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

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

Period covered: from 49 to 60 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

DIVERSIFY

New species for EU aquaculture

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

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

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1. Publishable summary

A summary description of project context and objectives

The European Union (EU) is the largest importer of fisheries and aquaculture products in the world. Of the seafood produced in the EU, aquaculture provides only 20% and capture fisheries provide the rest (Eurostat 2018), while the worldwide contribution of aquaculture towards seafood consumption is already >50%. This situation can be attributed partially to a lack of diversity of aquaculture products in Europe, since European demand increases for a diverse range of fish products, especially for fish fillets and other processed products. Nevertheless, aquaculture is undertaken in all EU states, and plays an important role in the supply of high-quality seafood to the European consumer. The EU aquaculture is a modern industry providing direct employment for 85,000 people, producing 1.3 million tons worth €4 billion (https://ec.europa.eu/fisheries/cfp/aquaculture/). Many world-class researchers and facilities exist in research centers and universities throughout Europe, while the private sector employs highly skilled and educated personnel, with modern production facilities. Therefore, the sector is well situated to become the world leader in the efficient and sustainable production of safe seafood of the highest quality and nutritional value, considering consumer preferences and lifestyles, and the immense diversity of aquatic products from the wild, to which the consumer is accustomed.

Even though some 35 aquatic species are cultured in Europe, finfish aquaculture production is dominated both in volume and value by a handful of species --such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*)-- that in turn limit the number of aquaculture processed products available in the market. In fact, the 10 most common species account for up to 90% of the production and 87% of its value (Eurostat 2018). An efficient, sustainable and market-oriented expansion of the EU aquaculture sector based on new species and products will reduce the dependence of the EU consumer on imports from countries of questionable production, health, environmental and social standards, and it will reduce the pressure on over-exploited fisheries in the EU.

The objective of DIVERSIFY was to support the EU aquaculture industry in diversifying its production with new/emerging species with important advantages over the ones cultured currently, such as fast growth, large size or low requirement in fishmeal and oil. In addition, the project identified the drivers for market acceptance of the new food prototypes in order to position the EU aquaculture sector in relation to imports from outside the EU. Although the emphasis of DIVERSIFY was on Mediterranean cage-culture, fish species suitable for cold-water, pond/extensive and fresh water aquaculture have been included as well. The fish species studied were **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 aquaculture systems (RAS).

A strong socioeconomic component was included in DIVERSIFY, in order to address important bottlenecks in aquaculture development, beyond biological/production issues. The socioeconomic part of the project had a science based applied market development approach, with a lot of components. These included the perception of aquaculture products in general and products specifically, market potential and demand factors, consumer and professional buyer preferences, new product development, creating added value in relation to raw products and market development. An important limitation in aquaculture consumption is that in many countries and/or segments of the EU market, aquaculture fish have a weaker

image than wild fish. Parallel to technological improvement of production methods for the new species, expansion opportunities for the EU aquaculture sector have been identified.

The combination of biological, technological and socioeconomic research activities developed in DIVERSIFY are expected to support the diversification of the EU aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets.

A description of the work performed since the beginning of the project and the main results achieved so far.

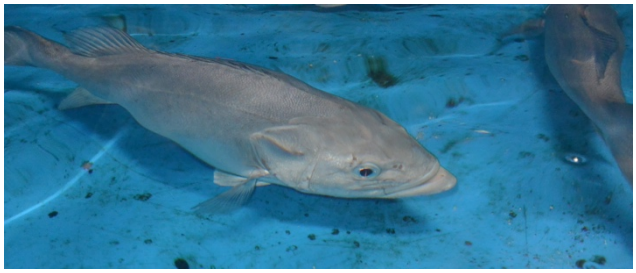
After five years of implementation, DIVERSIFY has acquired the knowledge needed to solve bottlenecks in reproduction, juvenile production, grow-out, nutrition and feeding husbandry, new product development and marketing of six new/emerging species.

In **meagre**, sufficient genetic variation for breeding programs was confirmed around Europe and protocols have been developed for paired spawning and *in vitro* fertilization. A protocol for early weaning was developed and the nutritional requirements of the species were better described, especially in relation to the pathological condition Systemic Granulomatosis. Feeding in sea cages can be carried out using optical and mechanical stimuli to improve feeding behavior. Immune markers have been established for the innate, adaptive and inflammatory responses of the immune system of meagre in order to develop vaccines. Methods to prevent Chronic Ulcerative Dermatopathy and to address parasitic and bacterial infections have been developed.

In **greater amberjack**, hormonal induction methods have been developed to induce spawning, producing large numbers of eggs of good quality for commercial larval rearing. Hatchery-produced (F1) individuals were shown to undergo reproductive maturation. The ontogeny of the digestive and vision system has been described. Significant breakthroughs were achieved in larval nutrition and husbandry, allowing the production of large numbers of juveniles for commercial production. On growing resulted in important information on feeding patterns and stocking densities. Identification of immune markers and health management tools under aquaculture conditions were developed, including probes for the early detection of epitheliocystis, and methods to control infestations of the parasites *Zeuxapta seriolae* and *Neobenedenia spp*.

In **pikeperch**, a genetic map comparing captive and wild broodstock was developed for use in breeding programs. Studies have identified the nutrient requirements of the species, and the optimal combinations of environmental, feeding and population factors to improve survival and growth during larval rearing in recirculating aquaculture systems (RAS). Low light intensity and red-light spectrum was shown to be less stressful and the effect was confirmed in RAS farm conditions. Domestication level was shown to influence stress responsiveness and immune response.

In **Atlantic halibut**, use of gonadotropin releasing hormone (GnRHa) implants advanced and synchronized spawning, resulting in improved egg production, though egg quality remained highly variable. First feeding of larvae in RAS systems resulted in improved growth and development compared to flow through systems. Metagenomic analyses of the microbial communities in the water and larvae of the two systems revealed interesting differences, which will be useful in industrial applications.



In **wreckfish**, the reproductive cycle of wild fish was described in captivity. Spontaneous spawning takes place in the Spring, with a periodicity between spawns of 3-5 days. Males may be in full spermiation throughout the year. A new broodstock diet resulted in successful maturation and production of high-quality eggs. The ontogeny of the digestive and vision system has been described. Successful larval rearing was implemented, producing a small number of juveniles.

In **grey mullet**, spawning was achieved using GnRHa and metoclopramide therapies. Algal addition during larval rearing provided beneficial effects on survival and growth. After metamorphosis, commercial feeds for juveniles should be designed for the omnivorous feeding of this species. Larvae have a high taurine requirement during rotifer feeding, and the benefit of this nutrient during early feeding was still apparent during juvenile growth. Diets with low fishmeal content can be used successfully for on growing.

In the **Socioeconomics** area, market research identified market potential in cross-cultural consumer segments, with increased-to-strong interest in new products in the main EU fish markets. Especially involved consumers are open to try new species. Most value added new products developed were positively perceived in terms of healthiness, convenience and overall quality, and were characterized by high nutritional value (protein and Omega-3). Buyers and consumers would welcome new species, if they are a) sustainably farmed, ideally in domestic or EU waters; b) fresh (especially southern-EU) or mildly processed (northern-EU); c) easy to prepare and/or ready to eat; and d) competitively priced. In Europe, greater amberjack shows the most promising market opportunities, given its large size, processing potential and superior sensory characteristics. Grey mullet is a very interesting species due to the higher sustainability of its production methods. No specific preference region has been identified for this species. Wreckfish has very firm flesh that discriminates it readily from other fish. The remaining species (Atlantic halibut, pikeperch and meagre) have certain advantages due to their biological and physical characteristic and are of interest to specific regions in Europe.

The expected final results and their potential impact and use (including the socio-economic impact and the wider societal implications of the project so far.

The DIVERSIFY consortium integrated a multidisciplinary group of research and academic institutions, small and medium-sized enterprises, large enterprises, five professional associations and one consumer NGO from 12 European countries. The acquired knowledge and developed methods will enhance the production of the selected emerging species (meagre, Atlantic halibut, pikeperch) by the European aquaculture and will enable the incorporation of some new species (greater amberjack and grey mullet).

In the area of **reproduction**, DIVERSIFY has provided improved understanding of the regulation of reproduction and the dysfunctions that occur when fish are maintained in captivity. The project has defined optimal broodstock management conditions and has developed species-specific spawning induction protocols for the acquisition of optimal gamete quality. In addition, DIVERSIFY has focused on the genetic characterization of various broodstocks of two species with current relevant industrial production, in order to overcome future inbreeding problems in these two species and solve current problems with variable growth rates (meagre) and stress sensitivity (pikeperch). Thus, it acquired the necessary knowledge and developed the required methods for the implementation of breeding selection programs, which are imperative for the development of an efficient, profitable and sustainable aquaculture industry.

The cost of **feeding in aquaculture** production is around 40-70% of total production cost; therefore, improving feed conversion efficiency and growth rates is directly related to the profitability of the industry. New species in aquaculture are fed with available diets designed for other well-established species, which may constraint their growth performance, welfare and health. For this reason, it is important to develop species-specific feeds that consider the nutritional requirements of each species at different stages of development and that can improve their performance, quality and health condition. To achieve this goal, DIVERSIFY has established the nutritional requirements of several macro- and micronutrients for most of the species considered in the proposal. In addition, specific live prey enrichment products have been developed. Specific formulated feeds, live prey enrichment products and feeding protocols will result in new products that can be commercialized worldwide.

A **larval and juvenile rearing system** is a complex artificial environment, with numerous factors influencing fish development and performance, as well as behavior and survival. These factors can be environmental, nutritional, social and genetic. For species such as meagre, pikeperch, grey mullet and Atlantic halibut, improvements in fish growth and husbandry have been addressed to refine the existing protocols and facilities in order to solve existing bottlenecks. In contrast, emphasis has been given to developing new species-specific larval rearing protocols in the case of greater amberjack and wreckfish, since these were species with important knowledge gaps in these areas. The output of these tasks is the development and refining of rearing protocols for the selected species that will result in the improvement of current practices, and will provide an increase in production yields.

Fish health is a key aspect to be optimized in cultured fish. The effect of the developmental stage, rearing conditions and nutrition on the capacity to modulate specific immune responses will help predict vaccine responsiveness and fish health. DIVERSIFY has characterized the immune system of meagre and greater amberjack to identify key immune molecules as potential markers of immune system development, and induction of antiviral and antibacterial responses in preparation for vaccine development for disease management. In addition, potential solutions for specific bacterial infections and parasitoses have been investigated, providing means to prevent and/or minimize these issues at an industrial scale. DIVERSIFY

has produced practical health manuals for meagre and greater amberjack, which are freely available and can be used immediately by the industry in order to improve their stock management.

Sustainability of aquaculture production is a major concern worldwide. DIVERSIFY has considered this issue from different points of view. For example, the acquired knowledge will support the growth and expansion of the sector based on different production systems that can be regarded as more sustainable: cage culture – no competition with land resources; RAS- ecologically friendly, with efficient use of water; extensive pond-lagoon culture, with very low environmental effects and in some cases even contributing to the restoration of ecosystems. Also, the introduction of an omnivorous fish into the aquaculture sector, such as the grey mullet, with positive influence in the environment where it is cultured and requiring low or close to no input from fish meal/oils, will contribute to the reduction of the pressure in the capture fisheries. The determination of species-specific dietary requirements, as well as feeding behavior will result in less waste of diets and nutrients into the environment. Altogether, these factors will ensure a sustainable growth and expansion of aquaculture within the EU and EEA member states.

In the **Socioeconomics** area, DIVERSIFY identified a market potential in cross-cultural consumer segments, with increased-to-strong interest in new products in the main EU fish markets. A number of new products have been developed and were perceived positively as healthy, convenient and of high quality and nutritional value. Buyers and consumers were shown to welcome new species, if some important requirements are met (see previous section). Introduction of the new species seems to have the most impact if it is done country by country instead of pan-European, because in each market other buying factors and motives are important.

So, overall, the main expected impact of DIVERSIFY will be the improvement of production technologies for the new/emerging species of the project. Furthermore, DIVERSIFY is expected to have also a significant impact on providing more insights in markets and consumer's perception and preferences, potentially resulting in increases in the EU consumption of aquaculture products. Such an integrated combination of biological, technological and socioeconomic activities will lead to a reduction in the dependence of the EU on imports from third countries of at times questionable production, health, environmental and social standards.

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

2.1 Project objectives for the period

Reproduction and Genetics

A total of eight objectives were completed during the 4th reporting period to complete all the objectives of the project in reproduction and genetics. Spawning induction methods were developed for wild–captive greater amberjack broodstocks in tanks and cages and for reared F1 greater amberjack in tanks. The availability of wreckfish broodstocks in captivity was increased, the reproductive cycle described and spontaneous tank spawning procedures were developed. The objectives to scale-up breeding of grey mullet and assess the effects of captivity on first sexual maturity were completed. All the objectives in reproduction and genetics for meagre, pikeperch and halibut were completed in the previous reporting periods.

Nutrition

The objectives of this period have focused in the:

a) development of grow-out diets for greater amberjack in order to maximize growth potential,
b) formulation of an experimental diet to improve greater amberjack broodstock reproduction,
c) determination of Ca/P dietary ratio in weaning diets for pikeperch,
d) formulation of a test diet for pikeperch based on information gathered by previous experiments with emphasis was on optimal dietary lipid class composition; LC PUFA levels and ratios, levels and ratios of vitamins (Vit A, E, C, D) and minerals (Ca/P, Se),
e) inclusion of soy lecithin in diets for Atlantic halibut juveniles with dietary phospholipid increased from 9 to 30% of total lipids,
f) design of two enriched products for live prey with two different arachidonic acid levels for wreckfish larvae,
g) formulation of a dry food for wreckfish broodstock with high content of proteins, low level of lipids, a high amount of n-3 PUFA and EPA/ARA ratio similar to the one obtained in wild female gonads,
h) effects of vegetable oil-based diet on grey mullet broodstock reproductive potential,
i) supplementation of carotenoids to grey mullet broodstock diets and the contribution to bottarga coloration.
j) effect of soybean or poultry meal inclusions on grey mullet on-growing diets and the impact of those ingredients on growth, digestive tract length and weight, and gut health.

Larval husbandry

In greater amberjack an industrial protocol based on the results of previous reports was tested in two commercial hatcheries in Greece, which produced 15,000 and 48,300 juveniles, respectively, for on growing. In pikeperch, the overall aim was to develop an industrial protocol based on an optimal

combination of rearing factors. The protocol was not applied fully due to technical limitations and problems with the pikeperch broodstock of the SME. However, six recommendations have been proposed to promote an industrial protocol for pikeperch larval rearing. In Atlantic halibut, all objectives, tasks and deliverables have been achieved and reported on. In wreckfish, no final industrial protocol was developed. On the other hand, a significant step forward was achieved as juvenile wreckfish were produced and weaned onto prepared diets. At the writing of this report, there are 25 juveniles of more than 150 dph. In grey mullet) semi-commercial production runs testing the P4.IOLR protocol in 6 m^3 tanks produced 78,704 juveniles while the entire production in 2017 was ca. 200,000 fish. When comparing the "greening" of larval mullet rearing tanks with effective levels of lyophilized or live *Nannochloropsis oculata,* the results showed very similar larval performances, in terms of rotifer ingestion rate, swim bladder inflation, growth and survival as well as larval digestive tract enzyme activity. The protocols for the production of *Tisbe* spp. copepods and their ozone disinfectant treatment were developed at P4.IOLR. However, testing the effect of co-feeding rotifers and disinfected copepods did not succeed due to the complete mortality of eggs, pre-larvae and end of rotifer feeding larvae, when transferred to the experimental aquarium system. These results suggest that mullet larvae are extremely sensitive and all handling should be avoided before 25 dph.

Grow out husbandry

The tasks related to meagre were targeted to the definition of a feeding methodology for grow out in cages. In particular the conditions related to the feeding distribution (period and method) and the understanding of potential deeding rhythms were studied. The work implemented for the greater amberjack had as objectives (a) to contribute towards the development of cage husbandry (b) to define the optimal ranges in terms of environmental temperature during the rearing and also the optimum ranges regarding the stocking density. During the third period, the objectives for pikeperch were (*a*) to characterize growth, immune and physiological status of pikeperch in farm conditions in order to validate the effects of the best identified rearing conditions (Task 22.2) and (*b*) to assess the effects of pikeperch domestication level and geographical origin on growth and stress sensitivity (Task 22.3).

Fish health

In this reporting period the objective was to complete the remaining outstanding tasks in the Fish Health GWP, in WP 24 and WP 25, and this was achieved. In WP24 this included completing a series of feeding trials aimed at mitigating the impact of Systemic Granulomatosis (SG) in meagre, and generating a diagnostics protocol for SG development. In addition, a (trivalent) vaccine trial will be undertaken to study the immune responses elicited in meagre and to determine the vaccine efficacy following challenge with the bacterial pathogen *Vibrio anguillarum*. Completion of the descriptions, diagnosis and treatment of bacterial diseases discovered in meagre over the project was a further task to be completed, together with determining the optimal antibiotic doses to use and establishment of challenge tests for future studies. The results were to be collated in a meagre health manual that can be downloaded freely from the project website. In WP25, a variety of ways to reduce parasitosis of greater amberjack were to be undertaken, including the effects of environmental parameters on the behaviour and reproductive success of the monogenean parasite (*Neobenedenia melleni*). In addition, methods to interfere with the binding of the parasite to amberjack and reinforcement of the amberjack immune system through faecal microbiota transplantation (FMT) were tested. As for meagre, the diagnosis and treatment of bacterial/viral infections of amberjack was then collated from the project period, with challenge tests and antibiotic usage optimised. As for meagre, this information was used to create a diagnostic manual for greater amberjack health that will be uploaded onto the project website (http://www.diversifyfish.eu).

Socioeconomics

In this period mainly work has been done in WP 28 and WP 30. On other WP's papers have been prepared and/or finished. In WP 28 work has been done on the following objectives (no. 3 and 4 in the DOW):

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

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

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

2.2 Work progress and achievements during the period

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

For each work package, *except project management, which will be reported in section 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

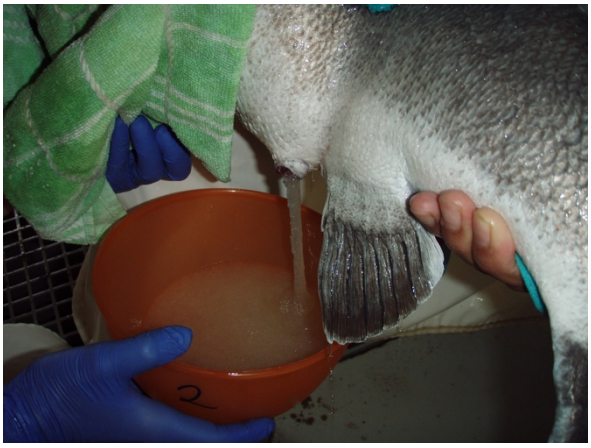
The progress and achievements during the 4th RP completed the project to offer solutions to the bottlenecks in reproduction and genetics that were identified.

Experiments on greater amberjack confirmed the capability of captive-reared greater amberjack to spawn "on demand" by administering GnRHa implants in sea cages and transferring broodstock to land based tanks, where it can produce eggs for a period of 2.5 months (**Task 3.2 Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean**). Results were good in terms of daily relative fecundity (26,505±5,846 eggs $kg^{-1}day^{-1}$), mean fertilization (43±7%), mean 24h embryo survival (36±9%), mean hatching (73±12%) and 5-days larval survival (7±5%). Experiments carried out on hatchery produced greater amberjack (F1 generation) in the eastern Atlantic, showed that they underwent normal gametogenesis and could be induced to mature oocytes, ovulate and spawn using GnRHa delivery systems (**Task 3.4 Development of an optimized spawning induction protocols for F1 greater amberjack in the eastern Atlantic**). These experiments demonstrated that repetitive handling of fish to administer GnRHa implants many times over a long period during the reproductive season did not affect fish welfare and reproductive performance

Three new wreckfish juveniles from Mediterranean Sea (43º10'N-05º36'E, France) were obtained and moved to the IEO facilities in August 2018. Bimonthly samples demonstrated excellent growth rates of the juveniles **(Task 6.1 Collect wild fish to establish new bloodstocks)**. Methods to determine plasma levels of luteinizing hormone (LH) were developed and used to determine that LH has a profile in relation to maturity stage similar to other species and to complete the **Task 6.2, Describe reproductive cycle.** The **Task 6.3 Development of spawning induction procedures**, was achieved to give clear conclusions that the best method to obtain high quality eggs and larvae was with spontaneous spawning in captivity.

The F1 grey mullet broodstock responded well to the hormonal treatment during natural and artificially shifted spawning seasons giving rise to tens millions of quality eggs and consequently mass production of robust fingerlings. The basic breeding units consisted of a single female and three males improved synchronization and increase the fertilization rate. The formulated broodstock diet containing fish oil (FO;

rich in n-3 LCPUFA), Marigold petal meal (MgM) as another carotenoid source and 3% dry Ulva to the diet, positively affected hatching success and larvae survival (**Task 7.3 Optimization and scale-up of a breeding protocol for grey mullet in captivity**). In **Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish**, the results indicated that: (1) the rearing conditions established at IORL allows a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea; (2) the reduction of the rearing density from 90 to 45 fish/m^3 has no effect on grey mullet growth and sexual maturity; (3) hatchery-produced grey mullet has a good potential to develop ovaries spontaneously up to a condition useful for bottarga production. Further assessment of the effects of fish origin (wild vs. domesticated) and culture conditions on advanced and spontaneous development of gonads comprising the required criteria for the production of high quality bottarga (i.e., minimal size of 100 g, bright yellowish color and chewy texture) indicated that: (1) traditional grey mullet farming procedure in freshwater ponds could be applicable, and also an advantage, for the roe production (2) domestication appears to have a favourable effect on spontaneous development of mullet ovaries up to a condition useful for bottarga production (3) pigment-enriched diets can enhance the roe coloration to meet the criteria for high quality bottarga. However, two stumbling blocks that may impaired the profitability of grey mullet farming for roe production are: (1) extended grow out to a minimum of 3 years (2) relatively low percentages (20-50%) of females developing ovaries at the appropriate size (≥ 100 g). Future studies therefore, should focus on genetic improvement programs giving rise to advanced sexual maturity and spontaneous ovarian development in captive grey mullet females



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

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

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

Summary of work reported in the previous Reporting Period (31-48 Mo):

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

families identified 5 QTLs on only two LGs, which exhibited significant evidence of linkage at the genome level, and multiple QTLs appeared to be related to differences in body weight and length.

Summary of progress towards objectives (49-60 Mo):

All work in the WP has been completed and reported in the previous Periodic Reports. No work took place during the 4th RP.

Details for each Task

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

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

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

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

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

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

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

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

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

This task has been completed during the previous reporting periods and the full description of the work and results have been since submitted as ***D2.1. Development of Single Nucleotide Polymorphisms (SNP) marker tools for the genetic characterization of fast and slow growers, D2.4 Construction of a genetic linkage map in meagre*** and ***D2.5 Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre through QTL mapping***.

Deviations from Annex I and their impact:

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

Manuscripts that resulted from this Task (indicate Published, Submitted or In Preparation)

Duncan, N.J., Mylonas, C.C., Milton Sullon, E., Karamanlidis, D., França Nogueira, M.C., Ibarra-Zatarain, Z., Chiumento, M., Aviles Carrillo, R.O., 2018. Paired spawning with male rotation of meagre *Argyrosomus regius* using GnRHa injections, as a method for producing multiple families for breeding selection programs. Aquaculture 495, 506-512.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture 464, 323-330.

Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2017. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472, 21-44.

Nousias, O., Tsakogiannis, A., Tzokas, K., Villa, J., Estevez, A., Duncan, N., Tsigenopoulos, C. S. Parentage assignment using microsatellite loci in a project for aquaculture of meagre *Argyrosomus regius*. (In preparation)

Ramos, S., González, W., Dutto, G., Mylonas, C.C., Fauvel, C., Duncan, N. Gamete quality and management for in vitro fertilisation in meagre (*Argyrosomus regius*). (In preparation)

Zamorano, M.J., Tsigenopoulos, C.S., Soula, M., Duncan, N., Roo, J., Corriero, A., Mylonas, C.C. , Fauvel, C., Pousao, P., Pastor, E., Manchado, M., Mazuelos, N. , Gamsiz K., Villa, J., Afonso , J.M. Genetic characterization of European meagre (*Argyrosomus regius*) captive broodstocks: evaluation of available variability". (In preparation)

WP 3 Reproduction & Genetics – greater amberjack

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

Objectives

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

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

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

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

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

produced the highest amount of eggs compared to the treated ones. Moreover, eggs obtained by natural spawning showed the highest percentage of fertilization, viability at 24 hours and hatching, and provided the highest percentage of larval survival at 4 and 8 days.

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

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

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

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

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

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

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

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

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

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

Summary of work reported in the previous Reporting Period (31-48 Mo):

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

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

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

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

Summary of progress towards objectives (49-60 Mo):

The proper dose for GnRHa treatment (25 *vs* 75 µg kg^{-1} body weight) and the right time to induce spawning in greater amberjack reared in captivity in the Mediterranean were established during the 3rd reporting period. During the 4th reporting period, another experiment was conducted in the Mediterranean to corroborate the reproducibility of the results obtained in the previous years. Broodstock was treated with GnRHa implants in a sea cage and transferred to a land-based tank for spawning. Results were good in terms of daily relative fecundity (26,505±5,846 eggs kg^{-1}day^{-1}), mean fertilization (43±7%), mean 24h embryo survival (36±9%), mean hatching (73±12%) and 5-days larval survival (7±5%). Moreover, a second experiment carried out in 2018 to provide adequate greater amberjack egg quantity for larval husbandry led to the production of a total of 283,666 eggs kg^{-1} with 28-94% of fertilization. These last experiments confirmed the capability of captive-reared greater amberjack to spawn “on demand” by

administering GnRHa implants in sea cages and transferring treated broodstock to land based tanks, where it can produce eggs for a period of 2.5 months.

In 2018 a new trial was also carried out in the Mediterranean to test the efficiency of a 5 mm-mesh net in minimizing potential foraging activity on the newly spawned eggs by any wild juveniles entering the sea cage. Greater amberjack broodstock was left to spawn spontaneously in a sea cage with good results in terms of eggs collection (total egg production of 1,195 – 1,265 g in 8 spawns), demonstrating the effectiveness of the net.

