In 2016 (Mo 31), better quantities and of better wuality eggs have been obtained, and we expect to make significant progress in this WP as well.

WP18 Larval husbandry – grey mullet.

The delays in the planned experiments in 2015 were due to few and poor quality spawns during this season and these studies have been postponed to the 2016 season. We do not expect any negative impact on the outcomes of this WP.

WP22 Grow out husbandry – pike perch.

Milestone and deliverable dates were delayed because (1) the adaptation of the P9. UL facilities to the multifactorial protocol requirements took more time than expected and (2) it was also necessary to perform two preliminary experiments in order to better define some methodological aspects appropriated to the multifactorial stress screening since there is limited information on stress response for pikeperch. Despite that delay, the results from those preliminary assays facilitated the full achievement of ***Deliverable D22.1*** and will not have any effect on the WP.

WP23 Grow out husbandry – grey mullet.

In Task 23.2 there was a deviation from Annex 1 due to unexpected Israeli customs bureaucracy and demands, which delayed the arrival of the extruded mullet feed from P31. IRIDA and did not arrive in time for the growth trial started in 2015. Now that the sell-by-date has been passed, a new feed shipment has been produced and will arrive in May 2016 and will be used to carry out a similar study in 2016-2017 at the P4. IOLR and P25. DOR facilities. We do not expect any impact on the WP, other than the dealy in submitting the relevant Deliverable.

WP24 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". These changes will be requested officialyl in the next amendment (3rd) of the ANNEX 1.

WP26 Fish health – Atlantic halibut.

There has been no deviation so far. However, the wet lab challenge facility that is required for the activities in ***Deliverable 26.3*** has been closed for over 2 years now. The activity has thus not yet been carried out, and there is some uncertainty about whether it will be possible to carry it out during Autumn 2016. This might possibly delay the deliverable 26.3 to month 48. This has been indicated in the previous report.

WP27 Socioeconomics – Institutional and organizational context. All three deliverable presented in this periodic report were delayed by few months. This was mainly due to the delay of ***Deliverable 27.1*** and due to convergence of work around Christmas 2014. However all deliverables have been finalized and submitted before March 2015.

WP28 Socioeconomics – New product development.

We have faced some delays mainly due to the difficulties to find the raw material needed for the different activities. ***Deliverable 28.1*** was the only one delivered on time in month 14. ***Deliverable 28.2*** had a delay of four months and was delivered in month 20 thus affecting ***Deliverables 28.3*** and ***D28.4*** that were submitted with a delay of five and three months, respectively. These delays have also affected some the related activities described in WP 29 (see later), although an important part of these delays has been absorbed and reduced by WP 29, thus minimising their impact in the correct development of the socio-economic tasks.

Consortium modifications

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 Lead Beneficiary (Leader) of WP 6 Reproduction and Genetics –wreckfish and WP 18 larval husdandry - wreckfish.

P11. AU. On 8 July 2016 (Mo 31), the PC was informed by Prof. A. Krystallis, the PI of P11. AU that he will relinquish his position at this Partner, and there will be a new person taking his position in the organization. The official letters required for such a change (**Figure 2.3.6** left) were already provided by P11. AU and have been forwarded to the EU Legal and Scientific Officers, with an email from the PC on 14 July 2016. The partner has also requested that some of their 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 (**Figure 2.3.6** right). This transfer will be examined by the PC and the partners involved (WP 29) and the request will be included in the 3rd Amendment that will be prepared soon.

AARHUS UNIVERSITY

Subject: PIC: 999997736
Legal entity name: Aarhus Universitet

CHANGE OF PERSON IN CHARGE OF SCIENTIFIC AND TECHNICAL/TECHNOLOGICAL ASPECTS IN DIVERSIFY PROJECT FOR BENEFICIARY NO. 11

I, Mr. Jesper Juel Holst, in my capacity as LEAR and authorised representative at Aarhus University and thereby authorized to legally represent my organization (as substantiated in the enclosed documents) confirm the shift in person in charge of scientific and technical/technological aspects in the DIVERSIFY project (contract no. 603121) for beneficiary no. 11 from being Athanasios Krystallis to being Klaus G. Grunert by November 1st, 2016.

Kindly reflect the change in your records.

Kind regards

Jesper Juel Holst
Authorised representative

AARHUS UNIVERSITET

Research Support Office
Jesper Juel Holst
Head of Section
Date: 13 July 2016
Mobile Tel.: +45 30587943
E-mail: jjh@au.dk
Sender's CVR no.: 31119103
Page 1/1

Research Support Office
Aarhus University
Katrinebjergvej 107
DK-8200 Aarhus N
Denmark

Tel.: +45 +45 8715 0000
Fax: +45 +45 8715 0201
E-mail: adm@au.dk
medarbejdere.au.dk/en/administration/au-administration/au-research-support-and-external-relations/research-support-office/

MAPP CENTRE - RESEARCH ON VALUE CREATION IN THE FOOD SECTOR
DEPARTMENT OF MANAGEMENT
AARHUS UNIVERSITY

Dr. Constantinos C. Mylonas
Institute of Marine Biology, Biotechnology and Aquaculture
Hellenic Centre for Marine Research
1 Aqualabs, Thalassokosmos
P.O.Box 2214, Iraklion
Crete 71003, GREECE

DIVERSIFY

Dear Dr. Mylonas

As Professor Athanasios Krystallis will be quit his position at Aarhus University by October 31 and join another DIVERSIFY partner, HRH S.A., by November 1, some elements of the work in tasks for which AU has responsibility will be moved from AU to HRH. This refers to the following tasks:

a) Sub-task 29_3.2: Online experiments with consumer samples of N=80 per country/segment at minimum, in order to define the best-matching extrinsic/intrinsic attribute combinations that can stimulate preference for the new fish products studied within consumer segments with high adoption potential (Deliverable D29.6: "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").
b) Task 29_4: An additional series of online experiments with similar consumer samples in order to test various communication parameters (i.e. message type and source) for their ability to influence consumer value perceptions and attitudes, as well as purchasing intention and willingness to pay for the best matching extrinsic/intrinsic attribute combinations of sub-task 29.3.2 (Deliverable D29.7: "Communication parameters as experimental stimuli" and Deliverable D29.8: "Report on the results of the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers attitudes and behavior").

On this background, I ask you to move 28,335 € plus overhead from the AU salary budget to the HRH budget.