Experiments carried out on hatchery produced greater amberjack (F1 generation) in the eastern Atlantic, showed that they underwent normal gametogenesis and could be induced to mature oocytes, ovulate and spawn using GnRHa delivery systems. These experiments demonstrated that repetitive handling of fish to administer GnRHa implants many times over a long period during the reproductive season did not affect fish welfare and reproductive performance, since treated fish produced high amount of high quality eggs, adequate for the implementation of larval rearing for commercial purposes.

Details for each Task

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

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in the following Deliverables:

D3.1 Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor);

D3.2 Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack;

D3.3 Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating and apoptotic germ cells, vitellogenin accumulation, yolk content in the oocytes and nutritional status;

D3.4 Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of greater amberjack sperm;

D3.5 Description of the process of oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH and LH, (d) plasma levels of FSH, LH, leptin, sex steroid hormones and Vg, and (e) nutritional status;

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

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

The full description of the work and results is provided in ***Deliverable 3.9. Development of a spawning induction therapy for captive reared broodstock in the Mediterranean Sea based on the use of GnRHa in the correct mode of administration (hormone/implant), dose and timing.***

The aim of the present task was to examine: a) the two methods of spawning induction using GnRHa either in the form of implants (sustained release) or injections (acute release), b) the proper dose for GnRHa treatment and c) the right time to induce spawning, in terms of spawning kinetics, egg production and quality, with the objective of delivering a sound and efficient protocol to the aquaculture industry.

The first two subtasks were completed and reported during the previous periodic reports. As regards the last subtask, in order to ensure the reliability of the results, two experiments were carried out at GMF, the first one during the spawning season 2017 and the second one during the spawning season 2018. Additional work was done in ARGO in order to produce adequate greater amberjack egg quantity for WP15 – Larval husbandry - greater amberjack- and to make an industrial application of the larval rearing protocol in an SME (GMF).

In ARGO, three females (mean body weight 24.7±2.0 kg) having oocytes in Vitellogenesis (Vg) or Oocyte Maturation (OM) (mean oocyte diameter 743±136 μm) and three males (mean body weight 17.8±2.2 kg) having intratesticular (IT) sperm were transferred after GnRHa treatment with implants from the sea cage to a 75 m^3 outdoor land-based tank, supplied with a mixture of filtered seawater and borehole seawater. Fish spawned the day after and continued spawning for 15 days after GnRHa treatment, producing a total of 283,666 eggs kg^{-1} with 28-94% fertilization success (**Fig. 3.2.1)**.

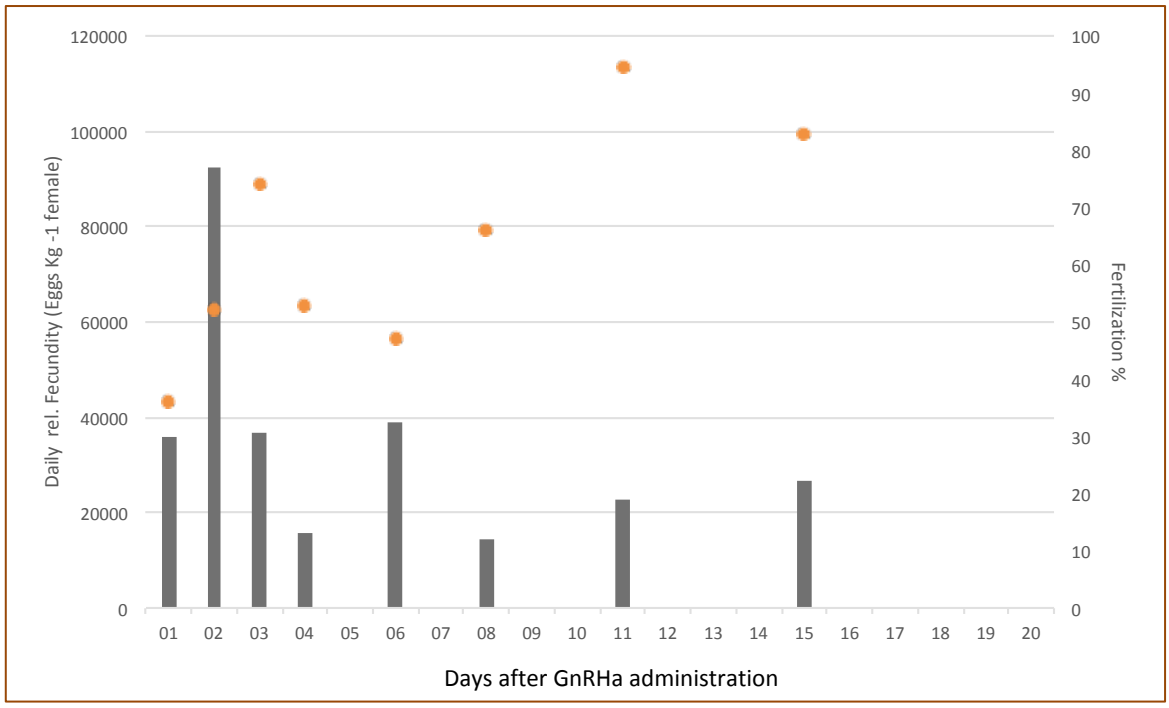


Figure 3.2.1. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) after GnRHa treatment of greater amberjack. GnRHa application was done on 31 May 2018.

In 2018, the broodstock in GMF showed a gonad development similar to the previous reproductive season. The females had mainly Vg oocytes whose mean diameter was 680-730 μm. One female, apart from the Vg oocytes, was in post ovulation stage (pOV). Two weeks later, on 27/6/2018, females had Vg oocytes 680-700 μm in diameter but the number of PO was increased. The reproductive evaluation on 12/7/2018 showed that the females still were in Vg with oocyte diameter 650-750 μm with some signs of early atresia (AT). One female was found at early oocyte maturation stage. Males had IT sperm from the first sampling to the last one.

Mean daily relative fecundity was not significantly different between the three periods of GnRHa treatment and was 26,505±5,846 eggs $kg^{-1}day^{-1}$ (**Fig. 3.2.2 and Fig. 3.2.3)**. The first two periods the number of spawns was five and the last one was three. Mean fertilization was 43±7%, mean 24h embryo survival 36±9%, mean hatching 73±12% and 5-days larval survival 7±5%, respectively (**Fig. 3.2.3)**.

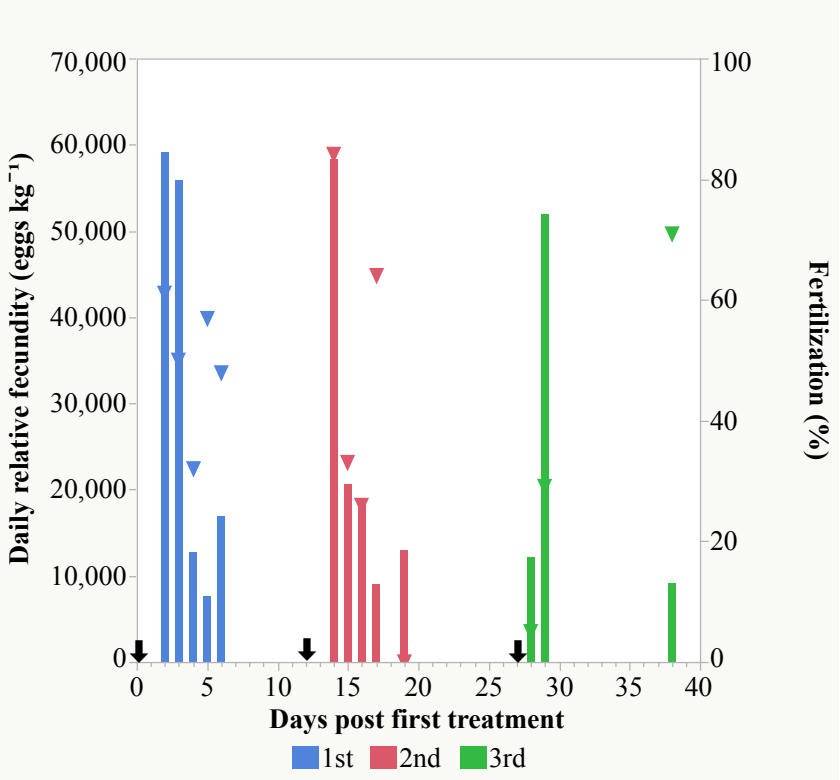


Figure 3.2.2. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of 1st (blue bars), 2nd (red bars) and 3rd (green bars) period of GnRHa treatment of greater amberjack. Arrows indicate the time of treatment. First application was done on 15 June 2018.

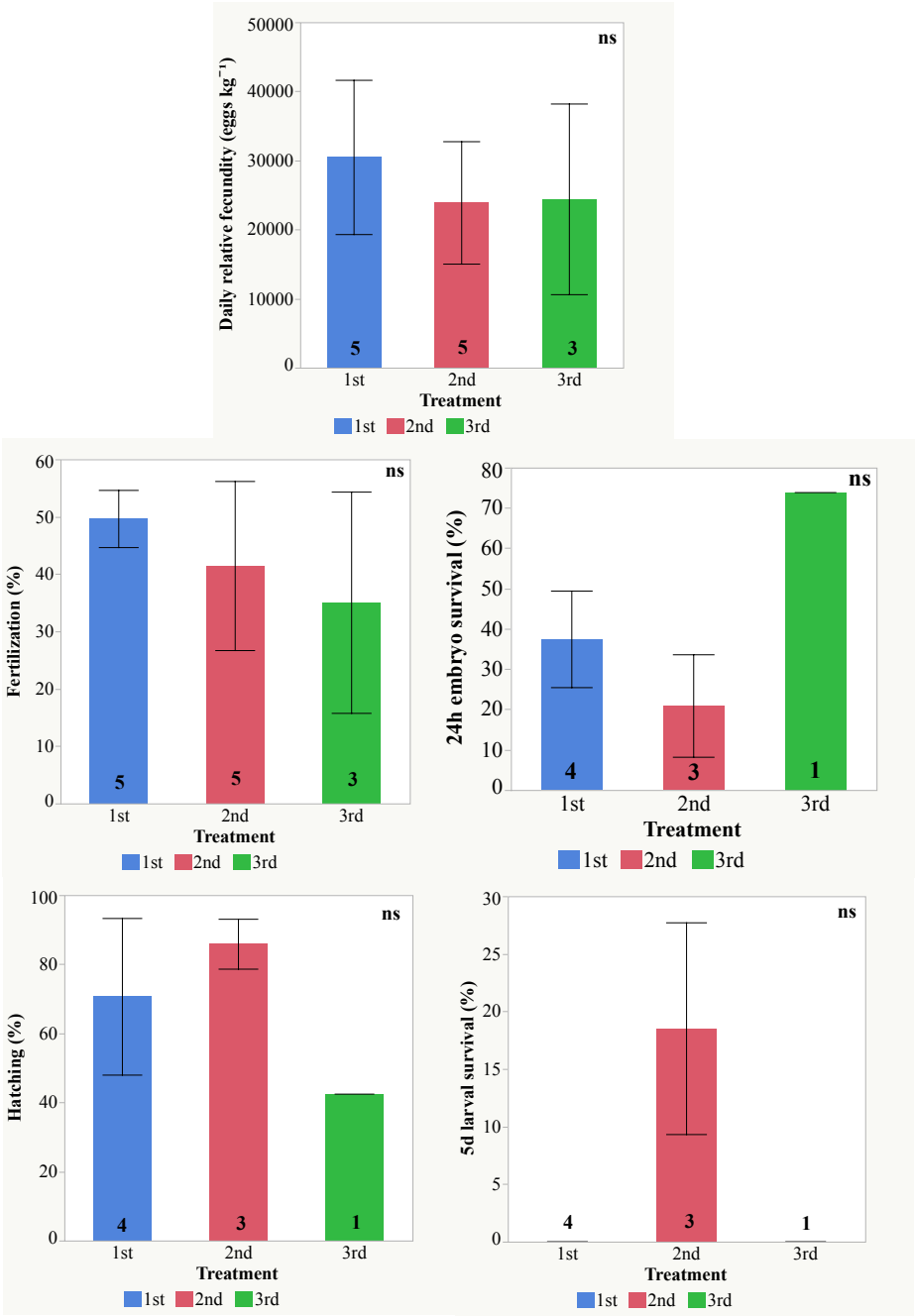


Figure 3.2.3. Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of 1st (blue bars), 2nd (red bars) and 3rd (green bars) period of GnRHa treatment of greater amberjack. Numbers in bars indicate the number of spawns that constitute each mean. No statistical differences were observed between different periods of GnRHa application (ANOVA, $P < 0.05$).

In conclusion,

- The greater amberjack produced adequate egg quantity of high fertilization success after GnRHa administration for the validation of the industrial larval rearing protocol.
- The greater amberjack in the Mediterranean **can produce eggs "on demand" after spawning induction with GnRHa for a period of 2.5 months** when keeping the broodstock in sea cages and transferring them in land based tanks for spawning.

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

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 3.7 Comparative effectiveness of a GnRHa injection vs GnRHa implant treatment for the induction of spawning of greater amberjack in the eastern Atlantic.***

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

The full description of the work and results have been provided in ***Deliverable D3.8 Dose response of GnRHa implant therapy for the induction of spawning in F1 generation broodstock of greater amberjack in the eastern Atlantic.***

The objective of this task was to develop a dose response of GnRHa implant therapy for the induction of spawning in F1 generation broodstock of greater amberjack in the eastern Atlantic. F1 greater amberjack females were treated with different doses of GnRHa in consecutive years while the males were implanted with the same dose of GnRHa. Repetitive implants with Ethylene-Vinyl acetate (EVAc) GnRHa were applied during each spawning season, according to the planned dose. The effects of the spawning induction treatments were evaluated in terms of reproductive performances.

Experimental conditions.

Rearing was undertaken in the facilities of the Centro Oceanográfico de Canarias, Instituto Español de Oceanografía, Tenerife, Spain. The broodstock consisted of 14 hatchery-produced fish, were maintained in two outdoor covered 50-m^3 tanks, supplied with well-water (10 renewals day^{-1}) at ambient water temperature and photoperiod until the beginning of the experiments. After the 1st GnRHa treatment, the selected fish were placed in a single outdoor covered raceway tank of 500 m^3 with continuous water supply under natural photoperiod. Fish were fed three times per week to apparent satiation with raw fish.

The fish were sampled monthly during the 2015 and 2016 spawning season (May, June, July and September). Fish were individually identified with PIT tags and biometric parameters of length and body weight were measured. Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula. A wet mount of the biopsy was examined first under a compound microscope to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes. A portion of the biopsy was also fixed 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 into the genital pore. At each sampling, blood was collected from all fish from the caudal vessel in order to measure sex steroid hormone concentrations and blood biochemical parameters. Blood was centrifuged and plasma was collected, frozen in liquid nitrogen and stored at -80°C until hormonal and biochemical analysis.

Fish were treated with an Ethylene-Vinyl acetate (EVAc) GnRHa implant (Mylonas & Zohar, 2001) loaded with Des-Gly^{10}, D-Ala^6-Pro-$NEth^9$-mGnRHa (H-4070, Bachem, Switzerland) at the sampling times of May, June and July. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and intratesticular sperm was obtained from males.Sperm quality parameters that were evaluated included (a) sperm density (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, a passive egg collector was placed in the outflow of the 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 at the same time by the presence of a viable embryo using a stereoscope. The diameter of ten randomly collected eggs and their lipid droplet were measured using a binocular microscope. Each spawning was incubated in a 90-l tank with gentle aeration and filtered water supply.

To monitor embryo and larval survival, eggs from each spawn were 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 sack 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.

Results for 2015 reproductive season.

In 2015, the fish were sampled in May, June, July and September, and the selected fish were implanted with the required dose of GnRHa in May, June and July. The mean (± SEM) diameter of the largest vitellogenic oocytes of the females biopsied varied between 560±80 and 760 ±110 µm with higher mean values in June and July. The selected females (oocyte diameter > 600 µm) were implanted with a dose of GnRHa (in the form of EVAc implant) about 50 µg GnRHa kg^{-1} BW in the successive spawning induction treatments. Mean sperm motility percentage was > 50%, and remained unchanged throughout the monitored period, while the duration of sperm motility was significantly higher in May (4.35±1.12 min) than in June (2.44±0.24 min) (P<0.05). The mean sperm density was 30.8±6.8 x 10^9 spermatozoa ml^{-1} in May and 78.0±72.2 x 10^9 spermatozoa ml^{-1} in September, although with elevated individual variability in September (**Fig. 3.4.1**).

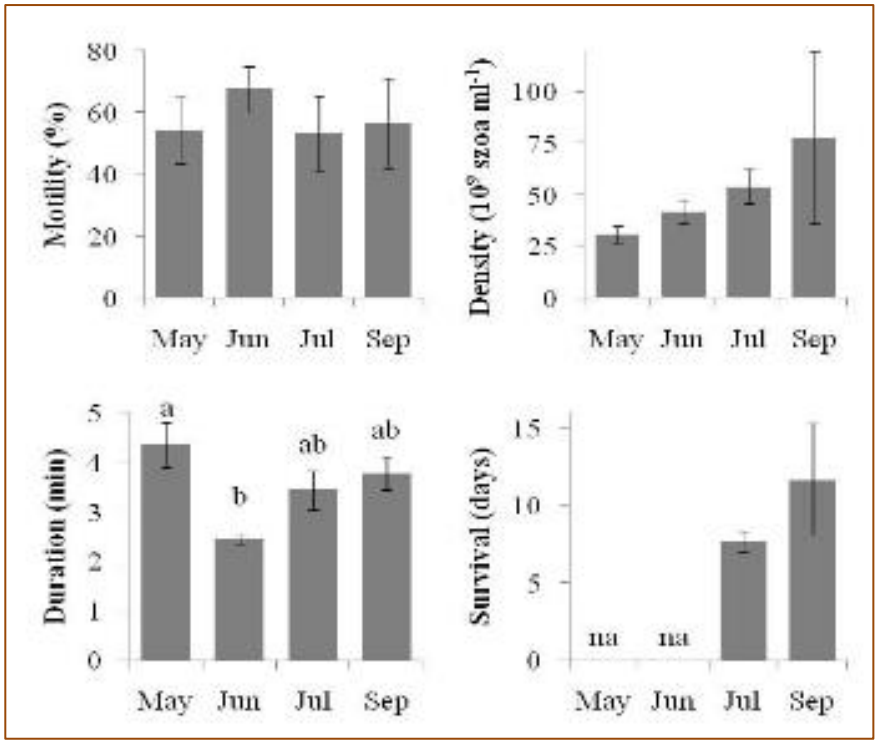


Figure 3.4.1. Mean (± SEM) sperm quality parameters of greater amberjack at different times during the reproductive season (spermatozoa forward motility, density, duration of motility and maximum survival during storage at 4°C). Statistically significant differences among months are indicated by different lower-case letters ($P \leq 0.05$). na = not available.

The first spawn occurred between 1 and 2 days after each GnRHa treatment (**Fig. 3.4.2**). A total of 52 spawns were obtained during a period of 72 days. The number of spawns and fecundity obtained after successive GnRHa implantations decreased. Moreover, the spawning events were concentrated around the application of each GnRHa treatment. The period from the 1st to the 2nd treatment, fish spawned 29 times. However, after the 2nd treatment, a total of 15 spawns were recorded during the first 16 days and no eggs were collected the following days. The eggs released after the 3rd GnRHa treatment were collected from 8

spawning events that were obtained during the following 9 days. The highest daily relative fecundity recorded was 5,539 eggs kg^{-1} fish after the 2nd GnRHa treatment, but the total egg production was higher after the 1st treatment, *i.e.* 60,540 eggs kg^{-1} fish compared to 40,180 eggs kg^{-1} fish after the 2nd treatment. No significant differences were found for daily relative fecundity between the three treatment periods. Almost 15 million eggs were produced from the three successive GnRHa applications.

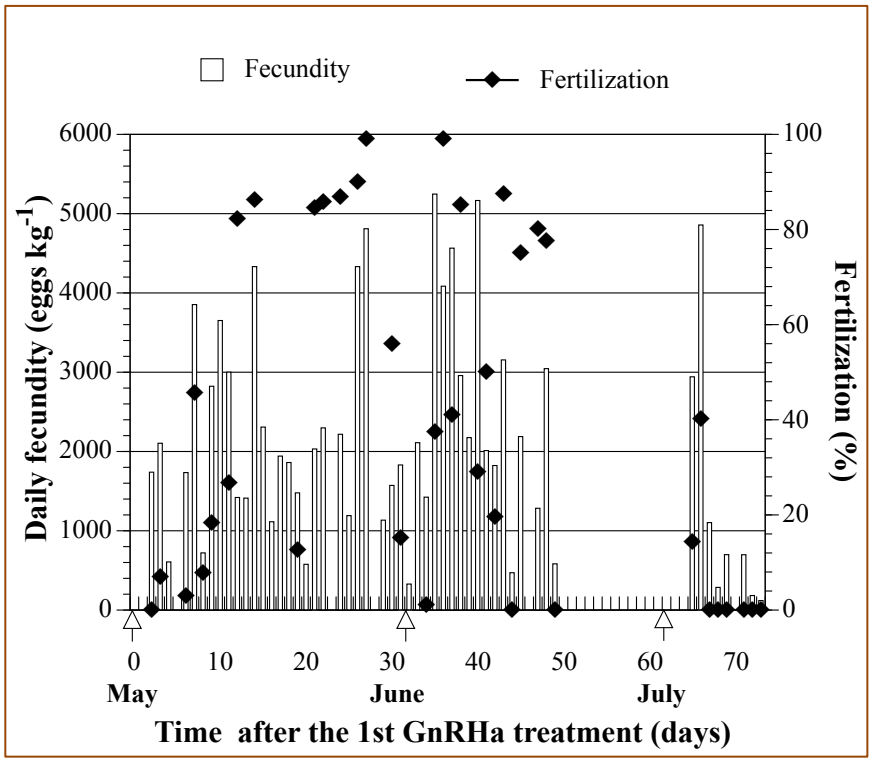


Figure 3.4.2. Daily fecundity (eggs kg^{-1}) and fertilization (%) of greater amberjack in response to three treatments with GnRHa implants in 2015. The white arrows on the X-axis indicate the times of the GnRHa treatments.

Mean fertilization changed through the spawning period after each treatment, reaching its highest values after the 1st and the 2nd GnRHa treatment, while a significant decrease was observed after the 3rd treatment ($P<0.05$). On the other hand, no significant differences were observed in hatching, 1-day embryo survival and 3-day larval survival after successive GnRHa treatment.

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 SEM, and declined not significantly during following months. Testosterone in the females' plasma was low along the spawning season and increased significantly ($P<0.05$) at the final sampling. Plasma 17,20β-P remained below 1 ng ml^{-1} during the spawning period. In males, both plasma T and 11-KT levels followed a decreasing trend from May to July, while they increased significantly in September ($P<0.05$). Also, 17,20β-P followed almost the same pattern along the spawning season, with the highest detected levels in September.

The studied blood parameters remained constant along the study and only the number of erythrocytes and plasma levels of protein, cholesterol, alkaline phosphatase and amylase changed during the experimental period. In both, females and males, a significant gradual decrease ($P<0.05$) in the number of erythrocytes was observed along the spawning season reaching the lowest level in September. Male plasma levels of cholesterol were high in June and July decreasing significantly in September ($P<0.05$). No significant differences ($P<0.05$) in cortisol levels were observed along the spawning season although a trend to diminish was observed at the end of the spawning season in females and males. Regarding other secondary responses to stress, no differences were found in glucose and lactate; however sodium showed lower values at the end of the spawning season in males ($P<0.05$).

Results for the 2016 spawning period.

In 2016, the number of available broodstock was reduced to eight (3 males and 5 females) after some mortality in the previous year. The fish were sampled in June, July, August, September, and October, and the selected fish were again implanted with the required GnRHa dose at three different times from June to September. The selected females were administered an effective dose of ~75 µg GnRHa kg^{-1} BW. The GnRHa dose for the males was the same as in 2015.

The mean oocyte diameter of the largest vitellogenic oocytes during the three samplings ranged between 571 µm in July to 776 µm in October, but no significant differences were observed. Sperm motility was 40-80% and remained unchanged throughout the monitored period, while the duration of sperm motility was significantly higher in September (3.9±0.2 min) than in June (1.8±0.1 min) and August (1.8±0.2 min) (P<0.05). The sperm density ranged from 0.86 ±1.28 x 10^{11} spermatozoa ml^{-1} in July and 2.24±1.82 x 10^{11} spermatozoa ml^{-1} in October, but no significant differences were observed during the monitored period.

The number of spawns was 20 after the 1st treatment, 23 after the 2nd treatment, 17 after the 3rd treatment and 1 after the final treatment (**Fig. 3.4.3a**). Mean fertilization and hatching exhibited similar trends during the three spawning periods, reaching their highest values in the second period (July) (**Fig. 3.4.3b**). However, no significant differences were observed between three first periods after successive GnRHa treatment.

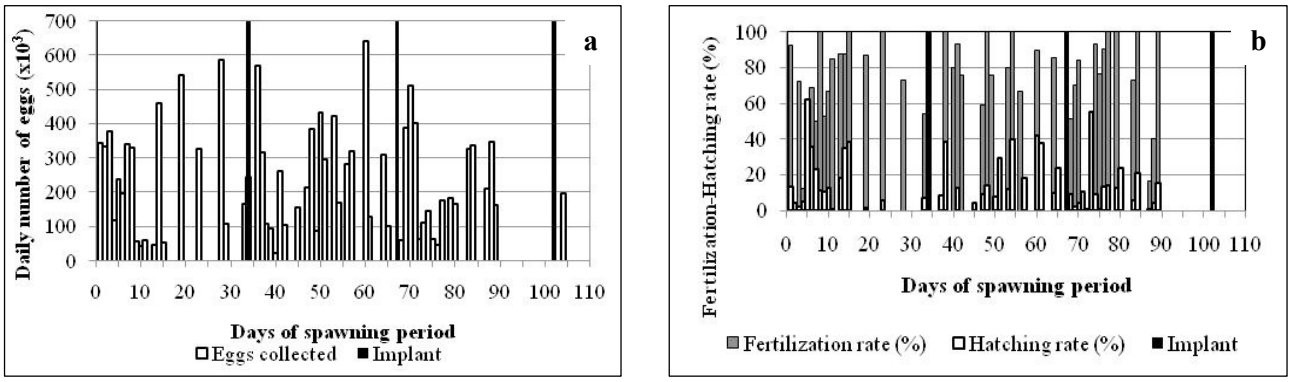


Figure 3.4.3 Daily fecundity (10^3 eggs) (**a**), daily fertilization and hatching success (%) during 2016 (**b**) spawning period. The black bars indicate different treatments.

Results for 2017 spawning period.

In 2017, only 3 males and 1 female (mean weight ± SD, 17.3 ± 6.9 kg) were available, and were maintained in an outdoor covered circular tank of 50 m3 with continuous water supply under natural photoperiod. At the expected onset of the spawning season (May 2017), a passive egg collector was placed in the outflow of the spawning tank and checked daily, in order to monitor the occurrence of any spontaneous spawning. According to the plan, the fish was supposed to be treated in the middle of June with the lowest dose of GnRHa proposed in the DOW (~25 µg GnRHa kg^{-1} fish). However, spontaneous spawns were obtained before the GnRHa treatment and it was decided not to handle the fish, in order to obtain valuable data on the kinetics, fecundity and egg quality of spontaneous spawns from captive F1 broodstock. In a period of 125 days (more than 4 months), 21 spawns were collected with 4-10 days interval amongst spawns. The larger intervals among the spawns were observed from mid-July to mid-August.

In summary, the present study showed that hatchery-produced greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning using GnRHa delivery systems of 50 and 75 µg kg^{-1}. Egg production is high and egg quality adequate for the implementation of larval rearing for commercial purposes. The use of consecutive GnRHa-delivery systems over a long reproductive period resulted in multiple spawns of fertilized and viable eggs. In addition to inducing OM after vitellogenesis is completed in females, the positive results obtained could be due to successful

synchronization of gamete release between males and females, but also to the stimulation of egg release by the females at the appropriate time after ovulation (Mylonas et al., 2004). Despite that repetitive handling required to administer the implants of GnRHa during the prolonged spawning season of F1 greater amberjack in the Canary Islands, the present study demonstrated that there was no negative effect on the welfare and reproductive performance of the fish, and seems to be an appropriate method for cultured fish in terms of welfare status. Additionally, spontaneous spawning of F1 greater amberjack in tanks in the Canary Islands is possible, as it was shown in 2017 spawning season. The successful reproduction of F1 greater amberjack broodstock, are a step towards the industrial aquaculture production of this valuable species.

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

The full description of the work and results is provided in ***Deliverable 3.10. Method for inducing spawning and collecting greater amberjack eggs in sea cages.***

In the present task we examined the potential of greater amberjack broodstock to spawn in sea cages and developed methods to collect eggs after spawning adapted to the cage facility, as it was shown with other large farmed species like Atlantic bluefin tuna *Thunnus thynnus* (De Metrio, et al., 2010; Mylonas, et al., 2007) and Pacific bluefin tuna (Masuma, et al., 2011).

2018 spawning season

In 2018 the HCMR stock already utilised during the previous spawning seasons, was used. The same trial carried out in 2016 was repeated leaving the fish to spawn spontaneously, without any hormonal treatment. This year, however, the net of the cage was changed. The standard 22 mm mesh net was replaced with a 5 mm mesh one. This resulted in avoiding the entry of any wild juvenile in the cage minimizing any potential foraging activity on the newly spawned eggs.

The fish were not sampled for the whole reproductive period. The total egg production was 1,195 – 1,265 g in 8 spawns (**Table 3.5.1**).

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

2018

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (g)
HCMR Souda	0	1	80-100 g
	0	2	750 g
	0	3	20 g
	0	4	20 g
	0	5	200-250 g
	0	6	100 g
	0	7	10 g
	0	8	15 g

In conclusion, egg collection in the sea cages is feasible in greater amberjack after spontaneous spawning.

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Deviations from Annex I and their impact:

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

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Jerez, S., Fakriadis, I., Papadaki, M., Martín, M., Cejas, J., Mylonas, C.C., 2018. Spawning induction of first-generation (F1) greater amberjack *Seriola dumerili* in the Canary Islands, Spain using GnRHa delivery systems. Fishes 3, 1-22.

Pousis, C., Mylonas, C.C., De Virgilio, C., Gadaleta, G., Santamaria, N., Passantino, L., Zupa, R., Papadaki, M., Fakriadis, I., Ferreri, R., Corriero, A., 2018. The observed oogenesis impairment in

greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is not related to an insufficient liver transcription or oocyte uptake of vitellogenin. Aquaculture Research 49, 243-252.

Sarih, S., Djellata, A., La Barbera, A., Fernández-Palacios Vallejo, H., Roo, J., Izquierdo, M., Fernández-Palacios, H., 2018. High-quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its comparison with GnRHa implants or injections. Aquaculture Research, 1-9.

Zupa, P., Fauvel, C., Mylonas, C.C., Pousis, C., Santamaría, C.A., Papadaki, M., Fakriadis, I., V., C., 2017a. Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack, *Seriola dumerili* (Risso, 1810). Journal of Animal Science 95, 4085-4100.

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

Fakriadis, I., Lisi, F., Sigelaki, I., Papadaki, M., Mylonas, C.C., 2018. Spawning kinetics and egg/larval quality of greater amberjack (*Seriola dumerili*) in response to multiple GnRHa injections or implants. General and Comparative Endocrinology, (https://doi.org/10.1016/j.ygcen.2018.12.007)

Fakriadis, I., Raftopoulos, A., Iakovopoulos, G., Papandroulakis, N., Papadaki, M., Siggelaki, I., Mylonas, C.C., 2018. Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared in tanks and sea cages. PLoS ONE. (in preparation)

Fakriadis, I., Miccoli, A., Raftopoulos, A., Sigelaki, I., Mylonas, C.C., 2019. Comparison of two GnRHa doses for spawning induction in greater amberjack *Seriola dumerili*. Aquaculture. (in preparation)

Fakriadis, I., Karapanagiotis, S., Tsele, N., Iakovopoulos, G., Mylonas, C.C., 2019. Timing of GnRHa treatment application for spawning induction in greater amberjack *Seriola dumerili*. Aquaculture. (in preparation).

Chrysovalentinos Pousis, Pasquale De Ruvo, Caterina De Virgilio, Constantinos C. Mylonas, Rosa Zupa, Letizia Passantino, Nicoletta Santamaria, Luisa Valentini, Aldo Corriero, 2019. Oocyte vitellogenin receptors in wild and captive-reared greater amberjack (*Seriola dumerili*) during three periods of the reproductive cycle. (in preparation).

WP 4 Reproduction & Genetics – pikeperch

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

Objectives

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

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

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

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

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

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

origins (German, Polish and Tunisian). The different captive broodstock populations presented different levels of genetic variability that ranged from wide variability greater than observed in wild populations to broodstocks that had reduced genetic variability that may have been the result of loss of variability through inbreeding. These broodstocks with reduced genetic variability should take measures to introduce greater variation into the base population for future breeding programs.

Summary of work reported in the previous Reporting Period (31-48 Mo):

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

Summary of progress towards objectives (49-60 Mo):

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

Details for each Task

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

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

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

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

Deviations from Annex I and their impact:

There were no deviations during the 4th Reporting Period.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Tsaparis, D., Lecocq, T., Fontaine, P., Economaki, K., Kyriakis, D., Darivianakis, S., Mylonas, C., Tsigenopoulos C. Evaluation of the genetic variation in domesticated broodstocks and wild populations of pikeperch Sander lucioperca in Europe as a tool for future breeding programmes. (In preparation)

WP 5 Reproduction & Genetics – Atlantic halibut

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

Objectives

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

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

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

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

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

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

Task 5.3 Fecundity regulation

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

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

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

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

Summary of work reported in the previous Reporting Period (31-48 Mo):

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

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

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

Summary of progress towards objectives (49-60 Mo):

All tasks were completed during the previous reporting periods and no work was done in the fourth period.

Details for each Task

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

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

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

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

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

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 5.3 Identification of potential disturbances in reproductive development in F1/F2 Atlantic halibut females.***

Deviations from Annex I and their impact:

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

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Norberg, B., Erstad, B., Bjelland, J., and Mylonas, C.C. GnRHa implantation advances and synchronizes spawning, but does not improve egg quality in female Atlantic halibut. *In preparation*

Norberg, B., Chauvigné, F., and Cerdá, J. Annual profiles of gonadotropins and sex steroids in female Atlantic halibut. *In preparation*

Norberg, B., Møgster, M., Mangor-Jensen, R., Olausson, S. K., Thorsen, A., and Hamre, K. Spawning performance and egg viability in farmed and wild-caught Atlantic halibut broodstock. *In preparation*

WP 6 Reproduction & Genetics – wreckfish

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

Objectives

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

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

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

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

During the 2nd Reporting Period, the work continued and advances were made with all tasks and objectives. **Task 6.1 Collect wild fish to establish new bloodstocks** has been complicated by the scarcity of wild wreckfish. Despite of these problems, new contacts have been established to catch wreckfish and two

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

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

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

Summary of progress towards objectives (49-60 Mo):

Regarding **Task 6.1 Collect wild fish to establish new bloodstocks**, during 2018 three new wreckfish juveniles from Mediterranean Sea (43º10'N-05º36'E, France) were obtained from Flying Sharks (company) and moved to the IEO facilities in August 2018. Bimonthly samples were done with excellent results in growth. Juvenile diet consist in dry commercial pellets (SPAROS) formulated based on the biochemical profile of the species. In the **task 6.2, Describe reproductive cycle**, during last period data obtained before allowed to know with more precision the reproductive cycle of the wreckfish, and to take advantage of the increase of spontaneous spawns to work with egg quality, fertility and relative fecundity of the females, obtaining important results for future studies of the reproduction of this species. Regarding **task 6.3 Development of spawning induction procedures**, our work was focus in to know the best method to be employed by the industry to spawn wreckfish, and achieving clear conclusions and knowing the best method is the spontaneous spawns in captivity and also a induction method with GnRHa implants with immature females.

Details for each Task

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

This task has been completed and the full description of the work and results has been provided in ***Deliverable D6.4 Establish reliable collection methods and protocols to form new wreckfish broodstocks.***

Three new wreckfish juveniles from Mediterranean Sea (43º10'N-05º36'E, France) were obtained from Flying Sharks (http://www.flyingsharks.eu/) and moved to the IEO facilities in August 2018. During 2018, the three fish were sampled twice, initial weights were 351.5, 351.8 and 609 g, with lengths of 32.5, 33.5 and 36 cm respectively **(Fig.6.1.1**.). During two months in captivity two juveniles have doubled weight, while the third achieved a weight of 1 Kg. Natural photoperiod and temperature cyles were maintained, with a mean sea water temperature between 15.4ºC and 19ºC. The ingestion rate was around 1% and FCR and SGR were 0.7 and 1% respectively. Fish were fed three times a week with a food based in a dry commercial pellet (SPAROS).

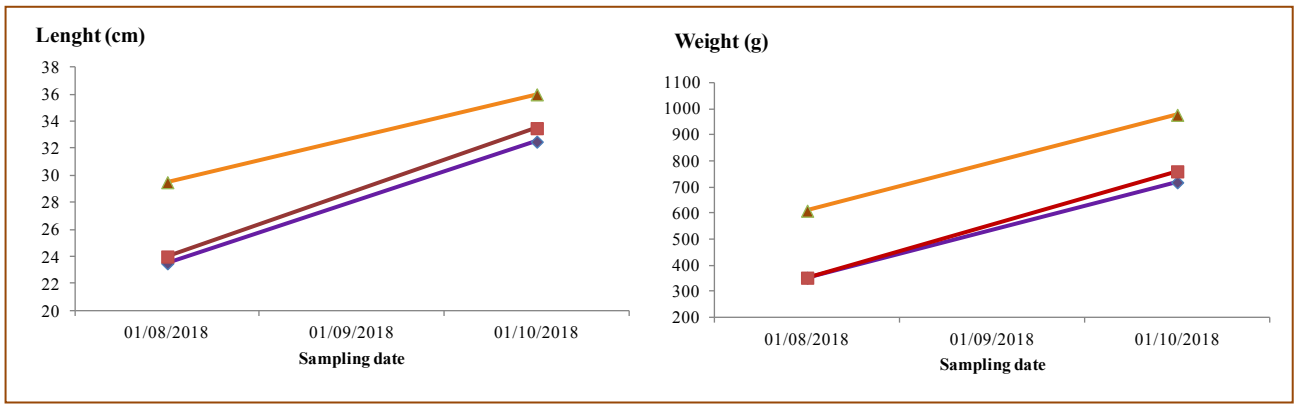

Figure 6.1.1. Length and weight of wreckfish juveniles in captivity that were captured in Mediterranean Sea in August 2018.

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

This task has been completed and the full description of the work and results has been provided in ***Deliverables 6.5. Describe the reproductive cycle of wreckfish.***

Most of the proposed work in the DOW was reported in the previous periodic reports. The remaining work according to the DOW was to analyse the gonadotropins (follicle stimulating hormone FSH / luteinizing hormone LH) of wild caught fish and evaluate the nutritional status of the fish.

Validation of the striped bass LH ELISA for wreckfish pituitary and plasma samples

In order to expedite the evaluation of the reproductive potential of wreckfish broodstock, the current study examined whether heterologous assay that was developed to measure striped bass (*Morone saxatilis*) LH (stbLH; Mañanós et al, 1997) and modified for tuna species (Rosenfeld et al., 2012, Berkovich et al., 2013) is suitable for monitoring LH profiles in the wreckfish. For that purpose, extract of pituitary derived from wild caught specimen was assayed at seven serial dilutions (range: 1:20,000 to 1,280,000).

For the plasma LH measurements 96- well polystyrene plates were coated with recombinant LH (r-LH; 2.4 ng per well) and incubated overnight at 4ºC. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl per well) for 0.5 h at 37 ºC. 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 ºC. Then the content in each tube was dispensed into the antigen-coated wells (100 µl per well in

duplicate). Following incubation (overnight at 4ºC), AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch laboratories, inc.) in 1% NGS-PBS T was added (100µl per well) for 0.5 h at 37ºC. The wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 µl per well). The reaction was stopped after 20 to 40 min at Room Temperature (RT) by the addition of 100µl of 1N phosphoric acid and the absorbance was read at 450 nm.

In order to test the possibility of using the stbLH ELISA for LH measurement in wreckfish, displacement curves obtained with serial dilution of pituitary extracts from wild wreckfish were compared with the rLH standard curve (**Fig. 6.2.1**). A clear linearity was obtained in the dilution of the wreckfish pituitary. Moreover, the dilution curves exhibited parallelism with the standard rLH enabling the determination of LH in this species. The sensitivity of the assay was 0.65 ng/ml and the respective inter- and intra- assay coefficients of variation were 8% and 15%.

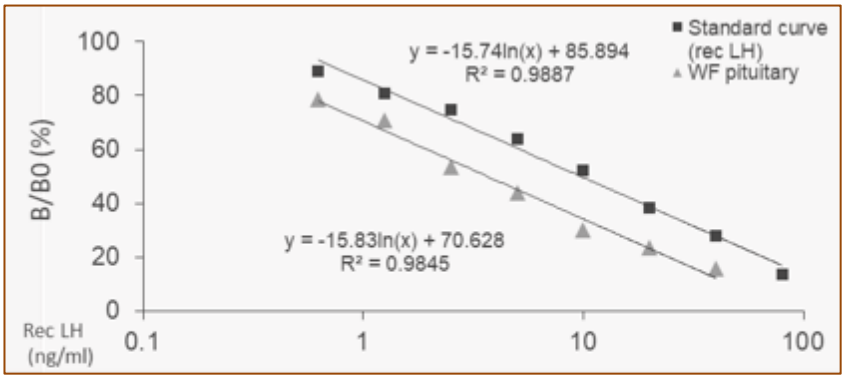

Figure 6.2.1. Displacement curves for standard rLH and serial dilutions of pituitary extract from wild wreckfish. The LOGIT function was utilized to transform standard curve to a linear plot. Each point is the mean of two determinations. (I cropped the figure to make it look a bit better, is it possible to put a figure with the scale clearly visible.)

The plasma LH levels, in both wreckfish females (**Fig. 6.2.2**) and males (**Fig. 6.2.3**) consistently increased in a stage dependent manner reaching their maximum concomitant with final gamete maturation, reflecting the classical roles of LH. Furthermore, the plasma LH levels in the ovulatory females (OV) were significantly ($P < 0.01$) higher than those measured in the spermiating males (S3). Interestingly, GnRH-implanted bluefin tuna (*Thunnus thynnus*) females and males exhibited similar plasma LH levels, however the pituitary LH content was significantly higher in females than in males (Rosenfeld et al., 2012).

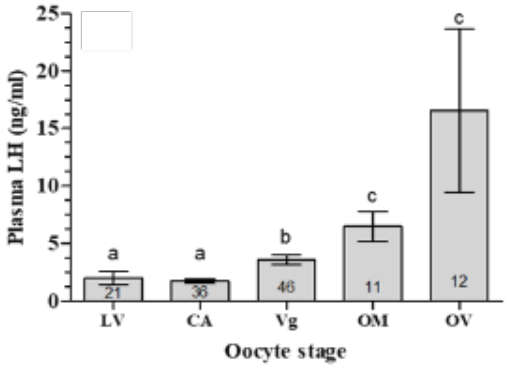

Figure 6.2.2. Mean (±SEM) plasma concentrations of LH at different stages of oocyte development (LV: lipid vesicle, CA: cortical alveoli, Vg: vitellogenesis, OM: oocyte maturation, OV: ovulation). Different letter superscripts indicate statistically significant differences in LH between different oocyte stages. The numbers inside the bars indicate the number of samples at each oocyte stage.

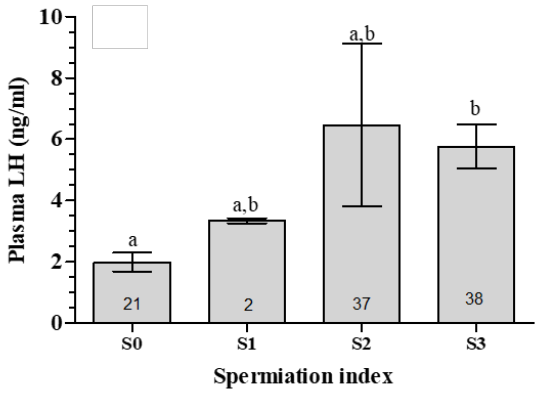

Figure 6.2.3. Mean (±SEM) plasma concentrations of LH of four different broodstocks of wreckfish in Greece and Spain at different spermiation index stages. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. Different letter superscripts indicate statistically significant differences in LH at different spermiation index stages, whereas numbers inside the bars indicate the number of male wreckfish found at each spermiation index stage.

References

Berkovich, N., Corriero, A., Santamaria, N., Mylonas, C.C., Vassallo-Aguis, R., de la Gándara, F., Meiri-Ashkenazi, I., Zlatnikov, V., Gordin, H., Bridges, C.R., and Rosenfeld, H. (2013). Intra-pituitary relationship of follicle stimulating hormone and luteinizing hormone during pubertal development in Atlantic bluefin tuna (*Thunnus thynnus*). Gen. Comp. Endocrinol. *194*, 10 23.

Mananos E. L., Swanson P., Stubblefield J., and Zohar Y. (1997). Purification of Gonadotropin II from a Teleost Fish, the Hybrid Striped Bass, and Development of a Specific Enzyme-Linked Immunosorbent Assay. General and Comparative Endocrinology 108, 209–222.

Rosenfeld, H., Mylonas, C. C., Bridges,C. R., Heinisch, G., Corriero, A., Vassallo-Aguis, R., Medina, A., Belmonte, A., Garcia, A., De la Gándara, F., Fauvel, C., De Metrio, G., Meiri-Ashkenazi, I., Gordin, H., Zohar, Y. 2012. GnRHa- mediated stimulation of the reproductive endocrine axis in captive Atlantic bluefin tuna, *Thunnus thynnus*. Gen. Com. Endocrinol. 175(1): 55-64

Biochemical analysis of nutrients

Analysis of carotenoids and vitamins of available samples from two wild female mature gonads and batches of eggs from the 3 Spanish centers, sent by CMRM, were performed at ULL facilities (P.15).

Carotenoids were extracted following the method described by Barua et al. (1993). Approximately 200-300 mg sample were homogenized with ethyl acetate/ethanol containing 0.01% BHT (10mL, 1:1 v/v), ethyl acetate (5 ml), and hexane containing 0.01% BHT (10 ml) as extracting solvents, respectively, in the presence of darkness and cold atmosphere. Afterwards, the solvent was evaporated and the extract re-dissolved in 1.5 ml hexane and maintained in the presence of nitrogen and darkness until their quantification by spectrophotometry (Beckman Coulter DU-800, IN, USA) at 470 nm.

Sample preparation and quantification of vitamins

Water-soluble vitamin C was extracted by homogenization of 100-200 mg sample in 2.5 mL metaphosphoric acid 0.5% containing 0.2% dithiothreitol (DTT). Homogenates were centrifuged at 1500 rpm, 4 ºC for 5 min and the supernatant diluted 1:10 using 0.5% metaphosphoric acid before injection into HPLC system (FAO, 1997).

Preparation of fat-soluble vitamins (FSVs), more specifically vitamins A, D and E, was carried out by hot saponification at 100 ºC for 20 min of approx. 100 mg sample, in a mixture of ethanol and 20% (w/v) aqueous KOH solution (8:1, v:v) in the presence of BHT as antioxidant. After a cooling period, FSVs were extracted with 3 mL hexane over 3 times, and centrifuged at 1000 rpm for 5 min. Finally, the solvent was evaporated to dryness with a gentle steam of N_2 and the residue reconstituted with 1 mL methanol and vigorously mixed for 5 min (Ball, 2006; Blake, 2007). Vitamin analysis were performed using a Thermo-Scientific ultra-high performance liquid chromatograph (Thermo-Fisher Scientific, San José, CA, USA) equipped with a Hypersil GOLD (100 x 2.1 mm, particle size: 1.9 µm, Thermo Scientific) column. An isocratic mobile phase composed of buffer acetate 0.2% DTT, pH 3.6: Milli-Q water: MeOH (1.5:94.5:4) or MeOH: Milli-Q water (91:9) was used for water-soluble and fat-soluble vitamins determinations, respectively. The injection volume was 5 µL and the flow rate 400 µL min^{-1}. All extracts were filtered through a 0.20 µm pore size polyester membrane filter prior to injection. The eluate was detected using an Accela photodiode array (PDA) detector (Thermo-Fisher Scientific) set at 245 nm (vitamin C), 265 nm (vitamin D), 292 nm (vitamin E) and 325 nm (vitamin A).

The concentration of vitamins in the samples was determined using an external standard method. Seven point calibration curves (n=3) were prepared with standard stock solutions of vitamins diluted in appropriate solvent mixtures at concentrations spanning those present in samples.

As shown in **Table 6.2.1**, the gonad and egg carotenoid and vitamin contents greatly differed according to their origin and sampling period. WFG (25-05-16) and WFG (02-03-17) are two of four samples of wild female mature gonads sampled on 2016 and 2017, respectively from Vigo market, as have been described and analyzed for proximate and fatty acid composition in ***D12.2 Recommendations for wreckfish broodstock feeds***. With 59.8±10.5% of protein and 20.7±4.8% of lipids (DW) and 7.07±1.4; 5.35±1.5; and 25.1±7.4, of ARA, EPA and DHA, respectively (see **Table 6**, **D12.2**). Although consistent values of Vitamin D, and A were measured in these two samples contents of vitamin E and also vitamin C and carotenoids were more variable. Average values of total carotenoids were of around 8mg per 100g of dry gonad.

Available samples of eggs corresponded to two batches from IEO stocks, two from IGAFA (CMRM) and two from AF. Sample IEO 1179 (20-03-15) eggs were sampled on 2015 from a S1 female fed a semi-moist diet consisting of a mixture of white and oily fish, fish meal and mussels, whereas sample IEO 7938 (17-04-17) were sampled from an S2 female fed for at least one year a dry formulated feed containing increased quantities of squid meal and also fish meal, krill meal, macroalgae mix and tuna oil among marine origin ingredients (**D12.2, Table 3**). Reported relative fecundity expressed in number of eggs per kilogram of female was of around 35000 in 2016, over 100000 in 2017 and over 120000 in 2018, for this second IEO female (**D12.2**). Compared to the mature wild gonads, the eggs from the S1 IEO female displayed marked lower contents of Vit C, nule of Vit A and particularly high levels of carotenoids, whereas those from the S2 IEO presented more comparable values of most of the nutrients, except for an apparent deficiency of Vit A and an excess of Vit E.

Sample IGAFA 3FF2 (14-06-16) eggs came from a squid fed female with 5000-10000 eggs released per Kg in 2016, not reported spawning on 2017 and 40000 of relative fecundity on 2018. Sample IGAFA 6D01 (22-03-17) eggs were sampled on 2017 from a squid and hake fed female who's reported relative fecundity was of around 40000 eggs on both 2016 and 2017 (**D12.2**). The two IGAFA egg batches lack of Vit A with the squid fed sample (3FF2) better resembling the average carotenoid and vitamins measured values in wild mature gonads.

Finally, as shown in **Table 6.2.1**, another two batches of eggs from MC2 facilities were available for the analysis. These eggs were sampled on 2015 and 2016, but since the feeding control became more variable (fresh and frozen marine origin food) and very difficult to control, this broodstock was not used for the feeding experiments described in **D12.2**. Therefore, no data is available on the specific fecundity of these two females. One more time, Vit A was not detectable in these eggs, with extremely high contents of Vit D and carotenoids in the sample MC2 (A. FINISTERRAE) 7B78 (16-07-15) compared to the wild gonads, and huge differences and much lower values of most nutrients measured with respect to the other MC2 batch 5853 (12-07-16).

Table 6.2.1. Concentration of vitamin C and fat-soluble vitamins (mg/100 g dry matter) and total carotenoids (µg/g dry matter) in wreckfish wild female mature gonads (WFG) and captive-reared wreckfish eggs from different culture facilities and batches.