Kind regards,

Klaus G. Grunert
Professor, Dr.oec.habil., Dr.h.c., MPP Director

Klaus G. Grunert
Professor, Dr.oec.habil., Dr. h.c.
Date: 25. juli 2016
Phone.: +45 87 165057
Email: kgg@mgmt.au.dk
Page: 1/1

MAPP Centre
Department of Management
Bartholins Allé 14
8000 Aarhus C

Tel.: +45 87 165057
Email: mapp@mgmt.au.dk
http://www.mapp.au.dk

Figure 2.3.6. Letters of request to change the PI of P11. AU (left), and to transfer activities and budget from P11. AU to P38. HRH (right).

P24. ITTICAL. On 11 January 20156 Dr Fulvio Cepollaro (Principle investigator, 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 (**Figure 2.3.7**). 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. As indicated in the Section "Deviation from the DOW" for WP2 Reproduction and Genetics – greater amberjack and WP 7 Reproduction and Genetics – grey mullet, implementation of all tasks has been very difficult with this Partner, and even though we trust that this partner has made every effort to comply with its obligations, it has been underperforming in the consortium and the PC had already decided to make a motion to the Steering Committee of DIVERSIFY to discontinue their participation in the project.

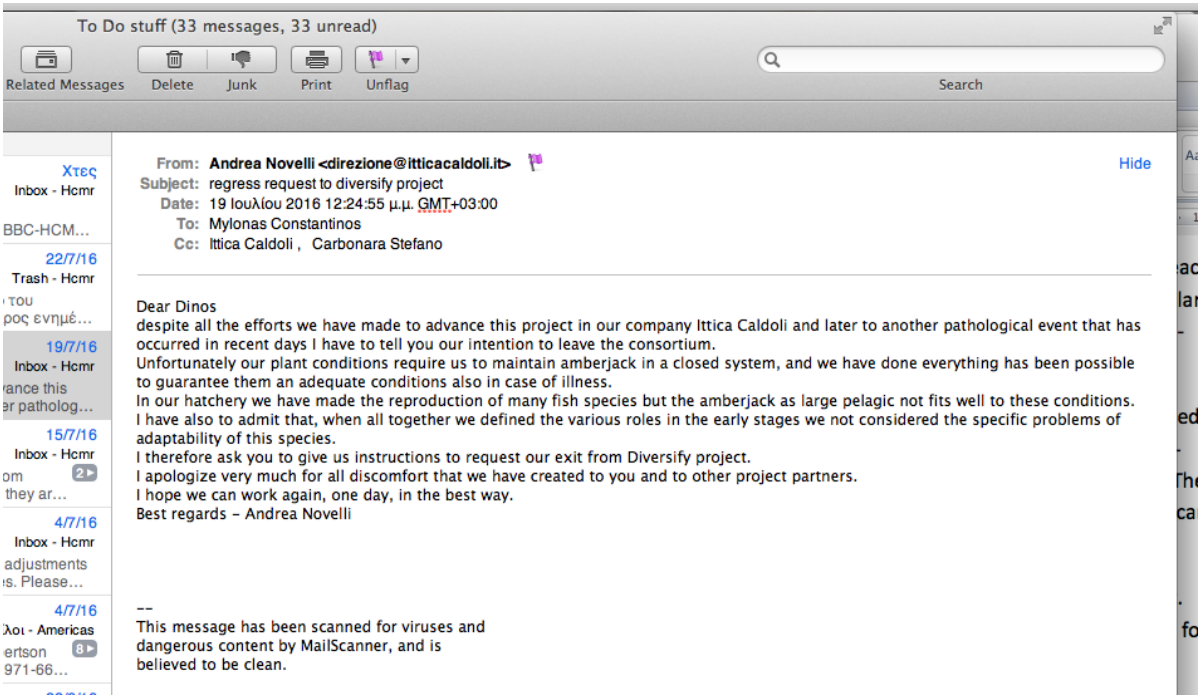


Figure 2.3.7. An email message from Mr Andrea Novelli, the CEO of P24. ITTICAL (SME).

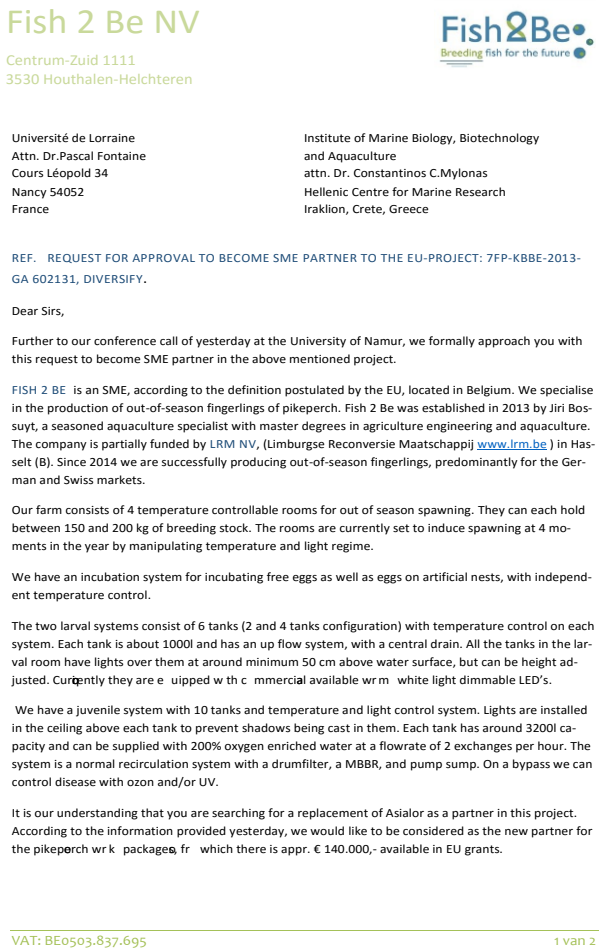
A contingency plan for the exit of P24. ITTICAL includes the transfer of this partner's activities to another partner within the consortium or to a new partner. Regarding WP2 Reproduction and Genetics – greater amberjack, the spawning induction tasks (Task 3.2) can be allocated to either P23. ARGO, which is already a partner in the consortium and has been performing very well so far. Alternatively, we can invite another SME such as as 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 will be able to participate also in the implementation and the industrial trials on the larval rearing and grow out.

Regarding WP7 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/or P26. GEI, who have already acquired wild fingerlings durin Y1 of the project, in order to implement other tasks. They have already been contacted and they are willing to undertake this responsibility.

The decision of inviting another partner (*i.e.* GMF) or allocating the remaing tasks of P24. ITTICAL to one of the existing partners will be made soon after the submission of the 2nd Periodic Report by the PC in coordination with the WP leaders, Task leaders and Steering Committee, considering (a) the best option for the successful implementation of the tasks, (b) the required (if any) budget transfers that need to be made, and (c) the most efficient utilization of the resources available within the CONSORTIUM and to any potential new partner. The EU Scientific Officer will be kept updated of this process and will be consulted prior to any decision, in order to ensure that the decision conforms to any official requirements.

P25. DOR. On 8 May 2016, we were informed that there was a change in the P.I. of the partner, from Mr Gilad Shafran to Mr Hagay Sarusi, who was also appointed as the LEAR of the partner. The official letters required for the change have been forwarded to the EU Legal and Scientific Officers, with an email from the PC on 9 June 2016.

P29. ASIALOR. In May 2016 (Mo 31) we were informed that the company has filed for buncruptcy and they are going to exit the consortium. The company has fulfilled so far their obligations and have been a valuable partner. Efforts have already been initiated to find another SME that could take on their remaing 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 (**Fig. 2.3.8**). 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 legal officers (Mrs Paula Wahlman Dakhiland Mrs Maria-Valeria Iliadou, sbout this consortium modification, in order to obtain all the necessary documents and take the required actions.



Fish 2 Be NV

Centrum-Zuid 1111
3530 Houthalen-Helchteren

Fish2Be
Breeding fish for the future

Université de Lorraine
Attn. Dr.Pascal Fontaine
Cours Léopold 34
Nancy 54052
France

Institute of Marine Biology, Biotechnology and Aquaculture
attn. Dr. Constantinos C.Mylonas
Hellenic Centre for Marine Research
Iraklion, Crete, Greece

REF. REQUEST FOR APPROVAL TO BECOME SME PARTNER TO THE EU-PROJECT: 7FP-KBBE-2013-GA 602131, DIVERSIFY.

Dear Sirs,

Further to our conference call of yesterday at the University of Namur, we formally approach you with this request to become SME partner in the above mentioned project.

FISH 2 BE is an SME, according to the definition postulated by the EU, located in Belgium. We specialise in the production of out-of-season fingerlings of pikeperch. Fish 2 Be was established in 2013 by Jiri Bossuyt, a seasoned aquaculture specialist with master degrees in agriculture engineering and aquaculture. The company is partially funded by LRM NV, (Limburgse Reconversie Maatschappij www.lrm.be) in Hasselt (B). Since 2014 we are successfully producing out-of-season fingerlings, predominantly for the German and Swiss markets.

Our farm consists of 4 temperature controllable rooms for out of season spawning. They can each hold between 150 and 200 kg of breeding stock. The rooms are currently set to induce spawning at 4 moments in the year by manipulating temperature and light regime.

We have an incubation system for incubating free eggs as well as eggs on artificial nests, with independent temperature control.

The two larval systems consist of 6 tanks (2 and 4 tanks configuration) with temperature control on each system. Each tank is about 1000l and has an up flow system, with a central drain. All the tanks in the larval room have lights over them at around minimum 50 cm above water surface, but can be height adjusted. Currently they are equipped with commercial available warm white light dimmable LED's.

We have a juvenile system with 10 tanks and temperature and light control system. Lights are installed in the ceiling above each tank to prevent shadows being cast in them. Each tank has around 3200l capacity and can be supplied with 200% oxygen enriched water at a flowrate of 2 exchanges per hour. The system is a normal recirculation system with a drumfilter, a MBBR, and pump sump. On a bypass we can control disease with ozon and/or UV.

It is our understanding that you are searching for a replacement of Asialor as a partner in this project. According to the information provided yesterday, we would like to be considered as the new partner for the pikeperch work packages, for which there is appr. € 140.000,- available in EU grants.

VAT: BE0503.837.695 1 van 2

Figure 2.3.8. Letter of request to join the consortium by an SME (Fish 2 Be, NV), in order to replace P29. ASIALOR.

Budget mofications

Budget modifications have been requested and approved as part of the 2nd Amendment (submitted on 11 November 2015 and approved in 15 February 2016). The budget amendment was the result of

- the exit of P30. CULMAREX from the consortium and the reallocation of its remaining budget,
- the transfer of WP3 tasks from P24. ITTICAL to P23. ARGO (mainly) and other Partners, and
- some minor transfers of budget among eligible catergories within some Partners.

P1. HCMR. This partner has requested a change in the equipment budget allocation, as we would like to obtain new sensory analysis booths that will be used within the Task 28 (subtask 28.2.1, subtasks 28.3.1 & 28.3.3) for sensory analysis (QDA) of the studied fish species and their products. This was not included in the initial proposal and DOW, as we already have four sensory booths in the HCMR taste panel room.

However, the need of an increased number of panelists that have to be trained simultaneously was not taken into account in the initial plan. Although 4-5 panelist are adequate for conducting the tests within this project, the need of a number of 2-3 times more panelists (than those conducting the tests) to be available, leads to the need of higher number of booths. The training has to take place simultaneously for all panelists and is a time- and resource-consuming process. Without the number of minimum 10-12 trained panelist there is a serious danger of not being able to conduct tests at the time fish or products will be available. The high perishability of those foods does not allow changing time of conduct for the tests, thus we have to ensure availability of panelists at certain time intervals. Coupled with the difficulty (at this stage) of obtaining fish of some of the species included in the DIVERSIFY (since these are either not cultured yet, or are produced in very small quantities and not in all countries), the availability of high number of trained panelists when the products become available, becomes imperative.

The total cost for these additional 8-place of sensory analysis booth is 3,650 Euros, and we request that this amount is considered eligible for funding by the project, as it will be used throughout the remaining 4 years of the project. It is pointed out that there is no need for changes in (a) either the total budget of HCMR, or (b) the total amount allocated for durable equipment for HCMR. The requested amount will become available by obtaining lower-cost software for sensory analysis and lower cost monitors (that have been already been included in the DOW within the requested tasks).

The above request was forwarded and approved by the EU Scientific Officer with an email of 21 January 2015, pending the approval of the EU Financial Officer (Mrs Elisabeth Aichinger), who unfortunately has not replied to the PC's email message of 2 February 2015.

Furthermore, P1. HCMR is expected to claim a higher number of staff effort in a number of WPs (*e.g.* WP2, 3, 15, 25, 31) to address issues of (a) repetition of experiments due to sub-optimal results, (b) underestimation of required staff effort to implement some proposed work/experiments and (c) additional tasks that have been included along the lifetime of the project. As for the above request, there is no need for changes in the total budget of HCMR and the additional staff effort will become available from savings in consumables. These proposed modifications will be included in the in the 3rd Amendment to be prepared soon after the submission of the 2nd Periodic Report.

P11. AU. Due to the fact that Prof. A. Krystallis, the PI of P11. AU will relinquish his position at this Partner, the partner has requested 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. This transfer will be examined by the PC and the partners involved (WP 29) and the request will be included in the 3rd Amendment that will be prepared soon.