	Vit C	Vit D	Vit A	Vit E	Carotenoids
Gonads					
WFG (25-05-16)	80.81	0.43	3.31	4.46	12.30
WFG (02-03-17)	34.39	0.72	3.77	0.12	4.46
Eggs					
IEO 1179 (20-03-15)	6.33	2.11	Nd	3.65	24.00
IEO 7938 (17-04-17)	21.85	1.58	0.70	8.08	9.41
IGAFA 3FF2 (14-06-16)	12.11	2.81	Nd	1.89	8.13
IGAFA 6D01 (22-03-17)	2.64	2.02	Nd	1.28	3.18
A. FINISTERRAE 7B78 (16-07-15)	38.15	17.99	Nd	2.26	68.26
A. FINISTERRAE 5853 (12-07-16)	Nd	1.04	Nd	3.19	8.17

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

This task has been completed and the full description of the work and results have been provided in ***Deliverable D6.3 Develop spawning induction methods with in vitro fertilization of wreckfish; Deliverable D6.6 Define an in vitro fertilization protocol to be employed by the industry to spawn wreckfish*** and ***Deliverable D6.7 Develop a spawning induction method for spontaneous spawnin of wreckfish in large tanks.***

In 2018 no induced spawning or in vitro fertilization trials were done, since natural spontaneous spawning was recorded. The only case where induced spawning with GnRHa administration was the MC2 facility in Spain and the fish left to spawn spontaneously (**Fig. 6.3.1)**.

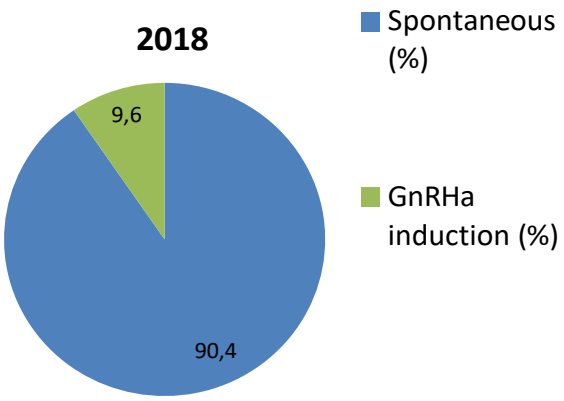

Figure 6.3.1. Number (%) of natural spontaneous and induction after hormonal administration (GnRHa implants) for the 2018 spawning season in the three Galician wreckfish broodstock.

The natural spawning behaviour was characterized by mature males chasing the mature females with liberation of eggs, which were immediately fertilized with the sperm released into the water by the male. Spawning mainly takes place during the night or very early in the morning. In 2018, spontaneous spawning in the IEO, MC2 and MCMR stocks produced a large number of fertilized eggs and achieved satisfactory fertilization success, establishing clearly a gap between one female spawns of 3-5 days in all stocks and verifying a time of spawns in early morning between 5-8 h, except for some that took place at mid day. The total number of annual spawns of all the Spanish stocks in the Diversify project increased substantially in 2018 (**Table 6.3.1, Fig. 6.3.2**).

Table 6.3.1. Number of spawns from 2015 to 2018 at IEO, MC2 and CMRM stocks.

STOCK	YEAR			
	2015	2016	2017	2018
IEO	10	9	13	43
MC2	14	23	5	19
CMRM	0	4	9	30
Total	24	36	27	92

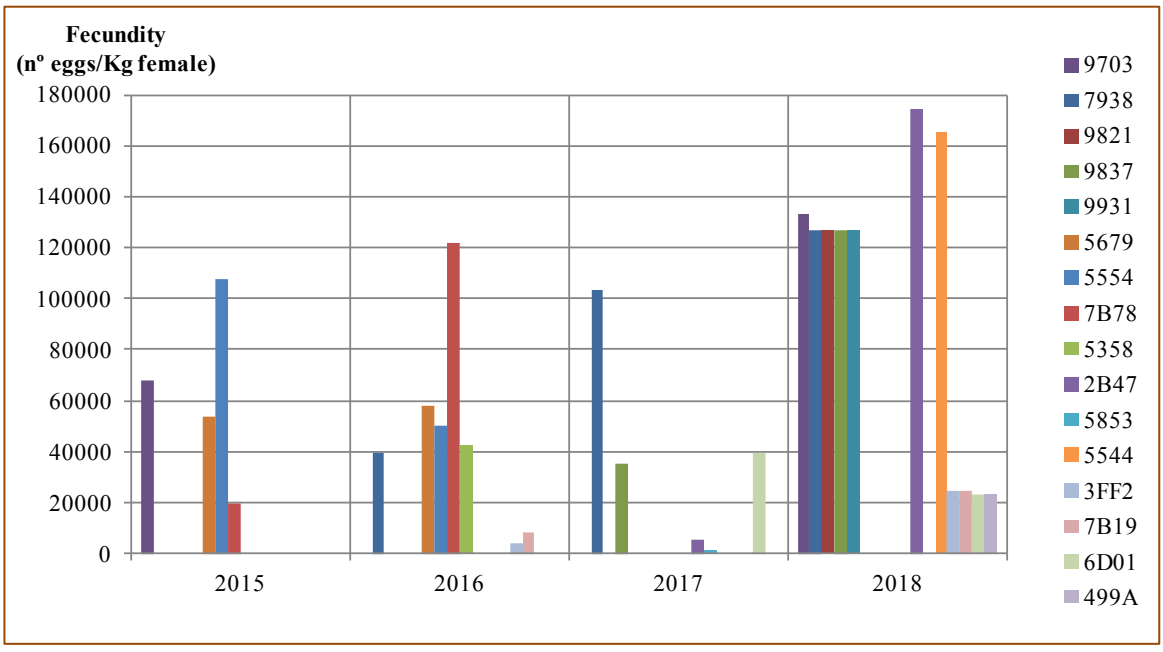

Figure 6.3.2. Relative fecundity (nº eggs/kg female) during 2015-2018 from the three Galician stock. The numbers in legend of the graph indicate the identification number tag of each female.

Percentage of egg fertility was between 50 and 100 % with better quality eggs at the medium-final part of the spawning period (**Fig. 6.3.3**). It has been found that females were able to spawn an average of 10 times per breeding season.

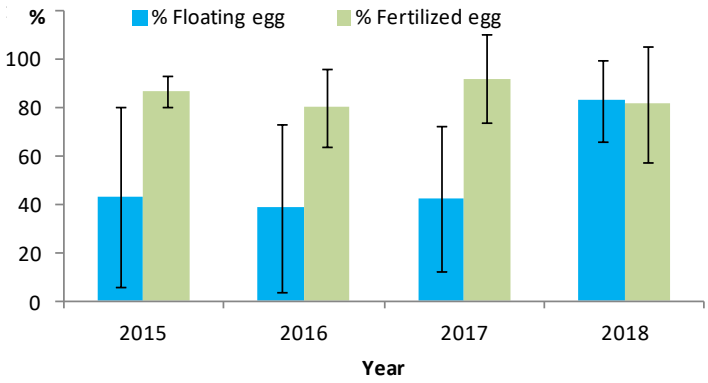

Figure 6.3.3. Total floating egg (% floating egg of the total egg spawned) and total fertility egg (% fertility egg in the total egg spawned).

During 2018 spawning season in the IEO facilities, it was observed that one male had the capacity to fertilise 30 spawn in a period of 150 days.

As shown in **Table 6.3.2** one female in MC2 facility was induced to spawn successfully in 2018 and spawned spontaneously 9 spawns with the volume of eggs exceeding 1100 ml and the fertilization success varying from 36 to 97%.

Table 6.3.2. GnRHa implant data of the spawning induction trial during 2018 in one female of the MC2 broodstock in Galicia.

YEAR	STOCK	FISH	WEIGHT (KG)	DATE IMPLANT	OOCYTES SIZE	Imp. GnRH (µg)	Dosis (µg/kg)	SPAWNING DATE	TOTAL EGGS (ml)	FECUNDATION (%)	OBSERV.
2018	MC2	2B47	20,1	20/06/2018	1,172	1750	87,0	26/06, 30/06, 4/07, 8/07, 12/07, 16/07, 19/07, 23/07 and 26/07/2018	1800, 1900, 1650, 1300, 1100, 1200, 1000, 1600 and 1600	97, 94, 97, 82, 77, 91, 83, 95, 36 and 76	ALL SPONTANEOUS

Spontaneous spawn management

The adaptation and development of a specific incubation system for the proper development of egg embryogenesis of this species has been essential to achieve very good results in the hatching rate, reaching values up to 80%.

During spawning season, a passive egg collector was placed in the outflow of the tank, in order to verify the occurrence of any spawning. Once a spawn was collected (**Fig. 6.3.4 *a***), it was transferred to a container with a sufficient volume of water to be able to separate the fraction of eggs that sank from floating eggs (**Fig. 6.3.4 *b*** and **Fig.6.3.5**).

The total egg volume must also be measured, as well as the floating and sinking volume, which was subsequently discarded. With the floating eggs, we measured the fertilization (%) and the stage of embryonic development using a stereoscopic microscope. The total number of eggs spawned was estimated by multiplying the observed egg volume by 150, which is the number of wreckfish eggs contained in 1 ml.

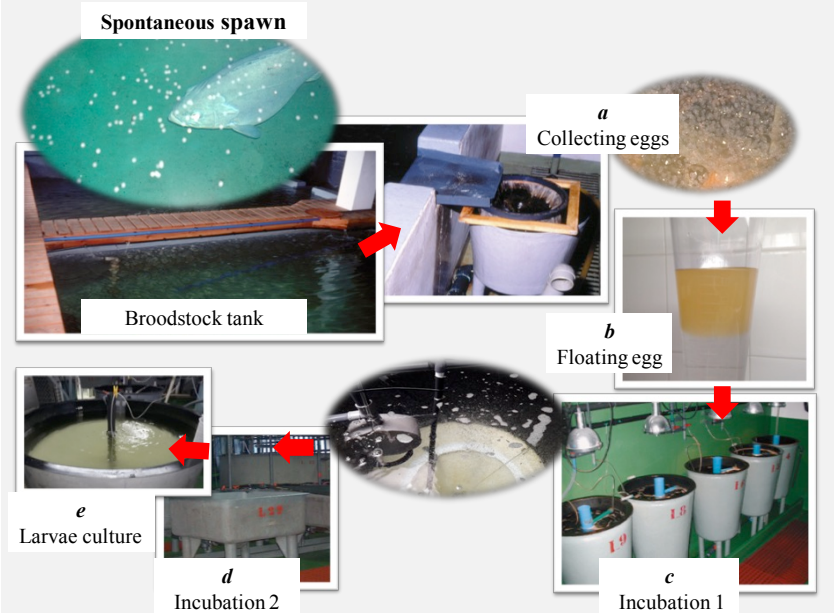

Figure 6.3.4. Spontaneous spawning management. Collecting eggs (***a***), separating viable floating eggs (***b***), incubating at first phase (***c***), the second phase (***d***) and finally transfer to the larval culture (***e***).

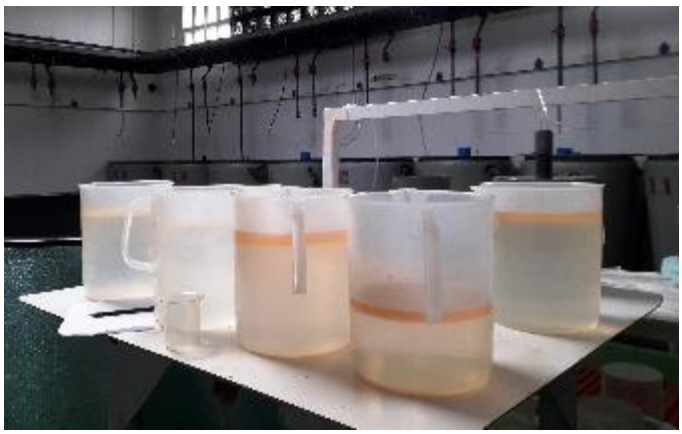

Figure 6.3.5. Total eggs of one spontaneous spawn separated in jars to measure the egg volume (floating and sunk) before incubation transfer to incubation tank.

The floating eggs were transferred into a cylindrical incubator tank with a conical bottom (**Fig. 6.3.4 *c*)** and a water flow at 16ºC, with enough air bubbles coming from the bottom to gently allow eggs distribution throughout the water column. During the first three days, one purge was made to collect dead eggs from the bottom of the tank, pulling out air and water inlet for few minutes so viable eggs raise to surface and dead eggs sank to bottom. On the third day of incubation (at 16ºC), when the embryogenesis begins, the air and water were removed and the floating eggs collected and transferred to another tank with different circulation and aeration system and similar water temperature (**Fig. 6.3.6**) until hatching. During this last stage of embryogenesis, egg density increased respect to water and the eggs lost buoyancy and tended to settle at the bottom of the incubator. Therefore, in a tank with a water flow from the bottom of the entire base of the tank the eggs remained in the water column. The air was arranged around the containment mesh to prevent the eggs sticking. Once larvae hatched (6dpf), they were transferred to the larval culture tank (**Fig. 6.3.4**), where husbandry continued.

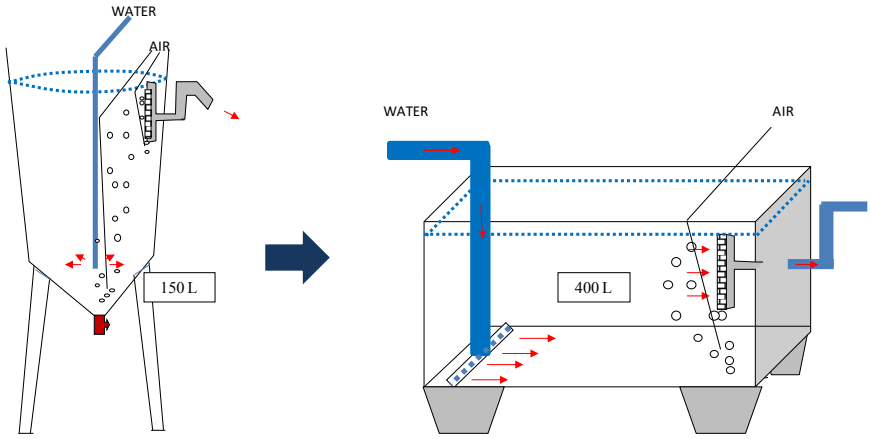

Figure 6.3.6. Scheme of incubation and hatching tanks for wreckfish eggs.

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

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

Deviations from Annex I and their impact:

The major obstacle to finish all the activities included in T**ask 6.2. Describe reproductive cycle**, was the lack of wreckfish pituitaries, which are crucial to validate and optimize the available heterologous ELISAs for this species. This is a prerequisite before analyzing plasma samples that we have already collected. Since we could not obtain pituitaries from episodically dead fish we validated the LH ELISAs using blood from some of the fish that we expected would have high LH levels. It was not possible to validate the FSH ELISA, so no results are provided.

The sharp drop in catches determined that the fulfillment of one of the objectives of the project that it was to obtain wild fish to increase wreckfish broodstocks of the different facilities involved in the project was very reduced, despite the efforts to contact with a large number of fishermen in different ports of Galicia. This objective can be improved in the coming years with the results obtained from the larval culture in 2018 with the provision of 25 juveniles of wreckfish, since in the future they can help to increase the number of individuals in the different wreckfish broodstock.

In respect to the nutritional status of the wild fish, the proximal composition and fatty acids profile of wild wreckfish are finished, but the carotenoids and C and D vitamins could not be measure due to a problem with the transport of the tissues samples. This problem was solved and the analyses were done, but because of the above problems the Deliverable 6.5 was delivered late.

The work completed on *in vitro* fertilization demonstrated that this method is complicated to apply to large expensive broodstock and that the manipulation has negative effects and caused some mortality. In addition, to successfully apply *in vitro* techniques it is necessary that the females reach advanced stages of maturity. In the initial stages of the project very few females reached advanced stages and this complicated the work with *in vitro* fertilization. Later in the project more females reached advanced stages of maturation and this coincided with these females spawning naturally and spontaneously. The over-all aim of the project for the reproduction of wreckfish was to achieve obtaining large quantities of fertilized eggs and this was achieved with the spontaneous spawning. The work on *in vitro* fertilization was therefore limited due firstly to few mature females and secondly due to the success of spontaneous spawning reducing the importance to research methods for in vitro fertilization.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Papadaki, M., Peleteiro, J.B., Alvarez-Blázquez, B., Villanueva, J.L.R., Linares, F., Vilar, A., Rial, E.P., Lluch, N., Fakriadis, I., Sigelaki, I., Mylonas, C.C., 2018. Description of the Annual Reproductive Cycle of Wreckfish *Polyprion americanus* in Captivity. Fishes 3, 1-20.

Pérez Rial, E., Linares, F., Rodríguez Villanueva, J.L., Vilar, A., Mylonas, C.C., Fakriadis, Y., Papadaky, M., Papandroulakis, N., Duncan, N., Robles, R., Lluch, N., Pazos, G., Méndez, B., Sigelaki, I., Gómez, C., Pérez, M. and Álvarez-Blázquez, B. 2019. Wreckfish (*Polyprion americanus*). New knowledge about reproduction, larval culture and nutrition. Promise as a new species for aquaculture. Fishes IP (Under review).

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	P25. DOR			
Lead Scientist preparing the Report (WP leader):	Hanna Rosenfeld			
Other Scientists participating:	Constantinos Mylonas (P1), Neil Duncan (P3), Sandra Ramos (P3), Aldo Corriero (P13), Christian Fauvel (P14), Covadonga Rodriguez (P15), 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 work reported in the previous Reporting Period (13-30 Mo):

Task 7.1 Evaluation of the effectiveness of hormone-based treatments on synchronizing gonadal development. The yeast expression system was used to produce large quantities of bioactive recombinant

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

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

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

Summary of progress towards objectives (49-60 Mo):

Task 7.3 Optimization and scale-up of a breeding protocol for grey mullet in captivity. The established breeding protocol (**D7.3** ***"Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet"***) was found to be effective for captive-born (G1) grey mullet broodstock (IOLR) and to a lesser extent for the wild caught broodstock (IRTA). The G1 broodstock responded well to the hormonal treatment during natural and artificially shifted spawning seasons giving rise to tens millions of quality eggs and consequently mass production of robust fingerlings. The basic breeding units consisting of a single female and three males, seems to improved synchronization and increase the fertilization rate. The formulated broodstock diet containing fish oil (FO; rich in n-3 LCPUFA) and Marigold petal meal (MgM) as another carotenoid source, apart from that provided by the 3% dry Ulva to the diet, positively affected hatching success and larvae survival (**D7.7** ***"Development of a breeding protocol for captive reared grey mullet broodstock based on optimized hormonal treatment, group structure and photo-thermal regime"***). In **Task 7.4 Assessment of the effects of captivity on first sexual maturity of wild-caught and hatchery-produced fish.** The aim of the present work was to assess the effects of captivity on

body growth, as well as on ovarian development and first sexual maturity on captive-reared and hatchery-produced grey mullet. The results indicated that: (1) the rearing conditions established at IORL allows a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea; (2) the reduction of the rearing density from 90 to 45 fish/m^3 has no effect on grey mullet growth and sexual maturity; (3) hatchery-produced grey mullet has a good potential to develop ovaries spontaneously up to a condition useful for bottarga production (**D7.5** ***"Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development"***). Further assessment of the effects of fish origin (wild vs. domesticated) and culture conditions on advanced and spontaneous development of gonads comprising the required criteria for the production of high quality bottarga (i.e., minimal size of 100 g, bright yellowish color and chewy texture) indicated that: (1) traditional grey mullet farming procedure in freshwater ponds could be applicable, and also an advantage, for the roe production (2) domestication appears to have a favourable effect on spontaneous development of mullet ovaries up to a condition useful for bottarga production (3) pigment-enriched diets can enhance the roe coloration to meet the criteria for high quality bottarga. However, two stumbling blocks that may impaired the profitability of grey mullet farming for roe production are: (1) extended grow out to a minimum of 3 years (2) relatively low percentages (20-50%) of females developing ovaries at the appropriate size (≥ 100 g). Future studies therefore, should focus on genetic improvement programs giving rise to advanced sexual maturity and spontaneous ovarian development in captive grey mullet females (**D7.6** ***"Culture procedure that identifies the ongrowing period for the production of grey mullet roe (bottarga) from wild and hatchery juveniles"***).

Details for each Task

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

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

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

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

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

The full description of the work and results is provided in ***Deliverable 7.7 "Development of a breeding protocol for captive reared grey mullet broodstock based on optimized hormonal treatment, group structure and photo-thermal regime".***

The current study aimed to develop a comprehensive and effective breeding management for captive reared grey mullet broodstocks. When reared in captivity, grey mullets display severe reproductive dysfunctions (Yashouv, 1969; De Monbrison et al., 1997): spermiating males are rarely observed and, in most cases, the produced milt is highly viscous and fails to fertilize the eggs; females are unable to finalize vitellogenesis or fail to undergo oocyte maturation once vitellogenesis is completed (De Monbrison et al., 1997). The observed failure in the attainment of vitellogenesis not only prevents ovulation and spawning. Previous

studies suggested that dopaminergic inhibition is a major barrier along the reproductive axis that arrests spontaneous spawning in captivity (Aizen et al., 2005). Consequently, a practical technique using dopamine antagonists and gonadotroping releasing hormone agonists (GnRHa) was developed. Nonetheless, the latter hormone-based treatment was found to enhance vitellogenesis in females, but fell short in stimulating spermatogenesis in males. To augment testicular development, grey mullet males were treated early on in the reproductive season with methyl testosterone (MT) administered *via* Ethylene-Vinyl acetate (EVAc) slow-release implants (Aizen et al., 2005). Despite the reported success, in most cases the treated males produced a very small volume of semen, which was highly viscous and failed to fertilize the eggs.

Reproduction is a very complex process that can be affected by several factors such as genetic background, environmental conditions including nutrition. In this task, therefore, we found it crucial to further improve the reproductive potential of captive grey mullet broodstocks fed experimental diets and to identify possible reproductive/metabolic dysfunctions through the characterization of essential nutrients including essential fatty acids (EFA), lipid and carotenoid profiles of captive broodstock gonads and eggs reared under different dietary regimes.

In view of the above, to further improve spawning success and reproductive outputs, the basic hormonal treatments have been used to test (i) the feasibility of extending the natural spawning period by the implantation of manipulated photo-thermal regime, (ii) the best performing breeder unit giving rise to maximal spawning success, and (iii) dietary effects on captive grey mullet reproductive performance and productivity. In addition, the latter hormonal treatments were implemented at both the IOLR and IRTA facilities, which enabled to compare treatments' effectiveness at different locations and on different broodstock origins, i.e., hatchery produced G1 vs. wild- caught captive-acclimated.

MATERIALS AND METHODS

Broodstock source and conditioning

IOLR broodstocks. Grey mullet breeders, consisted of 4-6 year old hatchery-produced (G1; first generation) fish that were individually tagged and maintained in 4-m^3 tanks supplied with ambient seawater at 40-ppt salinity (Gulf of Eilat, Red Sea) and subjected to either natural fluctuations of light and temperature conditions (elevation to 25°C in June, 28°C in August). In parallel, 4-year old G1 grey mullet females (n=18; av. BW= 1868 ± 74 g) and males (n=18; av. BW= 1154 ± 46 g) were acclimatized to a 4-month shifted photo-thermal regime. The experimental 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).

IRTA broodstocks. **IRTA broodstocks**. Grey mullet breeders were randomly assigned to the experiment from two groups: (1) wild caught fish from the Ebro Delta that had been acclimated in IRTA Sant Carles de la Ràpita for a period of over a year, and (2) a group from extensive culture in Seville (Spain) that had been acclimated in IRTA for 3 months. Three months or more before the experiment started, all fish were PIT tagged for identification and sexed. Fish were sexed depending on the presence of oocytes in the gonads obtained by cannulation. If an ovarian biopsy was obtained the fish were classified as female and fish that could not be biopsied were considered males. Fish were maintained in a 17 m^3 tank with seawater at 34-ppt salinity in recirculation (IRTAMAR®). Conditions before the experiment were natural temperature and photoperiod. During the experiment, temperature was controlled and maintained at 24ºC, whilst photoperiod and light conditions were natural. Breeders were fed daily at the rate of 1.5 % body weight with a broodstock commercial feed (Sole broodstock diet SPAROS, Portugal) and two days a week with frozen mussels (Sariego Intermares, Spain) and polychaetes (TOPSY Bait, Netherlands).

Hormonal acceleration of gonadal development

IOLR. To accelerate gonadal development mullet females and males were injected at the onset of gametogenesis (natural season: mid-July; shifted season: mid-October) with metoclopramide (15mg/KgBW) combined with r-FSH (5 µg per kg BW). One month later the males received MT-EVAc implant (5 mg/kgBW). Gonadal biopsies were timed with the advanced stages of gametogenesis (natural

season: September-October; shifted season: December-January). The relative abundance of fully mature females, and spermiating males were recorded. Females were considered fully mature once their oocytes reached an average diameter greater than 550 µm and more than 50% of sampled oocytes exhibit germinal vesicle migration. Sperm quality was classified into one of four categories based on its quantity, fluidity and ability to spread in the water.

IRTA. Nine grey mullet females and six males with a mean weight of 1,116.69 ± 395.78 g and 640.87 ± 293.90 g, respectively, were hormonally treated. The control group was formed with five females and two males with a mean weight of 909.76 ± 392.20 g and 532.60 ± 2.47 g, respectively. All fish were feeding well and in good condition with a condition factor that ranged between 1.18 and 1.86, with a mean of 1.6.

On the 24th July 2018, both males and females in the treated group received intramuscular injections of rFSH 5 µg/kg (provided by P4. IOLR), and 15 mg/Kg Metoclopramide (Metoc) (Sigma, Spain). On the same date, control fish were injected with a similar volume (0.5 mL) of saline solution. The fish were anaesthetized with 80 mg/L of MS222 for the application of treatments. No other sampling, maturity status or blood, was made on the fish. However, fish from the same stock that were not used in this experiment were sampled for maturity and blood. Two weeks (7th August) following the first injection to males and females, males received 17alpha-methyltestosterone (MT) loaded on EVAc slow-release implants at 5 mg/kg BW (provided by P1. HCMR). Males under 1 kg received one implant of 5 mg MT and males over 1 kg weight received two implants. Actual MT implant doses were higher than planned and ranged from 6.7 to 11.6 mg/kg. Males in the control group were injected with 0.5 mL of saline solution and a large bore syringe as used for the implants. At the beginning of September (4th), males received a second implant of MT following the same procedure. On the 1st October, the maturity status of all males and females were revised and determined. Ovarian biopsies were taken and the diameter of the 20 largest and most advanced oocytes were recorded. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure and spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = fluent but little sample can be obtained, 2 = fluent, 3 = very fluent). Total volume of sperm produced and sperm density was recorded. Percentage of sperm motility and duration of motility was checked in situ (a drop of sperm activated with 20 µL of clean seawater) with the fresh samples using light microscope (x10) and an hour later through videos recorded and analysed with Computer Assisted Sperm Analysis (CASA). Statistical comparisons were made between rFSH treated and control fish with a Student T-test.

Spawning induction trials

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

During the natural spawning season (2016) three breeding units varying in ratios of female to male and tank size, were tested. These include: **A)** 1 female and 3 males, in 1 m^3 tank, **B)** 2 females and 3 males, in 1 m^3 tank and **C)** 3 females and 6 males, in 3 m^3 tank. The rates of spawns per induction trails, attaining fertilized eggs per spawning event and hatching success were recorded.

Broodstock diet experiment

Based on the results of proximal and fatty acid (FA) composition in gonads from wild and domesticated mullets (see D13.3 Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression accumulat), the present study was aimed to improve the broodstock diet for grey mullet, through the: (i) replacement of soy oil (SO) with fish oil (FO) for increased supplementation of the n-3 LCPUFA, and (ii) addition Marigold petal meal (MgM; 3 mg kg^{-1} feed) as another carotenoid source, apart from that provided by the 3% dry Ulva to the diet. This meant that the total carotenoid level in the FO+MgM diet was *ca.*138 mg kg^{-1} while the SO diet was *ca.* 99 mg kg^{-1}.

During the onset of the reproductive season (early July 2017), 6-year old captive grey mullet broodstocks were divided into two groups, that were fed with either a mullet grow out diet containing soybean oil (SO)

that was previously developed by P4. IOLR, or with diet containing fish oil (FO+MgM), which is relatively rich in n-3 LCPUFA and carotenoids. The experiment was conducted in triplicates. Fish were maintained in 4 m^3 tanks supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40 ‰ salinity and subjected to natural fluctuations of light and temperature. Food was provided at the rate of 1-1.5% of their body weight. Fish were conditioned for spawning using protocols developed by P4. IOLR (as above).

In parallel, from each dietary group samples of ovaries, fertilized and unfertilized eggs were subjected to proximate composition, lipid classes, fatty acids and carotenoid analyses. Of note, the ovarian samples were taken from fully vitellogenic females that accidently died during the experiment (FO+MgM, n=1; SO, n=2).

RESULTS AND DISCUSSION

The aim of the present work was to systematically describe an expanded tool box for successful breeding of captive grey mullet, which includes hormonal, social (breeder unit) photo-thermal, and dietary conditioning compounds.

During several spawning seasons vast progress was made in optimizing hormonal treatments for alleviating reproductive dysfunctions among captive grey mullet broodstocks. In males, the combination of r-FSH injection and EVAc implant for sustained release of Methyltestosterone (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 (see D7.3 "*Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet*"). A previous study demonstrated that MT-EVAc treatment stimulates circulating levels of 11-KT, however in most cases the volume of the produced milt was relatively low (Aizen et al., 2005). In this regard, the value-added of the r-FSH-priming treatment administered as a single and to a greater extent double injections, seem to be attributed to the hormone's unique capabilities to regulate Sertoli cell activities (Schulz et al., 2010).

Aiming to improve the onset and progression of vitellogenesis among captive grey mullet, broodstock were treated with Metoclopramide (Metoc; an antagonist of dopamine D2 receptors) and rFSH. Indeed, the Metoc+r-FSH treatment enhanced and synchronized ovarian development in captive grey mullet females, giving rise to 91% post-vitellogenic females within the treatment-group. Relatively higher abundance of fully mature females and males (50-70%) could be found in untreated groups, compared to the relatively low percentages (10-20%) that were reported previously (Aizen et al., 2005). However, administration of Metoc+r-FSH to wild –caught breeders (IRTA) did not led to the development of vitellogenesis of wild grey mullet females, although in combination with MT implants wild-caught males produced good quality sperm. We tend to think that the enhanced spontaneous maturational process among captive grey mullet population also relates to the fact that all experimental fish at the IOLR (Israel) were hatchery-produced G1 broodstock, which have begun the process towards domestication. The fact that similar treatment was less successful in inducing vitellogenesis in wild-caught mullets at the IRTA (Spain) further emphasizes the discrepancy between the two broodstocks, i.e., hatchery produced vs. wild-caught. In that respect, a comparative study that evaluated pubertal development in hatchery-produced vs. wild-caught captive-reared grey mullet highlighted marked effects of domestication, with 3-year old hatchery-produced specimens exhibiting a more advanced ovarian development than fish caught from the wild and reared in captivity (see captivity (see D7.5 "*Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development*" and D7.6 "*Culture procedure that identifies the ongrowing period for the production of grey mullet roe (bottarga) from wild and hatchery juveniles*").

So far, regardless of the broodstock origin (i.e., wild- caught or G1) no spontaneous spawning have been documented in captivity. It is well established that dopamine can inhibit basal and GnRH-induced LH release (Peter et al., 1986, 1991; De Leeuw et al., 1986; Yu and Peter, 1992, Yaron et al., 2003), and also may modulate pituitary sensitivity to GnRH by decreasing the number of GnRH-receptors (De Leeuw et al., 1986, 1988; Omeljaniuk et al., 1989; Levavi-Sivan et al., 2004). Therefore, to induce grey mullet spawning, fully mature females and males were treated with two consecutive injections consisting of

GnRHa combination with Metoc. Results obtained so far, indicate up to 50% spawning successes following a treatment with priming (GnRHa 10 µg/kg; Met 15mg/kg) and resolving (GnRHa 20 µg/kg; Met 15mg/kg) injections given 22.5-h apart to selected fully mature females and males. Grey mullet seem to exhibit intricate social interplay in- and between- sexes, with dominant female(s) being capable of suppressing sexual maturation of conspecifics and enhancing males' spermiation (Aizen et al., 2005). Therefore, to promote the occurrence of successful spawning, several breeder group structure were tested. Our results (**Table 7.3.1; Fig. 7.3.1**) indicate that the best performing unit consists of a single female and 3 males, which prevents female intrasexual competition.

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

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

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

Figure 7.3.1. (A) Grey mullet experimental tanks consisting of 4 m^3 and 1 m^3 tanks for broodstock holding and spawning induction trials, respectively. (B) Breeder unit consisting of a single female and 3 males in a 1 m^3 tank, and (C) An egg collector.

The grey mullet, like other temperate fish species are annual spawners and mainly rely on annually cycling cues (temperature and photoperiod) to synchronise their reproductive cycle (Wang et al., 2010). The results of this study indicated that captive mullet responded very well to the 4-month shifted photoperiod giving rise to successful spawning events. Moreover, similar fecundities were documented during the shifted (2.22 ± 0.19 million eggs / kg BW) and the natural (2.5 ± 0.1 million eggs / kg BW) spawning seasons. These results suggest that photoperiod manipulation has significant potential for extending the availability of eggs and larvae for grey mullet aquaculture.

One additional way to overcome reproductive dysfunctions and/or improve gamete quality of captive broodstock is by providing an effective broodstock diet which should mimic body composition of breeders in the wild (Izquierdo et al., 2001; Rodríguez-Barreto et al., 2014). Following the same rational, the current study examined the effect of two diets differing in their oil source and carotenoid levels, on the grey mullet reproductive outputs. The basic diets that were used for the pigment-enrichment trial, consisted of the IOLR formulated pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid. Interestingly, despite of the difference of the PUFA in the diets (i.e., 18:2n-6 being higher in the SO diet), homeostatic mechanisms seem to allow the broodstock to maintain a balance resulting in a similar profile of polyunsaturated fatty acids (PUFA) in the gonads and eggs, independently of the diet, displaying

also a similar pattern to that of the wild counterparts (see **D13.3 "*Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression and accumulation*"**). Higher levels of 18:2n-6 and lower of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. In addition, the results of this study indicate that in both diets the ovaries described a predominance of neutral lipids (mainly triacylglycerol and wax-esters) over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs. This unique lipid composition, rich in wax esters is considered to play a role as metabolic energy resources for oocyte formation (Zudaire et al., 2014).

A comparison of proximate composition, lipid classes and fatty acid profiles in fertilized and unfertilized eggs revealed no marked changes regardless of the broodstock dietary regime (**Tables 7.3.2** and **7.3.3**). The only exaction was the noticeable disappearance (non-detectable levels) of sphingomyelin (SM), one of the four common phospholipids found in the plasma membrane of cells, in the viable fertilized eggs, independent of the treatment group. Lipid dynamic through the reproductive cycle is related to the functions of each lipid class during reproduction and a decrease in their quantity could limit fish productivity (Henderson et al., 1996; Marshall et al., 1999; Wiegand et al., 2007) and affect the viability of progeny (Rainuzzo et al., 1997). Indeed, similar decrease in SM levels was observed in *Xenopus laevis* fertilized eggs (Petcoff et al., 2008). In the latter study, it was found that during fertilization sphingomyelinase activation leads to SM hydrolysis and formation of ceramide, which can participate in a variety of cellular signalling, such as regulating differentiation, proliferation, and programmed cell death (PCD) of cells.

Carotenoids are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Miki, 1991; Guerin et al., 2003) and to ensure larval visual function and adequate chromatophore responses. Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. Since fish cannot synthesise either of the vitamins or carotenoids, the maternal dietary content of each prior to oogenesis is an important determinant of reproductive fitness and egg and larval quality. For this reason, we tested the effect of pigment-enriched diet on the reproductive outputs of captive mullet broodstock.

Table 7.3.2. Lipid content (% dry matter) and lipid of **eggs** from mullet fed a fish oil based diet (FO+MgM) or a soy oil based diet (SO)

	Unfertilized eggs		Fertilized eggs	
	FO+MgM	SO	FO+MgM	SO
Total lipid	28.49±5.85	23.15±9.71	25.88±2.75	25.85±5.07
Lipid class				
Sphingomyelin	0.21±0.30	0.42±0.14	nd	nd
Phosphatidylcholine	8.28±0.27	6.51±0.34	6.45±0.20	7.14±1.32
PS + PI *	0.68±0.13	0.76±0.07	1.11±0.61	1.02±0.21
Phosphatidylglycerol	0.60±0.17	0.35±0.28	0.53±0.12	0.59±0.41
Phosphatidylethanolamine	1.72±0.10	1.39±0.46	2.03±0.31	1.77±0.19
Total Polar Lipids	**11.48±0.11**	**9.81±0.94**	**10.13±0.91**	**10.72±1.34**
Diacylglycerols	nd	1.90±0.63	0.25±0.39	0.21±0.12
Cholesterol	5.01±0.38	5.69±0.06	5.38±0.29	5.19±0.71
Free fatty acids	2.35±1.01	0.64±0.05	nd	0.83±0.44
Triacylglycerols	11.37±0.55	9.93±0.75	12.35±1.89	10.24±1.47
Wax + Sterol esters	69.78±1.833	71.04±0.94	71.89±1.34	72.50±1.34
Total Neutral Lipids	**88.52±0.11**	**90.19±0.94**	**89.87±0.91**	**89.28±1.34**

Table 7.3.3. Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of **eggs** from mullet fed a fish oil based diet (FO+MgM) or a vegetable oil based diet (SO)

	Unfertilized eggs		Fertilized eggs	
	FO+MgM	SO	FO+MgM	SO
Total FA	**16.27±4.65**	**16.43±4.95**	**19.89±2.91**	**18.57±2.59**
14:00	0.34±0.01	0.26±0.01	0.38±0.04	0.30±0.02
16:00	8.78±0.48	8.11±0.17	8.72±0.73	8.37±0.48
18:00	2.80±0.10	2.77±0.14	2.84±0.16	2.87±0.17
Total SFA	**12.55±0.59**	**11.83±0.39**	**12.60±0.93**	**12.20±0.69**
16:11	7.61±0.25	5.39±0.03	5.80±0.15	6.61±0.77
18:12	38.95±2.30	34.36±0.23	39.77±2.75	34.45±0.74
20:12	1.21±0.08	1.22±0.02	1.12±0.18	0.80±0.05
22:13	0.12±0.17	nd	0.14±0.16	0.10±0.12
Total MUFA	**48.55±2.87**	**41.49±0.18**	**47.32±3.14**	**42.46±0.64**
18:2n-6	17.40±0.65	25.00±0.77	17.19±1.06	23.59±0.88
20:2n-6	0.79±0.04	1.03±0.00	0.79±0.04	0.90±0.07
20:4n-6	0.46±0.08	0.44±0.01	0.40±0.04	0.45±0.06
Total n-6 PUFA	**21.80±1.11**	**30.13±0.29**	**21.49±1.64**	**28.98±0.73**
18:3n-3	1.07±0.09	1.57±0.01	1.46±0.08	1.35±0.22
18:4n-3	0.10±0.14	0.26±0.01	0.27±0.04	0.28±0.03
20:5n-3	0.62±0.09	0.51±0.02	0.61±0.05	0.44±0.05
22:5n-3	2.02±0.14	2.60±0.21	3.79±0.29	2.64±0.40
22:6n-3	8.18±1.58	7.28±0.20	7.45±1.14	6.35±1.63
Total n-3 PUFA	**12.30±2.28**	**11.82±0.18**	**12.21±1.48**	**10.29±1.42**
DHA/EPA	13.07±0.65	14.20±0.88	12.13±1.17	14.42±2.83
ARA/EPA	0.74±0.03	0.86±0.04	0.65±0.04	1.02±0.09
n-3/n-6	0.56±0.08	0.39±0.00	0.57±0.04	0.36±0.05

Data are means ± SD (n=2 for unfertilized eggs and grey tank; n=4 for fertilized eggs). [1] mainly pn-7 isomer; [2] mainly n-9 isomer; [3] mainly n-11 isomer; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. nd, not detected.

Our results indicate that the dietary administration of marigold petal meal (MgM) pigment over 3 months, spanning the entire period of vitellogenesis, markedly contributed to the carotenoid levels in the ovaries and eggs giving rise to a bright yellowish color (**Table 7.3.4**; **Fig. 7.3.2**).

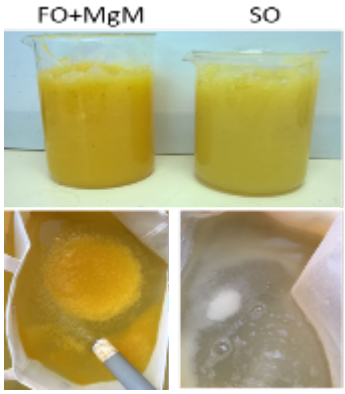

Figure 7.3.2. Effects of diets containing fish (FO+MgM) and soybean (SO) oil on grey mullet egg color.

Table 7.3.4. Total **carotenoid** content (mg kg^{-1}) of gonads and eggs from experimental mullets, and diets. Average values are expressed as mean ± SD. Different superscript letters indicate a significant (P<0.05) difference between mean values.

	Diets	Gonads	Fertilized eggs	Unfertilized eggs
FO+MgM	138.07	7.94	4.53 ± 0.50^{a}	4.74 ± 0.36^{a}
SO	99.25	5.68 ± 0.04	1.95 ± 0.48^{b}	2.87 ± 0.50^{b}

The MgM is a concentrated source of lutein and zeaxanthin (xanthophylls) pigments used to enhance the color of chicken eggs. The xanthophyll content of marigold petal meal (7000 mg/kg of meal) exceeds that of commonly used feed ingredients like yellow corn, corn gluten, alfalfa and algae by a factor which ranges from over 300:1 in the case of yellow corn (22 mg/kg) down to 3.5:1 for dried algae (2000 mg/kg) (North, 1984).

No dietary effects were observed on body weight or reproductive performance of the grey mullet broodstocks. However, the FO+MgM diet appears to increase the hatching success and survival rate of the larvae (**Table 7.3.5**). Furthermore, this diet contributed also to larvae tolerance of food deprivation and improved swim bladder inflation (see **D13.3** ***"Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression and accumulation"***). In that respect, Navas et al. (1997) found that egg quality and hatching rates were improved in seabass (*Dicentrarchus labrax*) by feeding appropriate amounts of n-3 LCPUFA during the vitellogenin period.

Table 7.3.5. Comparison of spawning data obtained from grey mullet broodstocks fed diets differing in their oil source: fish oil (FO) vs. soybean oil (VO).

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

The importance of species-specific effective levels of n-3 LCPUFA was also reported by Li et al. (2005) who determined that levels of these essential fatty acids below 2.40 or above 3.7% DW diet in crescent sweetlips, *Plectorhynchus cinctus*, had a negative effect, while between these values resulted in good egg

quality and larval performance. Zakeri et al. (2011) showed that replacing soybean oil with increasing levels of fish oil in the broodstock of yellowfin sea bream, *Acanthopagrus latus,* improved relative fecundity, percentage of buoyant eggs, hatchability, survival rate of larvae at 3 dph and higher starvation tolerance. Fish oil components, such as vitamin A and E as well as carotenoids may also affect egg quality and larval performance (Izquierdo and Koven, 2011).

CONCLUSIONS

1) The basic breeding units consisting of a single female and three males, seems to improved synchronization and increase the fertilization rate.
2) Shifted spawning season can be easily achieved via photo-thermal manipulation.
3) The established breeding protocol for captive grey mullet can be applied effectively during natural as well as shifted spawning seasons. During the past three years, tens millions of quality eggs were produced giving rise to mass production of robust fingerlings.
4) Broodstock diet enriched with pigment (MgM) and fish oil (FO), which is relatively rich in n-3 LCPUFA, positively affected hatching success and larvae survival.

Nevertheless, our results, which highlight episodic fertilization rates ranging between 0 to 98%, implicate the need to further fine tune and optimize the hormone-based and dietary regimes for successful captive breeding of grey mullet as a standardized technology for commercial venture.

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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)

The full description of the work and results are provided in ***Deliverable 7.5 "Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development"*** and ***Deliverable 7.6 "Culture procedure that identifies the ongrowing period for the production of grey mullet roe (bottarga) from wild and hatchery juveniles".***

The current study aimed to describe of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development: account of captive effects on pubertal development in grey mullet populations. In addition the study

evaluated the effects of diet and culture conditions on advanced and spontaneous gonadal development, prerequisites for mass production of the highly prized grey mullet roe (bottarga).

BACKGROUND

Puberty is the developmental period comprising the transition from an immature juvenile to a mature adult state of the reproductive system, i.e. the stage of development during which an individual becomes capable of reproducing sexually (Taranger et al., 2010). For fish farming industry, both early and delayed puberty may represent a major problem. The occurrence of a precocious puberty affects growth, health and welfare in salmonids (McClure et al., 2007), sea basses (Felip et al., 2008), flatfishes (Weltzien et al., 2003), cod fishes (Karlsen et al., 2006), tilapias (Longalong et al., 1999), sea breams (Gines et al., 2003, 2004) and perches (Shewmon et al., 2007). On the contrary, a delay or failure in the attainment of puberty may prevent reproduction and closure of the life-cycle in culture (Dufour et al., 2003; van Ginneken et al., 2007). In some cases (i.e. sturgeons), an advanced puberty increases farming economic sustainability by accelerating the production cycle of high-value products such as caviar (Taranger et al., 2010).

The roe of grey mullet, is known by a variety of names Bottarga, Sardinian caviar or Karasumi and is consumed either as salted individual eggs or as salted and dried whole ovaries or skeins. The catching of mullets and processing of its roe has a long tradition in Mediterranean countries on both the northern and southern coasts of the Mediterranean Sea. In addition countries in Asia and around the Australian continent capture sexually mature fish around the onset of their reproductive season and process the roe into this high cost fishery product for human consumption. The mullet roe production is a simple process involving (i) cleaning the roe sacs of any adhering blood or intestinal tissues after removal from the fish, (ii) coating the cleaned roe with fine salt for 6 – 12 hours, and (iii) pressing the roe to flatten it and drying it in the sun for about one week. At the end of this process, the roe having a yellowish-red color and rubbery texture is coated with beeswax and marketed. The unique chewy texture of the dried roe is due to a high content of wax esters, up to 60 – 70% of the lipid content, in the roe (Lu et al. 1979).

MATERIALS AND METHODS

Fish, Experimental System and Trial Procedure

First sexual maturity- In order to compare body growth in wild and farmed grey mullet, the age of 16 specimens sampled in the Lesina lagoon (10 females and 6 males) was determined through the count of growth marks observed in their scales (Meunier, 2002). Estimate of grey mullet theoretical growth in length was obtained by fitting the von Bertalanffy growth model (Bertalanffy von, 1938) to the mean lengths at estimated age: $TL_t = TL_\infty [1 - e^{-k(t-t_0)}]$ where, TL_t = predicted fork length at age t; TL_∞ = mean asymptotic fork length; k = growth constant ($year^{-1}$); and t_0 = theoretical age at which the fish would have been 0 cm in length.

For the analysis of body condition, 2 years old (hereafter referred to as age 2; n = 21) and 3 years old (hereafter referred to as age 3; n = 19) grey mullets were used. Each of the two age groups included two sub-groups: one of them was constituted by fish caught from the wild in the Ebro delta (northeastern Spanish coast) at an early stage, transferred to IOLR facility (Eilat, Israel) and reared in captivity for 2 or 3 years; the other group was made by fish produced in IOLR hatchery and reared in the same facility for 2 or 3 years before sampling. Moreover, both captive-reared and hatchery-produced fish were reared in 19 m^3 tanks at two different densities (low density =45 fish/m^3; high density = 90 fish /m^3) in order to evaluate the effect of rearing density on ovarian development and maturation.

The reproductive state was assessed by recording the most advanced oocyte stage for each specimen, according to commonly used classifications (Corriero et al., 2007; Zupa et al., 2017).

In order to compare oocyte yolk accumulation in wild, captive-reared and hatchery-produced individuals, the largest vitellogenic oocytes, 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 connected to a light microscope, using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK).

Effect of salinity regimes on gonadal development in wild-caught grey mullet. The fish origin: wild caught and reared in captivity vs. hatchery produced. The wild grey mullet used for this study were caught as fingerlings along the Israeli Mediterranean coast and reared in fresh water earthen ponds for two years. Then, the 2-year old fish (average body weight of 786 ± 33 g) were stocked in round 20 m^3 concrete tanks at densities of 4 kg/ m^3 provided with a continuous supply of either pumped freshwater or Red Sea seawater (40-ppt) and maintained at 24-28ºC and natural photoperiod regime throughout the experiment. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. Following a year of growth, fish (n=11, 15) of the two treatment groups were sampled during the species' natural spawning period (Mid-September). From the sampled fish, the following data were recorded: total length, TL in cm; body weight, BW in g; gonad weight, GW in g; liver weight, LW in g, viscera weight in g, and the weight of body fat in g. The gonado-somatic and hepato-somatic indices were calculated as GSI = $100xGWxBW^{-1}$ and HIS= $100xLWxBW^{-1}$, respectively. Additionally, to evaluate the ovarian reproductive stage the diameter of the most advanced oocytes were reordered.

Effect of a pigment-enriched diet on roe coloration. Three-year old hatchery-produced grey mullet were maintained in outdoor 4 m^3 tanks supplied with ambient Red Sea seawater (40 –ppt) which were exposed to natural fluctuations of light and temperature. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. During early June, concomitant with the onset of gametogenesis, the fish were divided into two groups and fed over 3 months with the IOLR pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid (see D13.3). However, the FO pelleted diet was also supplemented with Marigold petal meal (MgM; 3 mg kg-1 feed) as another carotenoid source, apart from the fish oil and 3% dry Ulva (produced at the IOLR). This meant that the total carotenoid level in the FO+MgM diet was ca.138 mg kg-1 while the SO diet was ca. 99 mg kg-1. During mid-September, coinciding with advanced stages of gametogenesis, 10 to 12 fish were sampled from each dietary group and the following parameters were measured; total length (cm), body weight (BW;g), gonadal weight (GW;g), liver weight (LW;g) and viscera weight (g). The gonado-somatic and hepato-somatic indices were calculated as above.

RESULTS AND DISCUSSION

The aim of the present work was to assess the effects of captivity on body growth, as well as on ovarian development and first sexual maturity on captive-reared and hatchery-produced grey mullet. This study was based on the analyses of: a) biometric data; b) age and growth; c) GSI and microscopic appearance of the ovaries. The analysis of the biometric data did not show any significant effect of fish origin and rearing density on the body growth of grey mullet reared for 2 and 3 years in captivity. In fact, fish caught from the wild and reared in captivity had the same body size, mass and condition index of hatchery-produced fish. Similarly, no difference was observed in body size, mass and condition index between fish reared at two different densities.

The study of the temporal trend of GSI and ovarian microscopic appearance of wild grey mullet sampled in different areas of the Mediterranean Sea showed a progressive gonadal development from April to August-September. Histologically, none of the ovaries showed signs of imminent (oocytes in final maturation) or recent (post-ovulatory follicles) spawning and late vitellogenesis was the most advanced oocyte stage observed in the analysed ovary samples. These findings seem to confirm previous studies indicating a late summer-early autumn spawning period from the wild grey mullet from the Mediterranean Sea (Assem et al., 2008; Bartulović et al., 2011). The occurrence of specimens with high GSI and fully vitellogenic ovaries captured during their migration from the Lesina lagoon to the Adriatic Sea confirms that the ovary ripening process (i.e. vitellogenesis) occurs in estuarine/brackish waters and then the fish move to sea waters to spawn (Thomson, 1955; Ibáñez and Gutiérrez-Benítez, 2004).