P18. CTAQUA. This partner requests a budget shift among WPs (**Fig. 2.3.9**). At the middle of the project, most of the work within WP28 Socioeconomics-New Product development (R&D activity), is finalized. The budget for WP28 for the partner was 105,829 € (79,372 € EU funded). Once the economic reporting has been done, part of this budget will no longer be required for these WPs. However, there are other WPs responsibilities of this partner that will need further economic support.

One of these cases is in WP23. 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", is still running and will last for another 5 months minimum. This task had a budget of 20,071 € (EU funded) and has consumed already 70% of the budget. 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 WP13, 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", has a budget of 15,000 € (EU funded) that will not be sufficient to correctly cover the obligations of the partner within this task. So the partner requests a 15,000 € 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, such as

- Tasks 31.5 "Full-day seminar on "Know-how Transfer" of the aquaculture of each of the DIVERSIFY species", led by CTAQUA and the Species Leader Partner,
- Task 31.6 "Promotional workshops in 4 strategic countries", led by CTAQUA, and
- Task 31.7 Dissemination to the food industry and consumers, ***Deliverable 31.24*** Technical leaflets with main technical information on the culture of DIVERSIFY fish species.

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 of another 15,000€ 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 (**Figure 2.3.9**), and among WPs will be modified. The full description of the budget and staff effort modification will be included in the 3rd Amendment to be prepared soon after the submission of the 2nd Periodic Report.

BUDGET IN DOW

		RTD (WPs 2-30)	Demonstration	Support	Management (WP 1)	Other (WP31 Dissemination)	Total
Personnel costs		107,400 €			2,400 €	30,640 €	140,440 €
Subcontracting							0 €
Other direct costs **	Consumables	34,703 €			0 €	2,595 €	
	Travel	7,433 €			3,550 €	7,175 €	
	Equipment						
	sub Total	42,136 €	0 €	0 €	3,550 €	9,770 €	55,456 €
Indirect costs (overheads)		89,722 €			3,570 €	24,246 €	117,538 €
Total budget		239,258 €	0 €	0 €	9,520 €	64,656 €	313,434 €
Requested EU contribution		179,443 €	0 €	0 €	9,520 €	64,656 €	253,619 €

REQUESTED

		RTD (WPs 2-30)	Demonstration	Support	Management (WP 1)	Other (WP31 Dissemination)	Total
Personnel costs		97,400 €			2,400 €	38,140 €	137,940 €
Subcontracting							
Other direct costs **	Consumables	24,703 €			0 €	5,095 €	
	Travel	7,433 €			3,550 €	12,175 €	
	Equipment						
	sub Total	32,136 €	0 €	0 €	3,550 €	17,270 €	52,956 €
Indirect costs (overheads)		77,722 €	0 €	0 €	3,570 €	33,246 €	114,538 €
Total budget		207,258 €	0 €	0 €	9,520 €	88,656 €	305,434 €
Requested EU contribution		155,443 €	0 €	0 €	9,520 €	88,656 €	253,619 €

Figure 2.3.9. Proposed budget shifts between RTD and DISSEMINATION activities.

P19. CMRM. This partner requests an increase in the staff effort in WP7. Due to the fact that the wreckfish stock expected to be used as breeders, did not mature the first year of the study (although they were of the right size, so this development was not expected), the partner had to put much more staff effort to this Task. 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, there is no request for additional budget, but solely for an increase in claimed staff effort from 13.6 in the original DOW to 18.8 PM now. The staff effort modification will be included in the 3rd Amendment to be prepared soon after the submission of the 2nd Periodic Report. The modification was discussed with the EU Scientific Officer, who approved it with an email of 21 December 2015.

P38. HRH. P38. HRH. Due to the fact that Prof. A. Krystallis, the PI of P11. AU will relinquish his position at this Partner, 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 of 28,335 plus overheads. The latter partner has accepted this transfer with a letter to the PC on 26 July 2016, and upon examination and approval by the EU Scientific Officer a request will be included in the 3rd Amendment that will be prepared soon.

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:

- 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.
- 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.

As a result of the above, P38. HRH request an additional Total budget of ~€ 25.000, in order to cover costs of subcontracting and other direct costs. There will be no increase in the personnel costs, which will be absorbed by the Partner. The additional budget will come from a Partner-to-Partner budget shift, and not from an additional request from the EC. The request will be made in the 3rd Amendment.

Various Partners. Other budget modifications are expected in the 3rd Amendment, addressing:

(a) minor within-Partner reallocation of budget among eligible categories, to address issues with higher than expected consumanble or personnel costs in some WPs or Tasks, or to address savings from originally planned durable equipment purchases,

(b) Major between-Partner money transfers, due to the request to exit the consortium by P29. ASIALOR and P24. ITTICAL,

(c) Invitation of a new partner, to take over the activities of exiting partners.

As before, every possible effort will be made to identify another appropriate partner to substitute the above exiting parnters, or if possible to reallocate the remaining tasks to one of the existing partners.

3rd Amendment (in preparation)

Various issues have arisen during the 2nd Reporting Period, necessitating the submission of a request for an Amendment to the Annex I. Some of the reasons include the following:

- Consortium modifications due to the exit of P24. ITTICAL and P29. ASIALOR, and the possible entry of a new partner that will take over their activities,
- Budget transfers among existing (and) new partners that will take the responsibility of the remaining activities of the exiting partners,
- Modifications in the methods that will be used to carry out some proposed work, such as for example in WP 2, Task 2.5. Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers (See Section Deviations from the DOW, WP3 Reproduction and Genetics - meagre),

- Modifications in the location (*e.g.* Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean) or the experimental design (*e.g.* Task 3.5 Development of an optimized spawning induction protocol for F1 greater amberjack in the Atlantic) of a proposed work (See Section Deviations from the DOW, WP3 Reproduction and Genetics - meagre),
- Modifications in the delivery of some Deliverables, due to problems in the implementation of the experiments (*e.g.* WP 18 Larval husbandry – wreckfish, See Section Deviations from the DOW, WP18),
- Budget transfers within Partners, among categories of eligible costs (*e.g.* personnel, consumables, travel or durable equipment) and between type of activity (*i.e.* RTD and DISSEMINATION) (*e.g.* P1. HCMR and P18. CTAQUA, See Section Budget modifications),
- Budget transfers between Partners, to address underestimation of proposed tasks, or modifications of implemented tasks (*e.g.* Partner 38. HRH, See Section Budget modifications),
- Increases in staff effort in some WPs by some Partners,

The amendment session will be requested as soon as possible after the submission of the 2nd Periodic Report and once a decision is made by the consortium on (a) the transfer of the remaining activities from P24. ITTICAL and P29. ASIALOR, and (b) the new partners (SMEs) that will be invited to enter the consortium.