In the present study, oocytes in late vitellogenesis were found in wild grey mullet having a minimum size of 38 cm TL. This body length, according to the TL-SL correlation provided by Guino-o II (2012) corresponds to 30 cm SL and falls within the first maturity size range reported by Ameur et al. (2003), i.e. 27–35 cm SL, for the grey mullet from the eastern Atlantic Ocean (Moroccan coasts). The histological analysis of the ovaries demonstrated that farmed grey mullet started to be reproductively active at the age of 3 years. In particular, 54% (7/13) of age 3 hatchery-produced grey mullets were sexually mature vs 25% (1/4) of captive-reared fish. This finding seems to indicate that hatchery-produced grey mullet have a good reproductive potential, as they were able to attain sexual maturity at the same age of the wild population (Bok, 1983; Ameur et al., 2003).

The analysis of GSI and oocyte diameter clearly confirmed that age 3 hatchery-produced specimens attained a more advanced ovarian development than fish caught from the wild and reared in captivity. Similarly, the amount of yolk of oocytes from age 3 hatchery-produced fish was similar to that of wild adults and higher than that of captive-reared fish (**Fig. 7.4.1**).

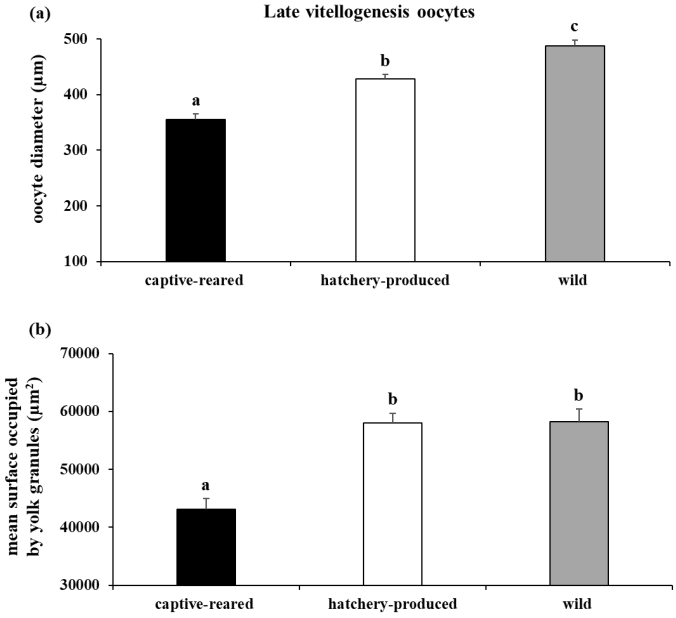

Figure 7.4.1. (a) Mean diameter of late vitellogenic oocytes and (b) mean surface occupied by yolk granules in oocytes of age 3 captive-reared and hatchery-produced grey mullets, and of wild adult females. Letters above bars indicate statistically significant differences ($P < 0.05$).

An additional aim of the present work was to assess the effects of fish origin (wild vs. domesticated) and culture conditions on advanced and spontaneous development of gonads comprising the required criteria for the production of high quality bottarga, i.e., minimal size of 100 g, bright yellowish color and chewy texture.

The results of the first experiment indicated that regardless of the water salinity, farmed grey mullet females can proceed through the late stages of vitellogenesis and gain relatively high GSI values (10-20%). These results further attest to the notion that in wild grey mullet the ovary ripening process (i.e., vitellogenesis) occurs in estuarine/brackish waters and then the fish move to sea waters to spawn (Thomson, 1955; Ibáñez and Gutiérrez-Benítez, 2004).

Thus, the ability to undergo vitellogenesis coupled with improved growth performance in freshwater relative to seawater conditions, highlight the relevance of conventional grey mullet farming methods, which include rearing in freshwater ponds, not just for the fish flesh but also for the bottarga production. However, to date farmed mullets are being harvested after 2 years of growth and destined to the fresh fish

market, before they reach sexual maturity. Based on our recent results (see D7.5 *"Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development"*), farmed grey mullet like the Mediterranean wild grey mullet populations, start being reproductive active at the age of 3 years (Bok, 1983; Ameur et al., 2003). Therefore, to facilitate bottarga production the grey mullet grow out period should be extended to a minimum of 3 years. In this respect, an advanced puberty due to domestication effects and/or genetic improvement program (Taranger et al., 2010) may increase the farming economic sustainability by accelerating the production cycle of this high-value "mullet caviar".

The fact that relatively low percentages (10-20%) of wild-caught captive reared females have reached late stages of vitellogenesis is in line with previous studies describing the difficulties of Mediterranean grey mullet broodstocks to proceed with gametogenesis in captivity (De Monbrison et al., 1997; Yashouv, 1969). Captive mullet female reproductive dysfunctions were confined to two critical phases, i.e., the early stages of vitellogenesis, and final oocyte maturation and ovulation (De Monbrison et al., 1997). Furthermore, Aizen et al. (2005) reported that some female mullets (approximately 20%) in untreated groups manage to go through the processes of vitellogenesis, while most females do not. It was suggested that low percentages of fully mature females, surrounded by undeveloped females (of the same age and size), typifies a state of social hierarchy, in which the dominant female(s) suppresses sexual maturation of conspecifics. The results of this study further indicate that (i) sexually undeveloped and developed mullet females have comparable body weights and (ii) sexually undeveloped females have remarkably higher energy reservoirs (i.e., visceral and body fat mass) compared with those of the sexually developed ones (**Fig. 7.4.2**). These characteristics exclude the incidence of food limitations as a possible cause for the failure of the vast majority of the captive females to develop gonads and support the possible involvement of social hierarchy.

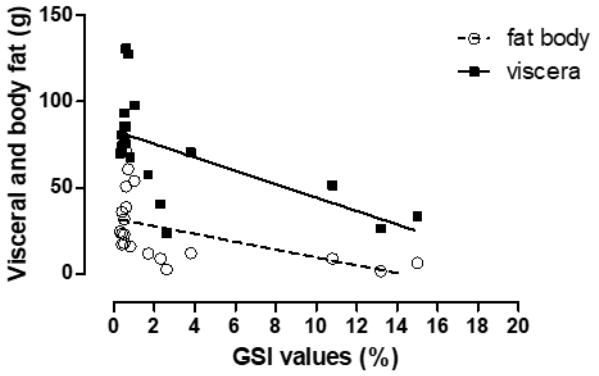

Figure 7.4.2. The relationships between GSI values and the weights of fat body and viscera in 3-year old wild-caught and captive-reared grey mullet.

The current study points to favourable effects of captive conditions on mullet females' competence to undergo and complete vitellogenesis. Accordingly, increased abundance (50%) of spontaneously developed gonads (GSI values 10% and above) could be detected among the G2 (second generation) captive-born mullet populations, relative to their wild-born captive-reared counterparts.

Mullet's cultured intensively in earthen ponds would be fed formulated feeds to ensure their receiving sufficient nutrients to foster optimal body growth and gonadal development. Natural food (detritus) which develops in the pond may act as a supplement to the formulated feed provided by the farmer and a rich source of microelements such as vitamins, minerals and pigments. Whether or not the detritus will supply sufficient pigments to generate the characteristic yellowish color of developed ovaries will be dependent on

a number of factors, two of which are the composition of the detritus and its abundance relative to the fish population. By supplying sources of natural pigments as part of the formulated feeds to the mullet, we have a greater chance of ensuring that they receive enough to enhance the color of their roe. Indeed, the dietary administration of marigold petal meal (MgM) pigment over 3 months, spanning the entire period of vitellogenesis, markedly contributed to the carotenoid levels in the ovaries (**Fig. 7.4.3**) giving rise to a bright yellowish roe color (**Fig. 7.4.4**).

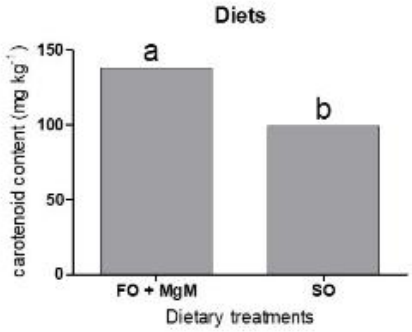

Figure 7.4.3. Carotenoid content of the fish oil (FO) + marigold petal meal (MgM) and soybean oil (SO) diets, based on the IOLR formula that were pelleted in a California pelleting mill.

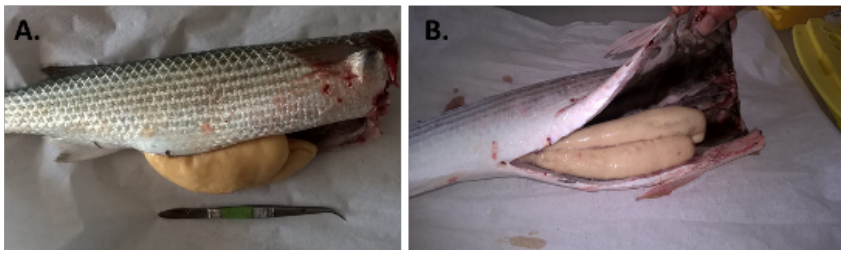

Figure 7.4.4. Roe derived of 3-year old hatchery produced grey mullet fed (A) a fish oil based diet (FO) + marigold petal meal (MgM) demonstrating a bright-yellow color and (B) a vegetable oil based diet (SO) showing a lack of color.

The basic diets that were used for the pigment-enrichment trial, consisted of the IOLR formulated pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid. Interestingly, despite of the difference of the PUFA in the diets (i.e., 18:2n-6 being higher in the SO diet), homeostatic mechanisms seem to allow the broodstock to maintain a balance and similar profile of polyunsaturated fatty acids (PUFA) in the gonads, independently of the diet, displaying also a similar pattern to that of the wild counterparts. Higher levels of 18:2n-6 and lower of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. In addition, the results of this study indicate that in both diets the ovaries described a predominance of neutral lipids (mainly triacylglycerol and wax-esters) over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs. This unique lipid composition, rich in wax esters is considered to play a role as metabolic energy resources for oocyte formation (Zudaire et al., 2014) as well as major contributor to the chewy texture of the dried roe (Lu et al. 1979; Bledsoe et al., 2003).

CONCLUSIONS

In conclusion, the present study indicates that: 1) the rearing condition established at IORL allows a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea; 2) the reduction of the rearing density from 90 to 45 fish/m^3 has no effect on grey mullet growth and sexual maturity; 3) hatchery-produced grey mullet has a good potential to develop ovaries spontaneously up to a condition

useful for bottarga production (advanced vitellogenesis). (4) traditional grey mullet farming procedure in freshwater ponds could be applicable, and also an advantage, also for the roe production (5) domestication appears to have a favourable effect on spontaneous development of mullet ovaries up to a condition useful for bottarga production (6) pigment-enriched diets can enhance the roe coloration to meet the criteria for high quality bottarga. However, two stumbling blocks that may impair the profitability of grey mullet farming for roe production are: (1) extended grow out to a minimum of 3 years (2) relatively low percentages (20-50%) of females developing ovaries at the appropriate size (≥ 100 g). Future studies therefore, should focus on genetic improvement programs giving rise to advanced sexual maturity and spontaneous ovarian development in captive grey mullet females.

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

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 7.4 Protocol for shipping grey mullet eggs***

Deviations from Annex I and their impact:

D7.7 Development of a breeding protocol for captive reared grey mullet broodstock based on optimized hormonal treatment, group structure and photo-thermal regime, includes additional work studying the effects of broodstock diet on gamete quality and reproductive outputs.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Rosenfeld, H., Corriero, A., Rodriguez, C.et al. Advances in broodstock nutrition of grey mullet enhances larval performance. (In preparation).

Corriero, A., Rodreguez, C., Rosenfeld, H. et al. Effect of captivity on the timing of the onset of first sexual maturity in grey mullet (*Mugil cephalus*). (In preparation).

Rosenfeld et al. Overcoming reproductive dysfunction in captive grey mullet (*Mugil cephalus*) broodstock. (In preparation).

Ramos, Sandra, François Chauvigné, Wendy Gonzalez, Hanna Rosenfeld, Joan Cerdà, Ignacio Giménez, Neil Duncan. Induced gametogenesis in flathead grey mullet (*Mugil cephalus*) using homologous recombinant follicle stimulating hormone. (In preparation).

Group Work Packages

Nutrition

The work done with greater amberjack was focused in on-growing diets and the formulation of an experimental diet for broodstock. ***The dietary lysine requirement determined for greater amberjack juveniles was previously determined to be 2.11% of diet***. A commercial diet containing this amount of lysine was ***tested in farm conditions***, with results on growth and FCR demonstrating that this amount of lysine covers the requirements of this amino acid in this species. The experimental diet for broodstock was based on the results obtained during 2nd and 3rd periodic report to contain ***higher proportions of polar lipids and a fatty acid profile***, and was assayed in comparison to a commercial diet or a mackerel supplementation. A Mackerel group, fed on frozen mackerel (composition in % dry weight: crude protein: 22.0%, crude fat: 9.6%, moisture 76.8%) supplemented with a vitamin premix. A Control Group, which was fed with a commercial Control pellet and an Experimental Group, feeding a diet formulated accordingly to previous results obtained for this species to contain higher proportions of polar lipids and a fatty acid profile. The three groups of breeders did not release eggs naturally or induced, after the successive hormonal treatments, during the study period, but based on the biopsy of some of the experimental fish, the results obtained on oocyte maturation and sperm quality, and the results on spawning quality of broods fed different levels of essential fatty acids, obtained in previous periodic reports, ***a diet containing 14-15 % EPA+DHA of total fatty acids (corresponding to 2.5-3 % in a dry diet) resulted in best spawning performance in greater amberjack broodstock, and increasing dietary EPA+DHA contents did not improve spawning performance.***.

A confirmatory experiment was carried out to establish effects on growth performance, enzymatic activity and deformities by inclusion of ***graded levels of dietary Ca/P ratio in weaning diets for pikeperch***. The effect of Ca/P ratio was examined not only varying one of the two minerals, but also by varying both. As so six diets with three Ca/P levels (0.3, 0.6 and 1.2). Low growth was observed in larvae fed the diet containing the highest Ca/P ratio and the lowest P %**,** suggesting a ***low Ca/P ratio in pikeperch larval diets***. No significant differences were observed in digestive enzymatic activities. A second experiment involved formulation of a test diet (based on information gathered by previous experiments) and test of the performance of larvae/juveniles reared on this diet against a high quality commercial control diet until 52 days post hatch. Emphasis was on optimal dietary lipid class composition; LC PUFA levels and ratios; levels and ratios of vitamins (Vit A, E, C, D) and minerals (Ca/P, Se). ***The formulated diet was superior in terms of larval growth at 35 DPH and 52 DPH to a commercial high quality performing diet, Otohime, when tested at commercial farm conditions***.

For Atlantic halibut, the inclusion of soy lecithin so that ***dietary phospholipid increased from 9 to 30% of total lipids had no effect on growth, but changed lipid metabolism*** in Atlantic halibut juveniles.

Gonads from females of wild wreckfish have a high level of ARA (7-10 %TFA) and EPA/ARA ratio nearly 1. Dry food specifically formulated for wreckfish broodstock must contain a big amount of proteins, low level of lipids, a high amount of n-3 PUFA and the EPA/ARA ratio must be similar to the one obtained in wild females gonads. The use of ***formulated diets specific for wreckfish increased relative fecundity*** over the years from 2015 to 2018. After the use of two enrichments with different levels of ARA, no differences in fatty acid composition of wreckfish larvae fed with the prey enriched with the two enrichment products were found at 11, 18 and 21 dph, ***larvae showing high amounts of PUFA specially DHA, EPA and ARA***.

Grey mullet fed on a commercial carp diet exhibited green dark spots on the surface of the liver where coloration of the liver in mullet fed the IRIDA diet was normal. There were no differences in sensory

parameters such as odor, flavor and texture between the commercial carp feed and the Irida diet. Besides, ***the use of poultry meal induced a significant improvement in the performance of grey mullet in terms of growth, digestive tract length and weight***. The utilization of soybean or poultry meal did not induced digestive tract disease or oxidative stress. ***Grey mullet broodstocks fed a fish oil (FO) based diet was significantly higher than eggs from broodstocks fed a vegetable oil (VO) based diet***. Larvae from the FO broodstocks showed 100% swim bladder inflation by 5 dph, and no swim bladder inflation was found in fish from the VO broodstock. When comparing the fatty acid and lipid class profiles between female and male gonads, there were highly marked differences. ***Higher contents of total lipids, triacylglycerols and wax and sterol esters in female gonads compared to male gonads*** while there were higher quantities of phospholipids in male gonads compared to female gonads. The results suggest that ***another FO component, possibly carotenoids, are responsible for the observed benefit of the broodstock fish oil*** diet on larval performance. The supplementation of carotenoids to brood stock diets not only appears to improve larval performance but also ***carotenoids contribute to bottarga coloration***.

WP 8 Nutrition – meagre

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

Objectives

1. Improve current larval weaning feeds for meagre,
2. Determine nutritional requirements to promote feed utilization, consistent growth rates and fish welfare to reduce size variation.

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

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

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

During the 2nd reporting period, two major studies were conducted. First, a study was conducted to determine optimum essential fatty acids and related micronutrient levels in weaning diets for meagre. The results of this study showed that 0.4% dietary HUFA is not enough to cover the essential fatty acid requirements of larval meagre and, since their elevation up to 3% markedly improved lipid absorption, essential fatty acids levels and growth, a high HUFA requirement in weaning diets is foreseen for this species. Besides, the results also pointed out the importance of dietary vitamin E and vitamin C to protect these essential fatty acids from oxidation, increase their contents in larval tissues and promote growth, suggesting as well high vitamin E and vitamin C requirements in meagre larvae (higher than 1500 and 1800

mg kg-1 for vitamin E and vitamin C, respectively). A second study was conducted to determine the importance of dietary vitamins A, K and D in weaning diets for meagre. Results obtained demonstrated the importance of supplementation of meagre weaning diets with 2.4 mg/kg vit K, since the absence of this vitamin markedly reduced larval survival. However, meagre seemed to be very sensitive to hypervitaminosis D and, only midly to hypervitaminosis A, since supplementation with these vitamins leaded to a growth reduction. On the contrary, taurine supplementation did not have any effect in meagre larvae performance.
Both experiments were included in Deliverable 8.1. "Improvement of larval weaning diets", delivered at month 24.

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

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

Summary of work reported in the previous Reporting Period (49-60 Mo):

No work was done in this WP, as all work was completed in earlier reporting periods

Details for each Task

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

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

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

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 8.2 Dietary requirements for essential fatty acids of meagre Argyrosomus regius fingerlings.***

Deviations from Annex I and their impact:

No deviations from the plan have been recorded.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M., 2018. Dietary requirement for n-3 long-chain polyunsaturated fatty acids for fast growth of meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture 488, 105-113.

El Kertaoui, N., Hernández-Cruz, C.M., Montero, D., Caballero, M.J., Saleh, R., Afonso, J.M., Izquierdo, M., 2017. The importance of dietary HUFA for meagre larvae (*Argyrosomus regius*; Asso, 1801) and its relation with antioxidant vitamins E and C. Aquaculture Research 48, 419-433.

Carvalho, M., Montero, D., Lencina, A., Lund, I., Izquierdo, M.S. The effect of different dietary n-3 LC-PUFA contents on response to an acute and prolonged stress of meagre (*Argyrosomus regius*, Asso 1801) juveniles. Aquaculture, submitted.

Carvalho, M., Castro, P.L., Montero, D., Peres, H., Acosta, F., Fontanillas, R., Rosenlund, G., Robaina, L., Izquierdo, M.S. Essential fatty acid deficiency increases hepatic non-infectious granulomatosis incidence in meagre (*Argyrosomus regius*, Asso 1801) juveniles. Aquaculture, submitted.

WP 9 Nutrition – greater amberjack

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

Objectives

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

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

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

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

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

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

- Treatments E1 and E3 produced similar survival than the commercial product (C), whereas treatment E2 produced lower survival.
- Overall, the experimental treatment E1 showed the best results in terms of survival and ovigerous females in the rotifer population.

- Longer enrichment protocols and higher total lipid levels in rotifers increased the proportions of TAG.

The results indicate that rotifer enrichment treatment E1 (100% marine lecithin) is the best protocol for LC-PUFA enrichment according to the lipid composition of wild greater amberjack viable eggs. To achieve objective 3, information on the nutritional requirements and spawning quality determination in greater amberjack and related species were collected in order to define a basal diet formulation for amberjack broodstock (Task 9.3).

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

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

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

Regarding greater amberjack juveniles, the effects of different dietary levels of lysine on growth, voluntary feed intake, nutrient utilization, body proximate composition and antioxidant capacity of fish fed diets with low fishmeal inclusion were studied. Result obtained indicate that the dietary lysine requirements, based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, was 2.11% of diet. Lysine supplementation affected the specific activity of CAT in liver and intestine of greater amberjack fed the diet containing 2.11% lysine. In broodstock diets, an experiment to determine the optimum level of essential fatty acids for reproductive success was conducted. Different groups of broodstocks were fed diets containing different essential fatty acids levels (from 2.8 to 0.96% of total fatty acids) in order to determine the effect on reproduction reliability. The effects on gonad maturation, frequency of spawns, fecundity, fertilization rates, hatching rates and larval survival rates were determined. Proximate composition of diets and eggs were also analysed. The diet containing 1.57% of total fatty acids induced a higher number of eggs per spawn and kg of female, with the highest percent of fertilization, egg viability, hatching rate and larval survival. The lipids and carotenoids egg profile of culture females in comparison with their wild counterpart was also studied. An experimental diet was formulated and the experiment will start by January 2018.

Summary of progress towards objectives (49-60 Mo):

During this experimental period, studies on broodstock nutrition were finished, with an experiment conducted during this last periodic report. An experimental diet was formulated based on the results obtained during 2nd and 3rd periodic report. Greater amberjack broods were distributed in three groups, which were fed on different diets: A Mackerel Group, fed on frozen mackerel (composition in % dry weight: crude protein: 22.0%, crude fat: 9.6%, moisture 76.8%) supplemented with a vitamin premix. A Control Group, which was fed with a commercial Control pellet manufactured by P20 (SARC). An Experimental Group, which was fed with a diet also manufactured by P20 (SARC), accordingly to previous results obtained for this species to contain higher proportions of polar lipids and a fatty acid profile. The three groups of breeders did not release eggs naturally or induced, after the successive hormonal treatments, during the study period, but based on the biopsy of some of the experimental fish, the results obtained on oocyte maturation and sperm quality, and the results on spawning quality of broods fed different levels of essential fattu acids, obtained in previous periodic reports, it could be concluded that a diet containing 14-15 % EPA+DHA of total fatty acids (corresponding to 2.5-3 % in a dry diet) resulted in

best spawning performance in greater amberjack broodstock obtained as wild caught juveniles and increasing dietary EPA+DHA contents did not improve spawning performance.

Regarding on-growing, the results from previous studies included in deliverable D9.2 by HCMR indicated that the dietary lysine requirements based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion was 2.11% of diet. Based on that lysine content in the described formulae for greater amberjack, a study was conducted in sea cages. The growth measured with the objective weight of one kg at sea cages was lower than that previously obtained in indoor on-growing facilities, FCRs measured were higher that the predicted ones) for the normal feed utilization obtained at indoor facilities, that could be related with oceanographic conditions and not well adaptation of fish to the cages, together with the high incidence of monogenea parasitic infection in fish held at sea cages. However, taking onto account the growth obtained, Lysine of 2.11% f diet is enough for on-growing in sea cages.

Details for each Task

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

Sub-task 9.1.1 (FCPCT, Marisol Izquierdo)

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

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

This Sub-task was completed during the previous reporting periods and results were submitted in ***Deliverable 9.1. "Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids in greater amberjack enrichment products".***

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

Sub-task 9.2.1 (HCMR, Yannis Kotzamanis)

This Sub-task was completed during the previous reporting periods and results were submitted in ***Deliverable 9.2. Lysine requirements of greater amberjack juveniles.***

Sub-task 9.2.2 (CANEXMAR, Alfredo Mormeneo)

Introduction

The results from previous studies included in deliverable D9.2 by HCMR indicated that the dietary lysine requirements based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion was 2.11% of diet.

Material and methods

Based on that lysine content in the described formulae for greater amberjack, a study was conducted in sea cages, belonging to CANEXMAR and sited in the east coast of Gran Canaria Island (Canary Islands, Spain) (**Fig. 9.3.1**).

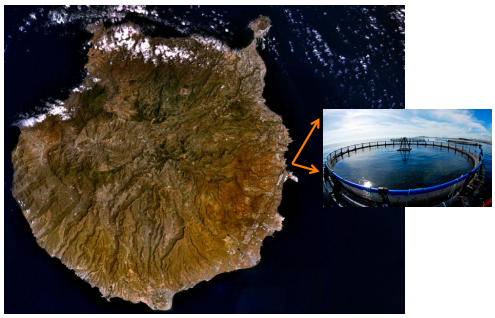


Figure 9.3.1. Site of CANEXMAR farm, at east coast of Gran Canaria Island (Canary Islands, Spain)

Greater amberjack juveniles were accordingly produced by P2 (FCPCT) in the aquaculture facilities form las Palmas de Gran Canaria University (ULPGC). Two different amberjack juveniles production (around 5000 individuals each) were placed in CANEXMAR facilities (**Fig. 9.3.2**). A total of 5,000 juvenile amberjack of 100 g body weight were produced with the standardized methodology for greater amberjack in 2015 and were placed in a experimental cage of 5x5 m. Same was done in 2016.

Figure 9.3.2. Transport of the amberjack juveniles from PCTM facilities to the experimental cage in CANEXMAR

These two productions (2015 & 2016) even when fish were very well acclimated (**Fig. 9.3.3**), failed to reach CANEXMAR farm for different reasons.

Figure 9.3.3. Different batches of greater amberjack juveniles acclimated in 5x5 cages waiting to be carried into CANEXMAR farm.

The first batch of fish did not survive to the transport to the off-shore cages due to operational reasons and most of the fishes died. The second batch of fish was lost due to cage breakdown during a big storm and hard weather. After those incidences, CANEXMAR then needed to make a new design and improvement in the fish transport system and cage construction for the sea. Then, a new batch of greater amberjack juveniles was produced at FCPCT at the end of 2016 and CANEXMAR focused in improvements of transport and viability in sea cages with this batch.