3 Deliverables and milestones tables

Deliverables

The deliverables due in this reporting period, as indicated in Annex I to the Grant Agreement have to be uploaded by the responsible participants (as indicated in Annex I), and then approved and submitted by the Coordinator. Deliverables are of a nature other than periodic or final reports (ex: "prototypes", "demonstrators" or "others"). ***The periodic reports and the final report have NOT to be considered as deliverables****. If the deliverables are not well explained in the periodic and/or final reports, then, a short descriptive report should be submitted, so that the Commission has a record of their existence. If a deliverable has been cancelled or regrouped with another one, please indicate this in the column "Comments". If a new deliverable is proposed, please indicate this in the column "Comments". The number of persons/month for each deliverable has been defined in Annex I of the Grant Agreement and cannot be changed. In SESAM, this number is automatically transferred from NEF and is not editable. If there is a deviation from the Annex I, then this should be clearly explained in the comments column. This table is cumulative, that is, it should always show all deliverables from the beginning of the project.*

TABLE 1. DELIVERABLE

Del. no.	Deliverable name	Version	WP no.	Lead benefi-ciary	*Nature*	Dissemination level[5]	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	1	1	1	Other	RE	2	10/02/2014	Submitted	Due to the project

[5] **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 "

	coordination meeting for Y1									starting in December, it was not possible to have the meeting during month 1 of the project.
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			30		Delayed	Submittion will follow the actual midterm evaluation, which will be scheduled by the EU-RTD (Scientific Officer)
2.1	SNP library and chip to genetically characterise meagre or to use in marker assisted breeding programs.	1.0	2	1	R	PU	18	01/06/ 2015	Submitted	
2.2	Genetic characterization of different meager captive	1	2	2	Report	PU	12	15/12/2014	Submitted	

	broodstocks and evaluation of available variability									
2.3	Protocol for paired spontaneous tank spawning of meagre.	1.0	2	3	R	PU	21	22/09/2015	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).	1.0	3	4	R	PU	12	17/03/2015	Submitted	
3.2	Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack	1.0	3	4	R	PU	18	31/8/2016 (Mo 33)	Delayed	We faced difficulties in the development of the expression plasmid for the production of the recombinant proteins, which will be used for the production of antibodies.
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	1.0	3	13	R	PU	24	26/01/2016	Submitted	
4.1	Genetic analysis of domesticated pikeperch broodstocks	1.0	4	9	R	PU	12	19/11/2014	Submitted	
4.2	Population genetic analysis of	1.0	4	1	R	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	1.0	5	7	R	PU	30	31/08/2016 (Mo 33)	Delayed	Deliverable delayed due to prolonged sick leave of the leading researcher
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	1.0	5	7	R	PU	30	12/05/2016	Submitted	
5.3	Identification of potential disturbances in reproductive development in F1/F2 Atlantic halibut females	1.0	6	14	R	PU	24	27/11/2015	Submitted	
6.1	Computer Assisted Sperm Analysis (CASA) for wreckfish sperm	1.0	6	14	R	PU	24	27/11/2015	Submitted	
6.2	Cryopreservation method for wreckfish	1.0	7	14	R	PU	12	12/12/2014	Submitted	
7.1	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of grey mullet sperm	1.0	7	4	R	PU	18	12/06/2015	Submitted	
7.2	Production of recombinant bioactive LH and FSH assay for grey mullet	1.0	7	4	R	PU	24	27/11/2015	Submitted	
7.3	Comparative effectiveness of hormonal treatments for	1.0	7	4	R	PU	24	30/11/2015	Submitted	

	spawning induction in captive grey mullet									
7.4	Protocol for shipping grey mullet eggs	1.0	3	4	R	PU	24	26/01/2016	Submitted	The Deliverable will be resubmitted on Mo 36 because of low egg quality, necessitating more trials
8.1	Improvement of larval weaning diets	1.0	8	2	R	PU	24	3/12/2015	Submitted	
9.1.	Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids	1.0	9	2	R	PU	24	16/12/2015	Submitted	
11.1	Report on nutrient profile of Artemia nauplii and ongrown Artemia from IMR and SWH	1.0	11	7	R	PU	24	28/11/2015	Submitted	
13.1	Determine changes in the essential fatty acid requirement as a function of developmental stage and ambient salinity in grey mullet	1.0	13	4	R	PU	18	30/11/2016 (Mo 36)	Delayed	The 2015 spawning season was poor (low egg quality), therefore this task could not be performed and will be repeated in the 2016 season (Sept-Dec)
13.2	Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet	1.0	13	4	R	PU	18	30/11/2016 (Mo 36)	Delayed	We faced difficulties in recruiting the student to perform the study, as well as technical problems in the design of the necessary primers.
14.1	Improved larval rearing protocol for meagre that includes weaning at an earlier		14	3	R	PU	30	16/05/2016	Submitted	

	age leading to reduced cost in live feed production and better quality juveniles									
15.1	Effective greater amberjack larval stocking densities	1.0	15	2	R	PU	16	09/05/16	Submitted	
15.2	Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing	1.0	15	8	R	PU	27	30/10/2016 (Mo 35)	Delayed	Severe mortalities during the first trial (2015) necessitate the repetition of this experiment
15.3	Optimum hydrodynamics and light conditions during greater amberjack larval rearing	1.0	15	2	R	PU	27	30/10/2016 (Mo 35)	Delayed	Severe mortalities during the first trial (2015) necessitate the repetition of this experiment
15.4	Ontogeny of greater amberjack larval visual and digestive system	1	15	1	R	PU	27	24/05/2016	Submitted	
16.1	Determine effect of environmental factors on pikeperch larval rearing	1	16	9	R	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 cause 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	R	PU	24	36	Delayed	High mortalities in the larval experiment necessitate the repetition of this experiment. The analyses are planned for the summer of 2016 and the deliverable will be prepared soon after that.
17.1	Production protocol of on-grown Artemia	1	17	7	R	PU	24	28/11/2015	Submitted	
19.1	Determine most effective type and concentration of algae used in grey mullet larval rearing	1	19	4	R	PU	24	36	Delayed	An equipment failure caused a delay in the experiment. The analyses have been completed and the deliverable is under preparation at the time the report was prepared.
20.1	Methodology to avoid size variability in meagre juveniles	1	20	3	R	PU	24	28/11/2015	Submitted	
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	R	PU	24	17/5/2016	Submitted	The delay was due to the necessity for adaptation of experimental tanks by P. 9 UL and to methodological refinement by P16. FUNDP.