Fish transport and acclimation

Following the fish transport protocol for similar species to the *S. dumerili*, the next points were carefully followed:

- ✓ The acclimatization of the fishes to face the transport. (**Fig. 9.3.4**).
- ✓ Equalize the water conditions between the exit tanks and the transport tanks.
- ✓ Controlling the pH level and ammonium in the transport tanks during its loading.
- ✓ Controlling the oxygen in the tanks before and during the fish loading to avoid important oscillations in its level.
- ✓ To double the transport capacity with more containers and minimize the fish density as possible as can.
- ✓ The fishes are deposited in containers covered with a cap to avoid the stress involved in the transport manage and in the transport (**Fig. 9.3.5**).
- ✓ The unloading is from a tank to another tank. In this process we renew the water inside two hours before the unloading, controlling the seawater temperature and equalizing the oxygen level.

Figure 9.3.4. Acclimation procedures and transport to off-shore sea cages

Daily feeding and fish studying of the feed and the environment conditions

The starting months of feeding were very hard due to a strong winter (more stormy than usual in the Canaries), being very difficult to see the fishes in the cage. The sea conditions generated additional stress and the feeding was very difficult. A feeding regime of three times per day to solve the situation was adopted. After the adaptation months, the fishes feed better and the feeding task is easier. This s the stressful situation could have had negative effects in the growth or the health of the fishes.

While the fishes grow, they improve its adaptation to the offshore conditions, in the sixth month, CANEXMAR decided to maintain to feeding twice per day (one on the morning and the other at the afternoon). There were no differences between the feedings, being the fish behavior equal during all the experimental time, with fish showing a nervous behavior when boats reach the cage.

Diets

Table 9.3.2 shows the different diets used during the whole on-growing cycle. A diet with the levels of Lysine proposed during the experiences done by HCMR and included in deliverable 9.2.

Table 9.3.2. Commercial diets used during the on-growing at sea cages. Levels of Lysine are according to the previously determined levels in deliverable 9.2.

		3 mm	4,5 mm	6,5/9 mm	12/15 mm
CRUDE PROTEIN	%	54	54	54	52
CRUDE LIPIDS	%	18	18	20	20
CARBOHYDRATES	%	12.3	12.3	10.4	13.1
CELLULOSE	%	0.6	0.6	0.5	0.6
ASH	%	9.2	9.2	9.5	8.8
PHOSPHORUS	%	1.4	1.4	1.4	1.3
CRUDE ENERGY	MJ/KG	21.7	21.7	22.2	22.1
DIGESTIBLE ENERGY	MJ/KG	19.1	19.1	19.7	19.4
DIGESTIBLE PROTEIN	G/MJ	25.1	25.1	24.3	23.7
VITAMIN A	I.U/Kg	10000	10000	10000	10000
VITAMIN D3	I.U/Kg	500	500	500	500
VITANIM E	MG/Kg	200	200	200	200
VITAMIN C	MG/Kg	100	100	100	100
LYSINE	**gr/Kg**	**36.5**	**35.3**	**36.7**	36.7

Sampling protocols

Four different sampling points were determined during the on-growing cycle. The sea conditions in the zone avoided more samplings with the security guarantees, because the fishes suffer more stress when we use the standard methods to pick them up and get the sampling with the fish tools (**Fig. 9.3.5**). The method used to calculate the weight was a dynamometer. The anaesthetic used to guarantee the welfare during the process was clove oil in a concentration of 25ml/1000l.

Figure 9.3.5. Sampling procedures at sea cages

The average temperature of the year was recorded around the sea cage located in CANEXMAR farm, being temperature according to the average temperature for the geographical region (**Fig. 9.3.6**).

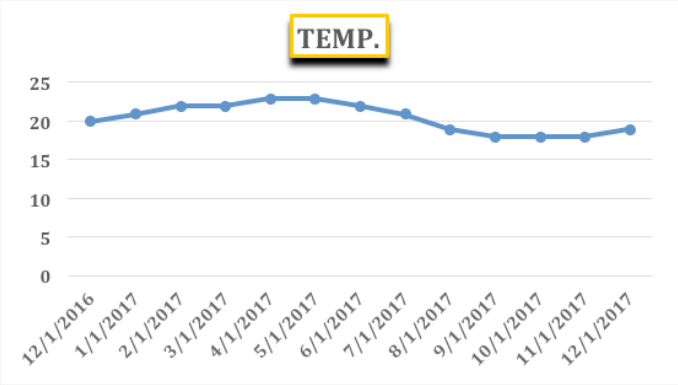


Figure 9.3.6. Temperature variations during the experimental period

In each sampling point, 107 fish were sampled (**Table 9.3.3**). Average body weigh was 121.9 in March 2017, increasing to 230 in May, to 303 in June and to 538.4 at the end of September. High variation on fish size was observed in each sampling point.

Table 9.3.3. Average body weight of experimental fish during the on-growing cycle.

DATE SAMPLING: 06/03/2017	
CAGE	J09X
FISH NUMBER SAMPLING	107
AVERAGE WEIGHT SAMPLING	121,9
MAX. WEIGHT	320
MIN. WEIGHT	100

DATE SAMPLING: 02/05/2017	
CAGE	J09X
FISH NUMBER SAMPLING	107
AVERAGE WEIGHT SAMPLING	230
MAX. WEIGHT	409
MIN. WEIGHT	110

DATE SAMPLING: 26/06/2017	
CAGE	J09X
FISH NUMBER SAMPLING	107
AVERAGE WEIGHT SAMPLING	303,4
MAX. WEIGHT	501,5
MIN. WEIGHT	196,5

DATE SAMPLING: 22/09/2017	
CAGE	J09X
FISH NUMBER SAMPLING	107
AVERAGE WEIGHT SAMPLING	538.4
MAX. WEIGHT	736.5
MIN. WEIGHT	431.5

The growth predicted and measured is shown in **Fig. 9.3.7.** MW L1 shows the growth data obtained from the samplings whereas MW L2 data from the theorical calculations with the objective weight of one kg, that is very easily obtained in FCPCT (P2) indoor facilities. FCRs measured were higher that the predicted ones (**Fig. 9.3.8**) for the normal feed utilization obtained at FCPCT indoor facilities. FCR L1 shows growth data obtained from the samplings whereas FCR L2 shows data from the theorical calculations with the objective weight of one kg.

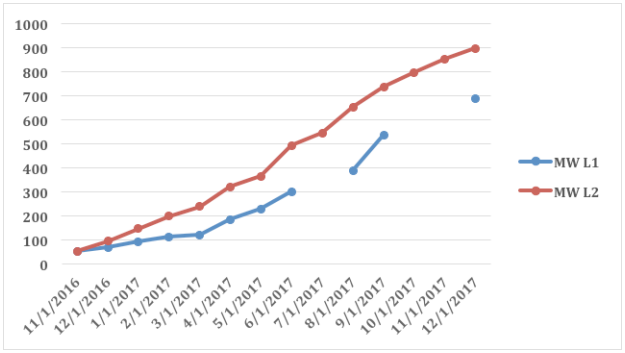


Figure 9.3.7. Comparison between growth projections *vs* growth from samplings.

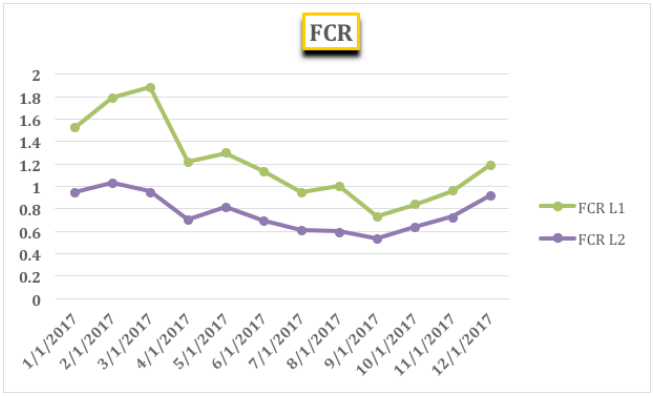


Figure 9.3.7. Comparison between FCR projections *vs* FCR from samplings.

Samples animals presented no parasitic infection during the three first samples, but at the fourth sampling point, monogenean parasite infection occurs, with a incidence at level 2 according with the scores defined by Fernandez-Montero et al. (2018), previously reported in a Deliverable (25.5) of amberjack health (**Fig. 9.3.8**)

Figure 9.3.8. Juvenile amberjack after sampling. Some ulcerative processes can be observed in the skin due to parasite infection.

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

Sub-task 9.3.1. Optimum essential fatty acids for reproductive success (FCPCT, Hipolito Fernandez Palacios and Daniel Montero)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D9.4 Recommended protein, carotenoids, Tau and EFA levels in greater amberjack broodstocks***

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

Materials and Methods

The greater amberjack broodstock consisted of 50 PIT-tagged hatchery-produced 4 years old F1 fish, reared in the facilities of Centro Oceanográfico de Canarias (IEO, P8), in Tenerife, Spain, since 2014. Fish had been fed with a commercial pellet for turbot (Initial diet) (Skretting Ltd, Norway; composition in % dry weight was: 52% crude protein, 20% crude fat, 8.7% ash, 1.7% crude cellulose and 1.4% total phosphorus), with size, frequency and quantity of pellet adjusted accordingly to fish weight.

In February 2018, the fish were distributed in three groups which were fed on different diets: A Mackerel Group (7 ♀ 6.6±0.9 Kg and 8 ♂ 5.9±0.8 Kg), fed on frozen mackerel (*Scomber colias*) (composition in % dry weight: crude protein: 22.0%, crude fat: 9.6%, moisture 76.8%) supplemented with a vitamin premix, to contain: 500 ppm of vitamin C and 200 ppm of vitamin E for each kg of raw fish in dry matter. A Control Group (7 ♀ 7.6±1.2 Kg and 12 ♂ 6.0±1.1 Kg), which was fed with a commercial Control pellet manufactured by P20 (SARC). Finally, an Experimental Group (5 ♀ 6.8±1.1Kg and 10 ♂ 5.6±0.8 Kg), which was fed with a diet also manufactured by P20 (SARC), accordingly to certain pre-requisites established by ULL (P15)/IEO (P8), to contain higher proportions of polar lipids and a fatty acid profile, with particular emphasis to contain essential fatty acids (EFA; ARA, EPA, and DHA and ratios) mimicking those from the wild mature gonads (**Table 9.4.7).** It was intended to maximize the presence of specific marine-origin ingredients, including carotenoids. This formula was based on the comparison of wild and captive greater amberjack broodstock composition obtained by IEO/ULL in several previous studies performed the last few years (Rodriguez-Barreto *et al*., 2012, 2014) and also those performed within Diversify Project (Zupa *et al*., 2017b; WP3; D3.3).

According to these results and specially at early gametogenesis, captive-reared fish gonads display clearly lower contents of specific lipid classes (PC, PE and PI) and lower contents of ARA and DHA and, consequently DHA/EPA and ARA/EPA ratios, all crucial factors for reproductive success and sperm, egg and larval quality (Zupa *et al.,* 2017b). These previous studies also demonstrated that the captive animals display much higher fat contents and also of 18:1n-9, 18:2n-6 and 20:5n-3, in gonads, muscle and liver.

For this reason, after several email contacts, the involved partners (IEO, ULL and SARC) held a Skype meeting on March 2017 to agree on the ingredients to formulate an experimental diet with a similar proximate composition to that of the control diet (51% crude protein /18% crude fat) and marine based lipid sources. The experimental diet was then formulated with commercial available ingredients to contain comparable lower levels of EPA and to maximize ARA and DHA contents (**Table 9.4.7**).

To maximize the inclusion of DHA, algae meal was added and the level of ARA was increased by adding a ARA-rich oil (10 % ARA). However, since the diets mainly contained marine raw materials, EPA could not be so low, as shown in Table 9.4.8. Finally, as a source of phospholipids, rapeseed lecithin

phospholipids were used to minimize the contribution of n-6 fatty acids. The resultant analysis showed that the experimental diet was lower in 18:1n-9 and 18:2n-6 and higher than the control one in ARA and DHA, and was still higher in EPA.

Table 9.4.7 Raw material and proximate composition of control and experimental diets (Skretting Aquaculture Research Centre, Stavanger, Norway).

Raw material	Control	Experimental
Rapeseed lecithin	1.00	1.00
Algae meal	0.00	2.50
krill meal	7.00	7.00
Wheat	18.49	17.46
Wheat gluten	17.00	18.00
Fish meal	43.17	43.17
Arachidonic acid, 10 %	0.26	0.60
Squid meal	3.00	3.00
Fish oil	9.09	6.27
Vitamin premix	0.50	0.50
Mineral premix	0.50	0.50
Proximate composition (analyzed)		
Total	100	100
Moisture	8.7	8.1
Crude protein	50.7	51.8
Crude fat	18.20	18.00
Ash	8.3	8.3

Table 9.4.8. Analysed main fatty acid composition (% of total fatty acids) of diets and mackerel.

Fatty acid	Initial Diet	Control Diet	Experim. Diet	Mackerel
16:0	14.0	18.2	18.5	16.1
18:1n-9	26.0	13.3	11.3	5.5
18:2n-6	10.4	6.3	5.6	1.3
20:4n-6 (ARA)	0.5	1.1	1.6	2.8
20:5n-3 (EPA)	6.8	11.1	12.0	5.5
22:6n-3 (DHA)	7.1	10.2	14.5	6.5
EPA/ARA	13.6	10.1	7.5	2.5
DHA/EPA	1.0	0.9	1.2	1.2
DHA/ARA	14.2	9.3	9.1	2,3
ARA+EPA+DHA	14.4	22.4	28.1	14.8
Carotenoids		50ppm	50ppm	

Fish were maintained in three outdoor covered raceway tanks of 500 m^3 with continuous water supply (10 renewals day^{-1}) under natural photoperiod and seawater temperature (19.2±0.3ºC), and hand-feeding once a day and 3 days a week to apparent satiation. Measurements of temperature and water quality (Dissolved Oxygen, NH_3-N and NO_2-N) were conducted once per week throughout the year. At the expected onset of the spawning season (May), a passive egg collector was placed in the outflow of the spawning tanks and daily checked, in order to collect the spawned eggs.

The fish were sampled monthly during 2018 spawning season (June, July, August, September, and October). Fish were starved for two days prior to sampling and were tranquilized initially in their tank with the use of chlorobutanol (0.1 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of chlorobutanol (0.3 ml l^{-1}). Fish were individually identified with PIT tags and biometric parameters of length and body weight were measured. Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula (Pipelle de Cornier). A wet mount of the biopsy was examined first under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10). 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 into the genital pore. The collected sperm was stored on ice and then transferred to a 4°C refrigerator until evaluation.

At each sampling, blood was collected from all fish from the caudal vessel using heparinized syringes, in order to measure the concentrations and blood biochemical parameters. Hematological parameters were estimated from fresh samples of blood. Total erythrocytes and leucocytes were determined by counting in 1/100 dilutions of blood in Natt and Herricks solution, using a Neubauer hemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation. Blood was centrifuged at 1400 rpm for 20 min and plasma was collected, frozen in liquid nitrogen and stored at -80°C until the biochemical analysis. Plasma levels of protein, triglycerides, cholesterol and glucose were measured in duplicates by enzymatic colorimetric assays (Biosystems, Spain).

Fish were treated with an Ethylene–Vinyl acetate (EVAc) GnRHa implant (Mylonas & Zohar, 2001) loaded with Des-Gly^{10}, D-Ala^6-Pro-$NEth^9$-mGnRHa (H-4070, Bachem, Switzerland) at the sampling times of July, August, September and October. There were variations in the effective GnRHa dose applied to each fish due to the fact that implants are loaded with fixed amounts of GnRHa. Moreover, the dose of GnRHa applied was increased in the September and October sampling. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and intratesticular sperm was obtained from males.

To evaluate broodstock nutritional status, available samples of oocytes and sperm from the three treatments, taken and immediately frozen and kept at -80ºC, in August and September were analyzed. Samples of mackerel, the commercial control diet and the experimental one were also analysed. Dry matter and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC, 2012). Moisture contents were determined in approximately 500 mg samples by thermal drying in an oven at 110°C, until constant weight. Protein was determined by sample digestion according to Kjeldahl method. Total lipid (TL) was extracted from the tissues and diet by homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch *et al*. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (Christie, 1982) and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and methyl acetate /isopropanol /chloroform /methanol/0.25% (w/v) KCl (5: 5: 5: 2: 1.8, by volume) as developing solvent system for the polar lipid classes and isohexane/diethyl ether/acetic acid (22.5: 2.5: 0.25, by volume), for the neutral lipid

separation. Lipid classes were visualized by charring at 160°C for 15min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, 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 with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/diethyl ether/acetic acid (90:10: 1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m 9 0.32 mm I.D. 9 0.25 lm; Sigma-Aldrich, Madrid, Spain). Helium was used as 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). Due to the small size of the biological samples, carotenoids were measured only in the diets, according to the method of Barua *et al*. (1993), using ethyl acetate/ethanol (10mL, 1:1 v/v), ethyl acetate (5 ml) and hexane (10 ml) as extracting solvents. Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

Statistical analysis

Differences in egg and sperm quality and blood parameters were tested using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. The data were checked for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test, and percentage data were Arcsine transformed prior to statistical analysis to normalize variances.

Pearson's correlation coefficients were used to assess the relationships between some egg quality variables. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean ± standard deviation (SD), unless mentioned otherwise. In all statistical tests used, differences with a *P* value of less than 0.05 were considered statistically significant. Analyses were performed with the IBM SPSS statistics package (version 20.0 for Windows).

Results

Fish conditions and reproduction performance

The feed intake (Food dry weight per body weight %) increased from March to May and declined thereafter with values in the overall period of 0.8±0.7 % for Mackerel Group, and 1.2±0.8 and 1.4±0.5 % for Control and Experimental Groups, respectively (**Fig. 9.4.12**).

The temperature from March to the first sampling (June) was stable (19.2±0.3ºC) and one degree below the usual in the month of June. From June to the end of the study (October), temperature increased by 4.4ºC with an average temperature in the period of 22.0±1.2ºC. The temperature was 1ºC lower than normally recorded from June to August.

The feed intake decreased significantly in all groups with the increase of temperature. However, experimental fish group showed a less marked decrease in intake (lineal slope = -0.043) than fish fed with control feed (lineal slope= -0.062) and mackerel fish (lineal slope= -0.053).

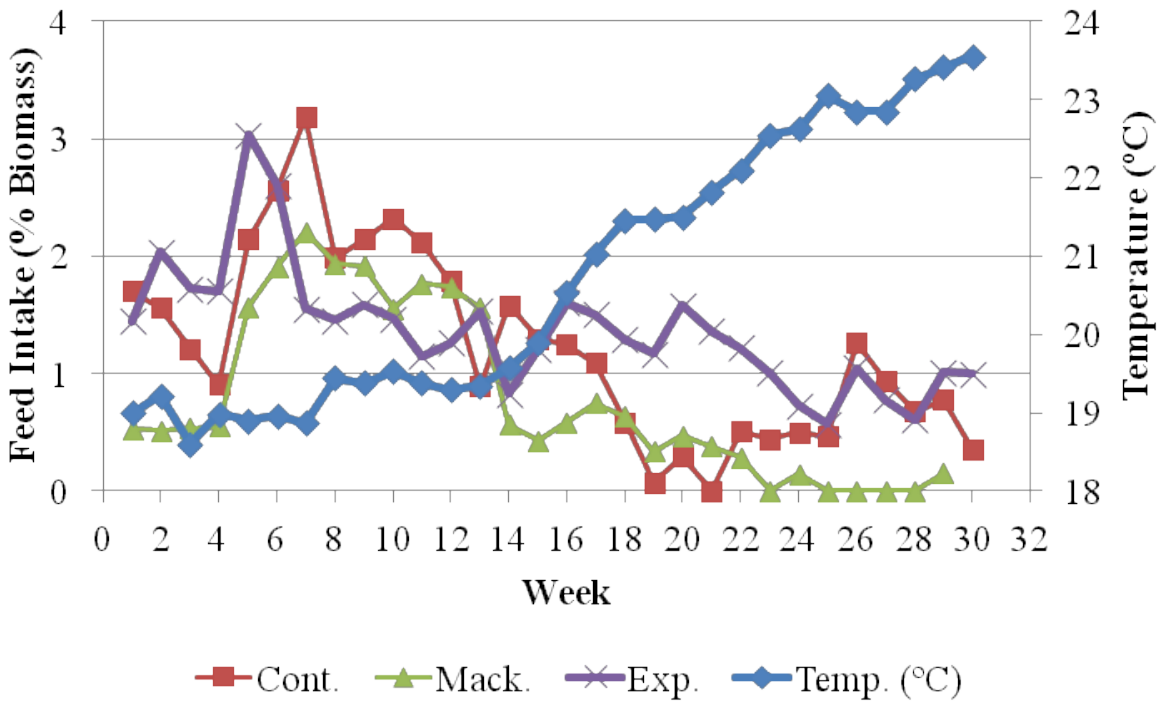


Figure 9.4.12. Weekly means of temperature (blue line) and food intake (dry weight) of Control (red line), Mackerel (green line), and Experimental (purple line) broodstock groups during the experimental period.

The weight of males and females of experimental groups in each sampling is shown in **Table 9.4.9**. The weights within each group were homogeneous in all sampling periods.

Table 9.4.9. Number of sampled fish and mean weight (± SD; kg) of the fish of each sex from the groups of breeders fed with commercial pellet (Control), frozen mackerel (Mackerel) and experimental diet (Experimental) at each sampling time. ns=not sampled.

	Treatment	**Experimental**		**Control**		**Mackerel**	
Month	**Sex**	n	Weight (kg)	n	Weight (kg)	n	Weight (kg)
June	**Females**	5	6.8 ± 1.1	6	7.7 ± 1.3	7	6.6 ± 0.9
	Males	10	5.6 ± 0.8	12	6.0 ± 1.1	8	5.9 ± 0.8
July	**Females**	5	6.6 ± 1.1	6	7.3 ± 1.3	7	6.2 ± 0.9
	Males	10	5.3 ± 0.8	12	6.1 ± 1.3	8	5.7 ± 0.8
Aug.	**Females**	5	6.6 ± 1.1		NS	6	6.1 ± 1.0
	Males	10	5.1 ± 0.8		NS	8	5.6 ± 0.9
Sep.	**Females**	5	6.1 ± 1.0	2	7.2 ± 0.4	4	5.3 ± 1.1
	Males	9	4.8 ± 0.8	5	5.8 ± 1.4	8	4.9 ± 0.8
Oct.	**Females**	5	5.8 ± 1.0	2	7.4 ± 0.6	3	5.1 ± 1.2
	Males	7	4.6 ± 0.7	5	6.0 ± 1.5	5	4.8 ± 0.9

During June, no spawns were registered and selected males and females (largest vitellogenic oocytes > 600 µm) were treated with a GnRHa implant at a dose of ~50 µg GnRHa kg^{-1} body weight (in the form of EVAc implant) in July. Because no spawns were obtained after the 1st treatment, the fish were treated with a higher dose (~100 µg GnRHa kg^{-1} body weight) in the successive spawning induction treatments (**Table 9.4.10**).

Table 9.4.10. Number of sampled fish, mean weight (± SD) and dose of GnRHa (µg kg^{-1} body weight) (mean ± SD) of implanted greater amberjack breeders fed with commercial pellet (Control), frozen mackerel (Mackerel) and experimental diet (Experimental) at each treatment/sampling time. All treated fish were given a GnRHa implant, and slight variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa. NS indicate fish no sampled.

Treatment		Experimental							Control							Mackerel						
Month	Sex	n	Weight (kg)			Dose (µg kg^{-1})			n	Weight (kg)			Dose (µg kg^{-1})			n	Weight (kg)			Dose (µg kg^{-1})		
Jul.	♀	2	6.4	±	0.5	55.1	±	4.5	2	7.9	±	1.3	64.3	±	10.7	2	5.8	±	0.4	63.4	±	25.6
	♂	2	5.4	±	0.0	46.5	±	0.2	2	7.1	±	1.3	35.6	±	6.5	2	6.5	±	1.3	56.4	±	16.4
Aug.	♀	3	6.8	±	1.0	111.6	±	15.8			NS			NS		3	5.7	±	1.4	137.3	±	32.4
	♂	3	5.4	±	0.6	92.6	±	10.8			NS			NS		3	5.4	±	0.4	93.3	±	7.3
Sep.	♀	3	6.6	±	1.0	115.3	±	17.8	2	7.2	±	0.4	103.9	±	6.3	3	5.1	±	1.3	152.6	±	36.5
	♂	3	5.1	±	0.6	98.1	±	11.0	2	5.7	±	1.0	89.3	±	15.1	2	5.1	±	0.1	98.4	±	2.2
Oct.	♀	2	5.9	±	0.7	105.8	±	17.2	2	7.5	±	0.5	83.3	±	18.2	1	6.1	±	-	81.4	±	-
	♂	2	4.8	±	0.5	104.8	±	11.1	3	5.3	±	1.8	101.0	±	34.0	1	4.8	±	-	103.3	±	-

The Control group was not treated in August, because there was a high mortality (50 %) probably caused by high incidence of parasitization in the group.

The percentage of females with oocytes larger than 500 µm was greater in the Experimental group during the first months (June-August) of the studied period (**Fig. 9.4.13)**.

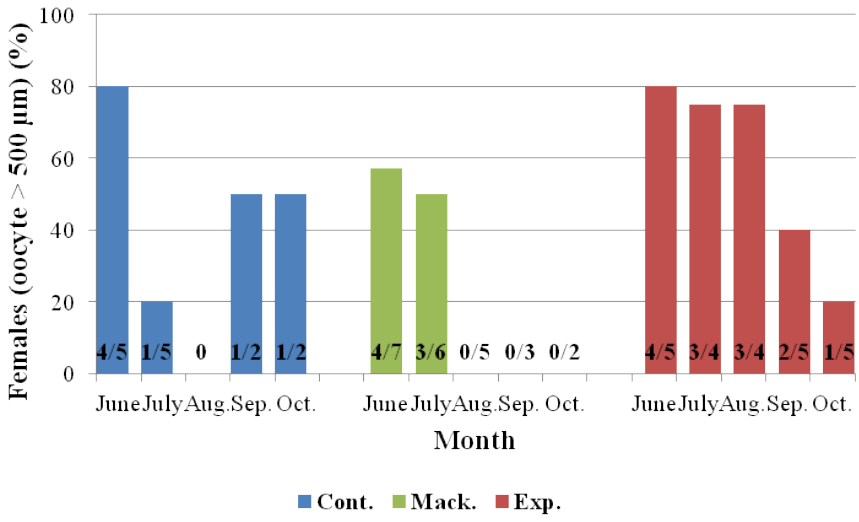


Figure 9.4.13. Percentage of Females with oocytes larger than 500 µm with respect to the total number of biopsied females in control (Cont.), frozen mackerel (Mack.) and experimental (Exp.) fed groups during the period of study. Numbers in bars indicate the number of females (oocytes > 500 µm) / number of females biopsied.