23.1	Cost-effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status	1	23	3	R	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	R	PU	18	31/5/2017	Delayed	Unexpected Israeli customs bureaucracy and demands delayed the arrival of the extruded mullet feed from P31. IRIDA and did not arrive in time for the growth trial planned for 2015
24.1	The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre	1	24	1	R	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	R	PU	24	15/6/2016	Submitted	
24.3	Cloning of key marker genes of innate and adaptive immune responses in meagre	1	24	5	R	PU	26	20/1/2016	Submitted	
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	R	PU	24	13/11/2015	Submitted	
27.1	Report on external environmental factors that affect or will affect the	1	27	6	R	PP	14	22/05/2014	Submitted	The time required for this Deliverable was

	production chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet									under-estimated
27.2	Report on current certification schemes and standards and their business dynamics in the fish supply chain	1	27	6	R	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	R	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	R	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	R	PU	12	28/11/2014	Submitted	
27.6	List of critical success factors for market acceptance	1	27	6	R	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	R	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	R	PU	14	14/4/2015	Submitted	
28.2	List of ideas for new product development	1	28	1	R	PU	16	21/7/2015	Submitted	

28.3	Report on product and process solutions for each species based on technological, physical and sensory characteristics	1	28	1	R	PU	18	5/10/2015	Submitted	
28.4	Physical prototypes of new products from the selected species meagre, greater amberjack, wreckfish, pikeperch and grey mullet	1	28	3	R	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	R	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	R	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	R	PU	28	4/4/2016	Submitted	
29.4	Report on the actual products' sensory profiling in the five countries investigated	1	29	3	R	PU	29	27/7/2016	Submitted	
29.5	Development of the product mock-ups for use in the experimentation with	1	20	11	R	PU	30	27/7/2016	Submitted	

	consumers in the five countries investigated									
31.1	Establishment of website (www.diversifyfish.eu)	1	31	18	R	PU	4	02/04/2014	Submitted	
31.2	Project logo and brochure	1	31	18	R	PU	6	24/06/2014	Submitted	
31.3	Publication of the first of two articles in Food Today	1	31	37	R	PU	6	30/05/2014	Submitted	
31.4	Production and release of audio-visual material	1	31	18	R	PU	6	24/06/2014	Submitted	
31.5	Collaboration agreement with food industry and consumer organization; linkage of websites	1	31	18	R	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	R	PU	9	27/10/2014	Submitted	
31.7	Production and release of audiovisual material	1	31	18	R	PU	12	15/01/2015	Submitted	
31.8	Production and release of audiovisual material	1	31	18	R	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	R	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	R	PU	21	16/11/2015	Submitted	
31.11	Scientific publications in relevant journals	1	31	1	R	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		31	18	R	PU	24	30/12/2015	Submitted	Production and release of audiovisual material
31.13	Production and release of audiovisual material		31	18	R	PU	30	25/6/2016	Submitted	Production and release of audiovisual material

Milestones

Please complete this table if milestones are specified in Annex I to the Grant Agreement. Milestones will be assessed against the specific criteria and performance indicators as defined in Annex I.

This table is cumulative, which means that it should always show all milestones from the beginning of the project.

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
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
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	
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
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	
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	No	01/12/2016	Experiments delayed and redesigned in response to results from MS43
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	No	31/8/2016	There have been some licensing delays in obtaining permission to use the P28. CANEXMAR sea cage for this species.
48	Experiment on the definition of optimal conditions for pikeperch on growing implemented	22	16	31/5/2016	Yes	31/5/2016	
50	Experimental trials of grey mullet in the three locations implemented	23	4	28/2/2015	Yes, partly	31/5/2016	Unexpected Israeli customs bureaucracy delayed the arrival of the extruded mullet feed from P31. IRIDA and did not arrive in time for the growth trial planned for 2015. The other two partners started on time their Tasks.
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	

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	
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 the organizations proposed in the DOW.

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)[6].

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 2nd Reporting period. As there were some retroactive modifications of the staff effort during the 1st Reporting Period, this staff effort is also presented here (**Tables 4.1c-d**).

[6] 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 2nd Reporting Period (13-31 Mo). The "Total Project" effort included in the DOW is shown as well.

DIVERSIFY	2nd Periodic Report (13-30 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
			667.76	166.62	144.90	28.53	25.05	18.84	5.54	17.23	14.27	19.46	1.31	16.80	0.99	19.00	6.79	23.04	27.25	8.60	17.45	8.82	1.37	6.59	8.85
WP1 Management	Management	1	29.63	23.00		0.61	0.75	0.25	0.69		0.14		0.05	0.10	0.18	0.10	0.12	0.09	0.25	0.54	0.10	0.30	0.14	0.04	0.30
WP2 Reproduction and Genetics			137.07	28.36	10.90	7.14	9.25	0.00	0.00	5.90	5.00	0.00	0.00	0.00	0.00	18.90	6.67	3.88	0.00	0.00	0.00	4.31	0.00	0.00	5.55
2.1	Reproduction - meagre	2	18.05	9.38		6.04											2.63								
2.2	Reproduction - amberjack	3	79.64	10.50	10.90		5.65				2.60					18.90	2.38	3.28							
2.3	Reproduction - pikeperch	4	3.68	3.68																					
2.4	Reproduction - halibut	5	11.75	0.30						5.90															5.55
2.5	Reproduction - wreckfish	6	14.05	4.20		0.70					2.40						1.30	0.56				4.31			
2.6	Reproduction - mullet	7	9.90	0.30		0.40	3.60										0.36	0.04							
WP3 Nutrition			97.43	3.50	37.60	0.57	10.90	0.00	0.00	2.41	1.60	0.00	0.00	0.00	0.00	0.00	0.00	9.06	13.00	7.68	0.00	2.55	0.00	6.06	0.00
3.1	Nutrition - meagre	8	15.66		12.80													1.24						1.62	
3.2	Nutrition - amberjack	9	16.94	3.50	9.10						1.20							3.14							
3.3	Nutrition - pikeperch	10	23.58															3.64	13.00					4.44	
3.4	Nutrition - halibut	11	11.12							2.41								1.03		7.68					
3.5	Nutrition - wreckfish	12	2.95								0.40											2.55			
3.6	Nutrition - mullet	13	27.17		15.70	0.57	10.90																		
WP4 larval husbandry			88.24	19.13	19.00	0.77	3.10	0.00	0.00	8.49	5.55	17.20	0.00	0.00	0.00	0.00	0.00	5.65	0.00	0.38	0.00	1.66	0.00	0.00	3.00
4.1	Larval husbandry - meagre	14	0.93			0.24												0.69							
4.2	Larval husbandry - amberjack	15	42.94	16.13	19.00						2.85							4.96							
4.3	Larval husbandry - pikeperch	16	21.73			0.53						17.20													
4.4	Larval husbandry - halibut	17	11.87							8.49										0.38					3.00
4.5	Larval husbandry - wreckfish	18	7.67	3.00							2.70											1.66			
4.6	Larval husbandry - mullet	19	3.10				3.10																		
WP5 Grow out husbandry			95.20	20.33	18.00	3.31	1.05	0.00	0.00	0.00	1.50	2.05	0.00	0.00	0.00	0.00	0.00	0.72	14.00	0.00	2.10	0.00	0.00	0.49	0.00
5.1	Grow out husbandry - meagre	20	36.11	16.33		2.48																			
5.2	Grow out husbandry - amberjack	21	22.82	1.00	18.00						1.50							0.72							
5.3	Grow out husbandry - pike perch	22	19.54									2.05							14.00					0.49	
5.4	Grow out husbandry - mullet	23	16.73	3.00		0.83	1.05														2.10				
WP6 Fish health			132.84	45.30	59.40	5.96	0.00	18.59	0.00	0.43	0.40	0.00	0.00	0.00	0.00	0.00	0.00	1.53	0.00	0.00	0.00	0.00	1.23	0.00	0.00
6.1	Fish health - meagre	24	75.20	34.30	24.40	5.96		9.31															1.23		
6.2	Fish health - amberjack	25	57.21	11.00	35.00			9.28			0.40							1.53							
N/A	Fish health - halibut	26	0.43							0.43															
WP7 Socioeconomics			76.47	23.00	0.00	9.62	0.00	0.00	4.85	0.00	0.00	0.00	1.26	16.70	0.00	0.00	0.00	2.11	0.00	0.00	11.90	0.00	0.00	0.00	0.00
7.1	Institutional and organizational context	27	0.00																						
7.2	New Product Development	28	37.79	15.00		6.56			2.55				0.88	2.70				2.11			6.25				
7.3	Consumer value perceptions and behavioral change	29	38.30	8.00		3.06			2.30					14.00							5.65				
7.4	Business model and marketing strategy development	30	0.38										0.38												
WP8 Dissemination	Dissemination	31	10.89	4.00		0.55					0.08	0.21			0.81						3.35				

Table 4.1b Staff effort per Partner (P23-38) and WP, during the 2nd Reporting Period (13-30 Mo). The "Total Project" effort included in the DOW is shown as well.

DIVERSIFY	2nd Periodic Report (13-30 months)																		
KBBE 2013.1.2.09. Diversification of fish species and products in aquaculture																			
Calculation of staff effort per parter and sub WP																			
				Example	SME partners									New Partners						
Work Package (Proposal)	Title	DOW WP	Total	1. HCMR	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
			667.76	166.62	39.80	6.44	4.00	6.10	0.20	1.50	9.65	0.00	1.85	0.96	0.55	0.18	0.96	1.07	0.00	7.21
WP1 Management	Management	1	29.63	23.00	0.20	0.11		0.10	0.10		0.15		0.10	0.07	0.05	0.04	0.34	0.44		0.18
WP2 Reproduction and Genetics			137.07	28.36	22.30	6.33	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.00	0.00	0.00
2.1	Reproduction - meagre	2	18.05	9.38																
2.2	Reproduction - amberjack	3	79.64	10.50	22.30	3.13														
2.3	Reproduction - pikeperch	4	3.68	3.68																
2.4	Reproduction - halibut	5	11.75	0.30																
2.5	Reproduction - wreckfish	6	14.05	4.20										0.58						
2.6	Reproduction - mullet	7	9.90	0.30		3.20	2.00													
WP3 Nutrition			97.43	3.50	0.00	0.00	0.00	0.00	0.00	0.00	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.1	Nutrition - meagre	8	15.66																	
3.2	Nutrition - amberjack	9	16.94	3.50																
3.3	Nutrition - pikeperch	10	23.58								2.50									
3.4	Nutrition - halibut	11	11.12																	
3.5	Nutrition - wreckfish	12	2.95																	
3.6	Nutrition - mullet	13	27.17																	
WP4 larval husbandry			88.24	19.13	0.00	0.00	0.00	0.00	0.00	0.00	4.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00
4.1	Larval husbandry - meagre	14	0.93																	
4.2	Larval husbandry - amberjack	15	42.94	16.13																
4.3	Larval husbandry - pikeperch	16	21.73								4.00									
4.4	Larval husbandry - halibut	17	11.87																	
4.5	Larval husbandry - wreckfish	18	7.67	3.00										0.31						
4.6	Larval husbandry - mullet	19	3.10																	
WP5 Grow out husbandry			95.20	20.33	17.30	0.00	2.00	6.00	0.10	1.50	3.00	0.00	1.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.1	Grow out husbandry - meagre	20	36.11	16.33	17.30															
5.2	Grow out husbandry - amberjack	21	22.82	1.00					0.10	1.50										
5.3	Grow out husbandry - pike perch	22	19.54								3.00									
5.4	Grow out husbandry - mullet	23	16.73	3.00			2.00	6.00					1.75							
WP6 Fish health			132.84	45.30	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	75.20	34.30																
6.2	Fish health - amberjack	25	57.21	11.00																
N/A	Fish health - halibut	26	0.43																	
WP7 Socioeconomics			76.47	23.00	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	7.03
7.1	Institutional and organizational context	27	0.00																	
7.2	New Product Development	28	37.79	15.00																1.74
7.3	Consumer value perceptions and behavioral change	29	38.30	8.00																5.29
7.4	Business model and marketing strategy development	30	0.38																	
WP8 Dissemination	Dissemination	31	10.89	4.00											0.50	0.14	0.62	0.63		

Table 4.1a Staff effort per Partner (P1-22) and WP, during the 1st Reporting Period (1-12 Mo). The "Total Project" effort included in the DOW is shown as well.

DIVERSIFY	1st Periodic Report (1-12 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																	
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
			243.19	32.95	19.08	20.46	19.40	3.94	13.46	6.65	4.25	3.30	6.91	2.96	9.68	5.00	3.10	6.15	2.00	1.20	3.10	5.25	1.30	2.42	8.40
WP1 Management	Management	1	11.72	7.50		0.07	0.10	0.12	0.58	0.08	0.10	0.20	0.22	0.11	0.12	0.20	0.10	0.06		0.10	0.10		0.14	0.22	
WP2 Reproduction and Genetics			78.45	9.40	4.00	6.78	5.50	0.00	0.00	1.59	2.80	0.60	0.00	0.00	0.00	4.80	3.00	0.55	0.00	0.00	0.00	3.15	0.00	0.00	6.40
2.1	Reproduction - meagre	2	13.32	2.50	4.00	6.72											0.10								
2.2	Reproduction - amberjack	3	39.98	3.20			3.20				0.80					4.80	0.40	0.21							
2.3	Reproduction - pikeperch	4	1.60	1.00								0.60													
2.4	Reproduction - halibut	5	8.29	0.30						1.59															6.40
2.5	Reproduction - wreckfish	6	9.70	2.10		0.04					2.00						1.70	0.20				3.15			
2.6	Reproduction - mullet	7	5.57	0.30		0.02	2.30										0.80	0.15							
WP3 Nutrition			26.72	0.00	6.85	0.00	9.30	0.00	0.00	0.66	0.35	0.00	0.00	0.00	0.00	0.00	0.00	2.66	0.00	1.10	0.00	2.10	0.00	1.70	0.00
3.1	Nutrition - meagre	8	6.43		6.21													0.22							
3.2	Nutrition - amberjack	9	2.37								0.30							2.07							
3.3	Nutrition - pikeperch	10	3.89															0.19						1.70	
3.4	Nutrition - halibut	11	1.94							0.66								0.18		1.10					
3.5	Nutrition - wreckfish	12	2.79		0.64						0.05											2.10			
3.6	Nutrition - mullet	13	9.30				9.30																		
WP4 larval husbandry			23.88	0.25	0.90	3.03	4.50	0.00	0.00	2.95	0.80	2.40	0.00	0.00	0.00	0.00	0.00	2.08	0.00	0.00	0.00	0.00	0.00	0.25	2.00
4.1	Larval husbandry - meagre	14	2.93			2.74												0.19							
4.2	Larval husbandry - amberjack	15	4.84	0.25	0.90						0.80							1.89							
4.3	Larval husbandry - pikeperch	16	6.44			0.29						2.40												0.25	
4.4	Larval husbandry - halibut	17	4.95							2.95															2.00
4.5	Larval husbandry - wreckfish	18	0.22																						
4.6	Larval husbandry - mullet	19	4.50				4.50																		
WP5 Grow out husbandry			21.90	3.00	2.90	3.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	2.00	0.00	0.00	0.00	0.00	0.25	0.00
5.1	Grow out husbandry - meagre	20	8.04	3.00		3.24																			
5.2	Grow out husbandry - amberjack	21	4.85		2.90													0.33							
5.3	Grow out husbandry - pike perch	22	4.25																2.00					0.25	
5.4	Grow out husbandry - mullet	23	4.76			0.51																			
WP6 Fish health			23.43	7.80	4.43	4.53	0.00	3.82	0.00	1.37	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	1.16	0.00	0.00
6.1	Fish health - meagre	24	13.73	4.60	1.53	4.53		1.91															1.16		
6.2	Fish health - amberjack	25	8.34	3.20	2.90			1.91			0.10							0.23							
N/A	Fish health - halibut	26	1.37							1.37															
WP7 Socioeconomics			48.78	4.00	0.00	2.30	0.00	0.00	12.88	0.00	0.00	0.00	6.69	2.85	9.02	0.00	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7.1	Institutional and organizational context	27	26.41						10.70				6.69		9.02										
7.2	New Product Development	28	7.54	4.00		1.80												0.24							
7.3	Consumer value perceptions and behavioral change	29	14.83			0.50			2.18					2.85											
7.4	Business model and marketing strategy development	30	0.00																						
WP8 Dissemination	Dissemination	31	8.30	1.00							0.10	0.10			0.54						3.00				

Table 4.1d Staff effort per Partner (P23-38) and WP, during the 1st Reporting Period (1-12 Mo). The "Total Project" effort included in the DOW is shown as well.

DIVERSIFY	1st Periodic Report (1-12 months)																	
KBBE 2013.1.2.09. Diversification of fish species and products in aqua																		
Calculation of staff effort per parter and sub WP																		
				SME partners									New Partners					
Work Package (Proposal)	Title	DOW WP	Total	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
			243.19	22.40	6.05	4.30	2.10	1.10	1.72	7.60	1.10	0.30	0.78	0.55	0.22	0.45	0.16	2.40	11.00
WP1 Management	Management	1	11.72	0.10	0.18	0.30	0.10	0.10	0.10	0.10	0.10	0.05	0.05	0.05	0.06		0.07	0.04	0.20
WP2 Reproduction and Genetics			78.45	21.50	5.87	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00
2.1	Reproduction - meagre	2	13.32																
2.2	Reproduction - amberjack	3	39.98	21.50	5.87														
2.3	Reproduction - pikeperch	4	1.60																
2.4	Reproduction - halibut	5	8.29																
2.5	Reproduction - wreckfish	6	9.70										0.51						
2.6	Reproduction - mullet	7	5.57			2.00													
WP3 Nutrition			26.72	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.1	Nutrition - meagre	8	6.43																
3.2	Nutrition - amberjack	9	2.37																
3.3	Nutrition - pikeperch	10	3.89							2.00									
3.4	Nutrition - halibut	11	1.94																
3.5	Nutrition - wreckfish	12	2.79																
3.6	Nutrition - mullet	13	9.30																
WP4 larval husbandry			23.88	0.00	0.00	0.00	0.00	1.00	0.00	3.50	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.00
4.1	Larval husbandry - meagre	14	2.93																
4.2	Larval husbandry - amberjack	15	4.84					1.00											
4.3	Larval husbandry - pikeperch	16	6.44							3.50									
4.4	Larval husbandry - halibut	17	4.95																
4.5	Larval husbandry - wreckfish	18	0.22										0.22						
4.6	Larval husbandry - mullet	19	4.50																
WP5 Grow out husbandry			21.90	0.80	0.00	2.00	2.00	0.00	1.62	2.00	1.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5.1	Grow out husbandry - meagre	20	8.04	0.80							1.00								
5.2	Grow out husbandry - amberjack	21	4.85						1.62										
5.3	Grow out husbandry - pike perch	22	4.25							2.00									
5.4	Grow out husbandry - mullet	23	4.76			2.00	2.00					0.25							
WP6 Fish health			23.43	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	13.73																
6.2	Fish health - amberjack	25	8.34																
N/A	Fish health - halibut	26	1.37																
WP7 Socioeconomics			48.78	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	10.80
7.1	Institutional and organizational context	27	26.41																
7.2	New Product Development	28	7.54																1.50
7.3	Consumer value perceptions and behavioral change	29	14.83																9.30
7.4	Business model and marketing strategy development	30	0.00																
WP8 Dissemination	Dissemination	31	8.30											0.50	0.16	0.45	0.09	2.36	