The mean diameter of the largest vitellogenic oocytes of the females biopsied is shown in the **Fig. 9.4.14**. Of the females biopsied, all those fed with Mackerel showed decrease in oocyte diameter with time, and value higher than 500 µm only in June and July. In the Control group, all females, except one, showed a decline in the diameter of the oocyte with time, and with the highest general values (> 500 µm) in June. In the Experimental group, two of the five females showed an increase in the diameter of the oocytes with time, while the oocyte diameter of the other females decreased.

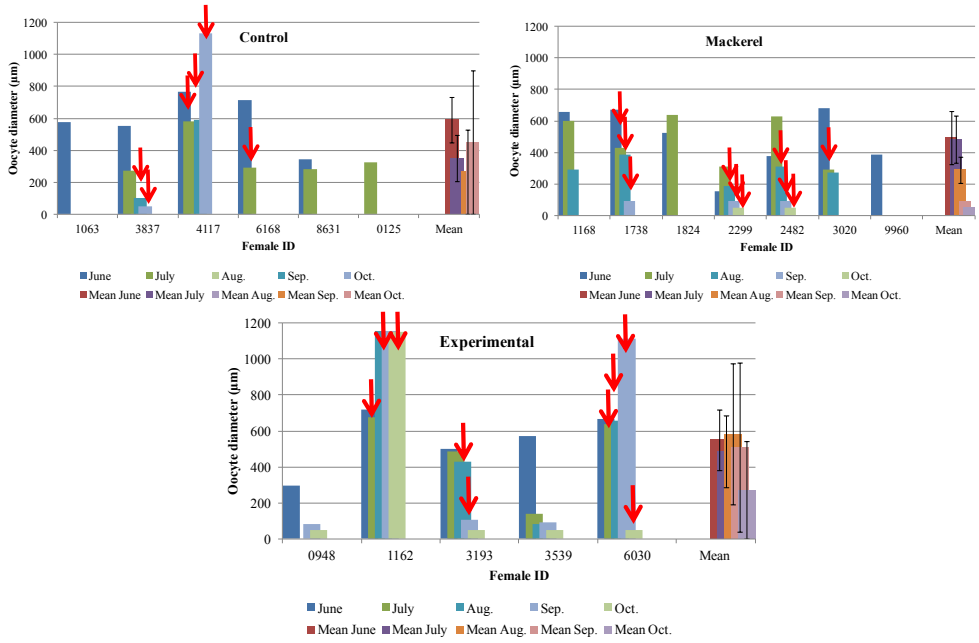


Figure 9.4.14. Oocyte size of individual females (ID) biopsied in each group at different samplings/treatment during the reproductive season. The red arrows indicate the female implanted in each group at each sampling time.

Sperm quality parameters of sperm motility (%) and sperm motility duration (sec.) tended to decrease along experimental period except in the Experimental Group, which maintained similar values in all sampling points **(Fig. 9.4.15 a b**).

In the experimental group, mean sperm motility percentage (~ 30%) (**Fig. 9.4.15a**) and the duration of sperm motility (~ 120 sec.) (**Fig. 9.4.15b)** remained unchanged throughout the monitored period, while in the Mackerel group, and less evident in the Control group, the mean sperm density (**Fig. 9.4.15c**) and duration of sperm motility decreased at each sampling time, except for motility duration in control group in October that increased with respect to September.

The Mackerel group mean sperm density was slightly higher (17.7 ± 14.5 x 10^9 spermatozoa ml^{-1}) than Control and Experimental groups in June (**Fig. 9.4.15c**). However, the Experimental group showed the highest sperm density in September and October (> 25 x 10^9 spermatozoa ml^{-1}), although with higher individual variability.

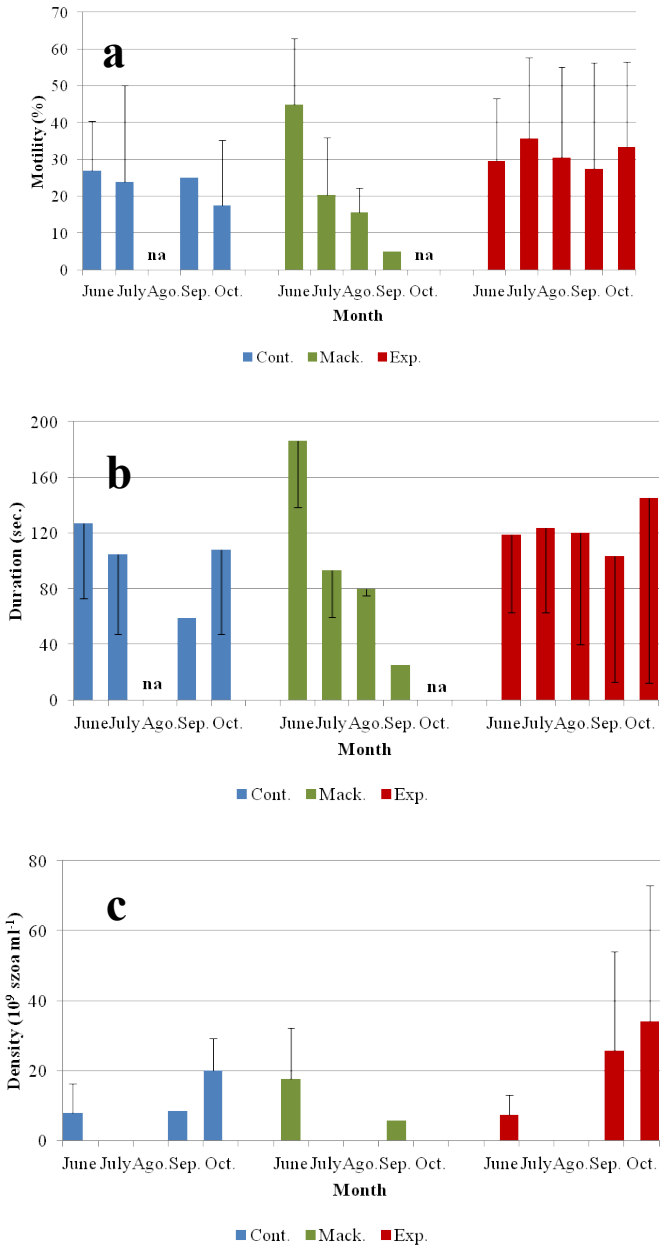


Figure 9.4.15. Mean (± SD) sperm quality parameters of greater amberjack in control (Cont.), frozen mackerel (Mack.) and experimental (Exp.) fed groups at different times during the reproductive season of 2018 (spermatozoa forward motility (%) (**a**), duration of motility (sec.) (**b**) and density (10^9 spermatozoa ml^{-1}) (**c**)). na = not available.

In general, the motility percentage of treated or not treated males of Control and Mackerel groups decreased during the repetitive samplings **(Fig. 9.4.16)**. However, in the Experimental group, both the implanted and non-implanted males showed an increase in the percentage of motility.

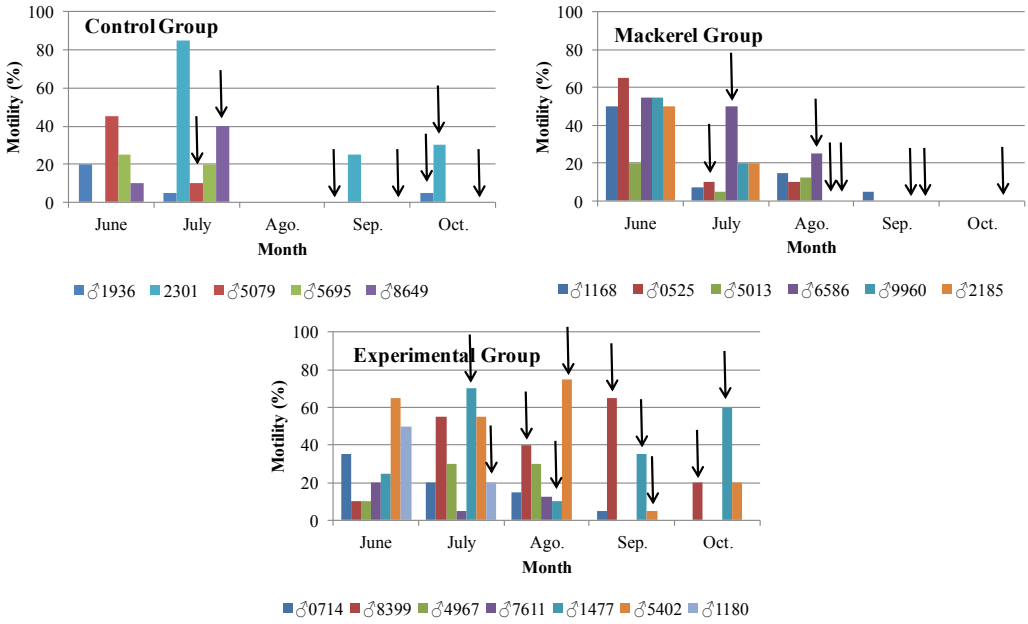


Figure 9.4.16. Motility percentage (%) of individual greater amberjack males in Control, frozen Mackerel and Experimental fed groups at different times during the reproductive season of 2018.

Regarding to the motility duration, Control and Mackerel groups showed a similar trend to that described for the motility percentage, with an important decrease during the consecutive samplings (**Fig. 9.4.17**). However, in the Experimental group, both the implanted and non-implanted males showed an increase in the motility duration.

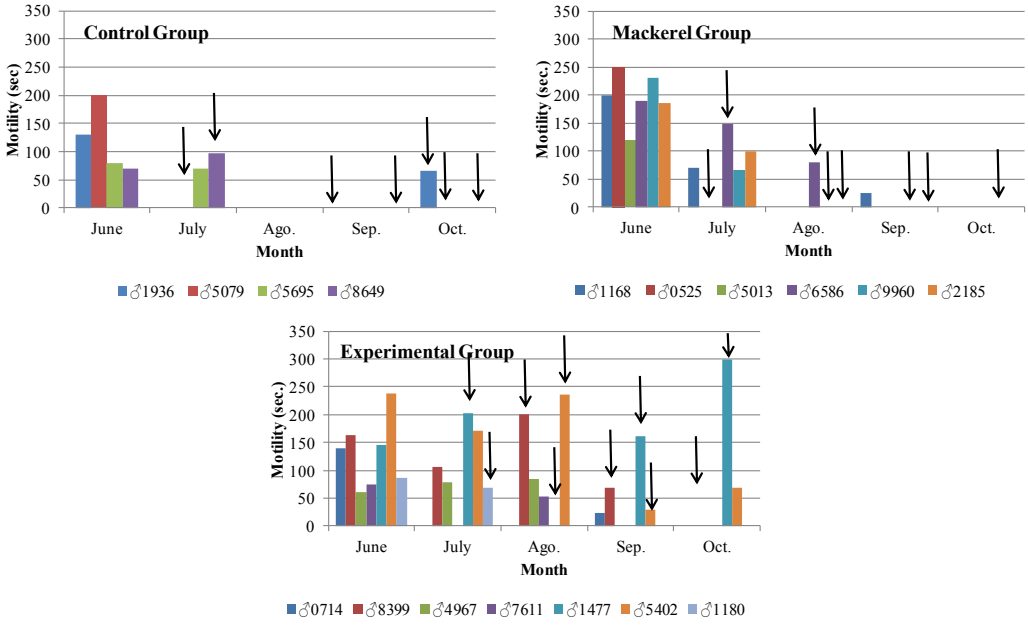


Figure 9.4.17. Motility duration (sec.) of individual greater amberjack males in Control, frozen Mackerel and Experimental fed groups at different times during the reproductive season of 2018.

The three groups of breeders did not release eggs naturally or induced, after the successive hormonal treatments, during the study period, although the biopsy of one female from the Control group and two females from the Experimental group showed mature eggs (>1100 µm) in October. In addition, one of the females of the Experimental group (ID 1162) showed mature eggs since August in the repetitive monthly samplings (**Fig. 9.4.18**).

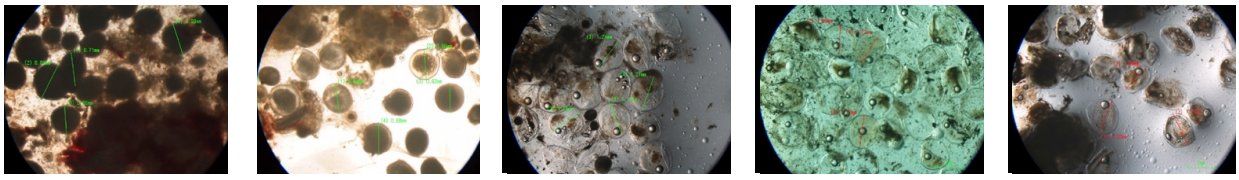


Figure 9.4.18. Oocyte diameter in the successive samplings of the greater amberjack female (ID 1162) of the Experimental group.

Lipid composition of oocytes and sperm

In the absence of spawns, the biopsies of females and males of each group, sampled in August and September were also subjected to analysis of lipid composition (**Tables 9.4.11-9.4.14**). Although samples taken the last month (October) are kept at -80ºC, they could not be analysed on time for the present Deliverable.

Tables 9.4.11 and 9.4.12 respectively show the female and male samples contents of total lipids and their corresponding lipid classes.

According to the standard deviation of data, it can be highlighted that de composition of oocytes was more variable for the control group. That means that some of the samples were very high and other very low in terms of total lipid contents, a condition that is reflected in each single lipid class. In addition, no samples from this group were available for comparisons in August. The most consistent results at both sampling points were obtained for the experimental group, where total lipid contents ranged between 2.1-2.2, with PC as the main representative phospholipid and the sterol esters as the most abundant neutral lipid class. In spite of the high deviation of the data and that oocytes in the control group had a higher content of total fat, overall the lipid class profile was similar to that of the experimental group. Oocytes from the mackerel group were more different between August and September and generally displayed some higher contents of cholesterol and lower of PC than oocytes from those of the two the extruded diets-fed groups.

Table 9.4.11. Total lipid content (% wet weight) and main lipid class composition (% total lipid) of oocytes from greater amberjack broodstock fed different diets

	Experimental		Control	Mackerel	
	August	September	September	August	September
TL (%ww)	2.29±1.11	2.12±1.01	3.85±3.60	2.74±1.51	1.19±0.40
SM	2.00±0.94	1.59±0.92	2.22±2.08	2.68±0.66	3.01±0.49
PC	12.35±1.14	11.85±3.52	15.39±9.14	9.68±2.52	10.08±2.37
PS	1.04±0.14	0.94±0.64	1.97±1.90	1.25±1.32	2.03±1.06
PI	0.73±0.60	1.59±0.93	2.12±1.62	1.18±0.40	1.25±0.70
PE	2.17±1.65	3.47±4.34	4.68±4.48	4.13±2.91	6.05±2.45
TPL	**21.68±6.78**	**20.59±9.53**	**27.34±20.3**	**19.84±7.65**	**23.13±7.16**
PAG	5.07±1.57	2.69±0.91	4.47±2.71	5.50±1.69	6.94±4.13
Chol	13.88±6.45	14.27±8.95	13.75±5.74	16.08±3.30	20.87±4.12
TG	23.14±5.81	22.75±5.99	19.03±12.8	22.56±1.38	21.37±5.46
SE	35.94±9.68	39.03±9.73	34.86±16.6	35.13±9.94	27.41±14.6
TNL	**78.32±6.78**	**79.41±9.53**	**72.66±20.3**	**80.16±7.65**	**76.87±7.16**

Data are means ± SD (n=3, 4). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; PAG, partial acyglycerols; Chol, Cholesterol; TG, triacylglicerides; SE, sterol esters; TNL, total neutral lipids.

Table 9.4.12. Total lipid content (% wet weight) and main lipid class composition (% total lipid) of sperm from greater amberjack broodstock fed different diets sampled in September.

	Experimental	Control	Mackerel
TL (%ww)	1.45±0.76	2.33	9.38
SM	1.25±0.46	0.48	1.99
PC	20.75±2.14	21.42	13.47
PS	5.79±2.52	3.94	4.14
PI	2.87±1.85	2.04	1.73
PE	15.21±2.87	15.68	2.43
TPL	**48.06±5.90**	**46.17**	**26.87**
PAG	7.12±1.83	10.24	36.17
Chol	33.13±2.87	36.08	25.06
TG	8.18±6.23	5.72	10.59
SE	2.40±1.18	0.59	nd
TNL	**51.94±5.90**	**53.83**	**73.13**

Data of the experimental diet are means ± SD (n=2). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE phosphatidylethanolamine; TPL, total polar lipids; PAG, partial acyglycerols; Chol, Cholesterol; TG, triacylglicerides; SE, sterol esters; TNL, total neutral lipids. Nd, not detected.

As shown in **Table 9.4.12**, and although only one sample was available for the control group, one more time the composition is quite similar between the sperm from the experimental and the control fed groups. In both cases PC was the most abundant phospholipid, followed by PE and cholesterol the most prominent lipid class. This situation completely changes however with regard to the sperm sampled from the mackerel fed fish which contained a very high level of lipids, with much less proportions of polar lipids. Main fatty acid composition of total lipids from the above mentioned samples are given in **Tables 9.4.13 and 9.4.14**. As shown in **Table 9.4.13**, composition of oocytes from the experimental group was quite stable, with 16-17% of DHA, 5-6% of EPA and 2-3% of ARA in % of total fatty acids. Although much more variable, particularly for the ARA contents, overall, the fatty acid profile was also similar for the control group oocytes. However, oocytes from the mackerel fed fish displayed higher contents of DHA and ARA at both sampling points, and also lower levels of 18:2n-6, and EPA, compared to the oocytes from the two extruded diet fed fish.

Table 9.4.13. Main fatty acid composition (% total fatty acids) of oocytes from greater amberjack broodstock fed different diets.

	Experimental		Control	Mackerel	
	August	September	September	August	September
16:0	16.53±0.75	10.03±2.34	15.52±2.86	15.89±1.78	17.52±0.37
18:1[1]	23.24±2.09	26.17±3.29	26.34±6.64	21.21±2.16	21.65±2.00
18:2n-6	9.20±2.41	10.03±2.34	10.00±4.26	2.77±0.36	4.41±1.19
20:4n-6	2.57±1.21	2.16±0.31	3.30±2.81	5.78±1.52	7.13±1.46
20:5n-3	6.13±0.84	5.43±0.89	4.97±0.57	2.85±0.42	2.91±1.01
22:6n-3	17.31±1.91	15.77±1.71	17.13±2.34	28.29±2.04	22.52±4.60
DHA/EPA	2.87±0.53	2.98±0.70	3.44±0.08	9.17±2.03	8.67±4.00
ARA/EPA	0.40±0.20	0.37±0.07	0.64±0.49	1.89±0.95	2.75±1.36

Data are means ± SD (n=3, 4). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Table 9.4.14. Main fatty acid composition (% total fatty acids) of sperm from greater amberjack broodstock fed different diets sampled in September.

	Experimental	Control	Mackerel
16:0	23.16±0.96	23.15	12.86
18:1[1]	19.20±2.60	24.00	36.36
18:2n-6	4.68±0.73	5.92	5.74
20:4n-6	4.37±0.74	2.56	1.97
20:5n-3	5.49±0.89	4.63	1.38
22:6n-3	23.90±3.15	18.69	11.76
DHA/EPA	4.39±0.38	4.03	8.53
ARA/EPA	0.80±0.03	0.55	1.43

Data of the experimental diet are means ± SD (n=2). [1], mainly n-9 isomer. DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6.

Haematological and plasma biochemical parameters

Hematological and plasma biochemical parameters of fish from experimental, control and mackerel groups are shown in **Tables 9.4.15** and **Table 9.4.16**. In general, the number of erythrocytes tended to decrease along the experimental period in all treatments while the number of leucocytes tended to increase. When we compare between treatments, most of studied parameters were similar although the number of erythrocytes was lower ($P<0.05$) and leucocytes higher ($P<0.05$) in blood from mackerel group compared to other groups in July. No significant differences were observed in hematocrit.

Regarding biochemical parameters, triglycerides diminished significantly ($P<0.05$) along de experimental period in all treatment groups. A similar trend was observed for cholesterol but only significant for Mackerel group. In addition, fish from mackerel group presented lower levels of triglycerides compared to the other groups in all periods. Several differences were detected in glucose levels between treatment groups in July and October.

Table 9.4.15. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}) and hematocrit (%) in blood from greater amberjack of Experimental (Exp,), Control (Cont.) and Mackerel (Mack.) groups during experimental spawning period. Values are means ± SD. Different letters indicate significant differences between month in each group (ANOVA, $P<0.05$). Different capital letters indicate significant differences between treatments (ANOVA, $P<0.05$).

Parameter		Erythrocytes					Leucocytes					Hematocrit			
Treat.	Month	Mean ± SD					Mean ± SD					Mean ± SD			
Exp.	Jun.	232.99	±	110.75	ab		31.77	±	19.33	b	B	44.01	±	9.00	
	Jul.	330.42	±	56.92	a	A	76.33	±	47.01	ab	B	46.75	±	5.93	
	Ago.	240.94	±	45.39	ab		95.21	±	60.24	ab		40.67	±	8.94	
	Sep.	138.88	±	55.04	bc		107.90	±	21.38	a		45.14	±	12.11	
	Oct.	89.75	±	34.79	c		154.54	±	26.29	a		40.69	±	6.85	
Cont.	Jun.	210.63	±	20.37	b		79.18	±	33.47		A	55.10	±	7.11	a
	Jul.	289.64	±	31.84	a	A	111.31	±	69.08		B	47.94	±	3.41	ab
	Sep.	138.33	±	73.36	c		71.85	±	11.65			45.54	±	4.55	c
	Oct.	122.50	±	21.64	c		151.98	±	45.96			35.56	±	4.07	bc
Mack.	Jun.	246.88	±	30.32	a		27.23	±	8.50	b	B	50.63	±	1.96	
	Jul.	216.25	±	49.27	a	B	171.00	±	78.02	ab	A	52.08	±	7.36	
	Ago.	139.79	±	33.01	b		121.46	±	39.98	ab		42.75	±	12.47	
	Sep.	62.00	±	17.96	c		110.03	±	28.31	ab		37.74	±	10.60	
	Oct.	134.06	±	55.20	bc		212.20	±	63.70	a		37.51	±	13.29	

Table 9.4.16. Triglycerides (mg dl^{-1}), cholesterol (mg dl^{-1}), protein (g l^{-1}) and glucose (mg dl^{-1}), in blood from greater amberjack of Experimental (Exp,), Control (Cont.) and Mackerel (Mack.) groups during experimental spawning period. Values are means ± SD. Different letters indicate significant differences between month in each group (ANOVA, $P<0.05$). Different capital letters indicate significant differences between treatments (ANOVA, $P<0.05$).

Parameter		Protein				Glucose					Triglycerides					Cholesterol			
Treat.	Month	Mean ± SD				Mean ± SD					Mean ± SD					Mean ± SD			
Exp.	Jun.	43.76	±	8.06		133.75	±	25.67	a	A	430.37	±	78.49	a	AB	290.57	±	59.34	
	Jul.	52.27	±	3.38		78.80	±	27.80	b		161.01	±	124.80	bc		276.84	±	19.24	
	Ago.	43.74	±	4.01		102.68	±	32.92	ab		168.71	±	28.52	bc	*	256.10	±	34.93	
	Sep.	42.47	±	8.39		81.11	±	31.00	b		340.66	±	221.64	ab	B	239.52	±	47.93	
	Oct.	43.12	±	5.16		98.18	±	23.24	ab	A	108.68	±	60.53	c		226.33	±	23.20	
Cont.	Jun.	49.71	±	5.92	ab	116.31	±	32.62		AB	566.98	±	161.59	a	A	301.74	±	26.96	
	Jul.	52.34	±	7.87	a	111.96	±	50.84			78.68	±	20.82	b		282.88	±	42.39	
	Sep.	47.87	±	8.41	ab	118.73	±	34.19			611.45	±	267.70	a	A	114.34	±	39.40	
	Oct.	38.06	±	5.43	b	65.87	±	13.71		B	170.68	±	8.21	b		201.58	±	35.53	
Mack.	Jun.	45.51	±	9.29		74.06	±	22.65		B	339.72	±	84.13	a	B	299.04	±	68.22	a
	Jul.	48.92	±	5.79		119.31	±	49.14			75.61	±	41.46	b		258.83	±	24.24	ab
	Ago.	43.17	±	7.28		106.69	±	23.72			28.39	±	4.61	b		254.67	±	47.24	ab
	Sep.	40.89	±	5.18		111.29	±	43.10			31.67	±	15.41	b	B	203.85	±	23.01	b
	Oct.	36.94	±	7.17		107.64	±	19.01		A	44.41	±	15.48	b		189.75	±	28.02	b

Deviations from Annex I and their impact:

Project Coordinator's Comments: In the **Sub-task 9.2.2. Performance of grow-out diets for greater amberjack developed in order to maximize growth potential**, the study was not carried out for the full duration so that fish would reach market size (2-3 kg) and allow the analysis of fillet quality, as committed in the DOW.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Sarih, S., Djellata, A., Roo, J., Hernández-Cruz, C.M., Fontanillas, R., Rosenlund, G., Izquierdo, M., Fernández-Palacios, H., 2019. Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack (*Seriola dumerili*, Risso, 1810). Aquaculture 499, 72-79.

Sarih, S., Djellata, A., LaBarbera, A., Fernandez-Palacios Vallejo, H., Roo, J., Izquierdo, M.S., Fernandez-Palacios, H. 2018. High-quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its comparison with GnRHa implants or injections. Aquaculture Research 49, 3442-3450. DOI: 10.1111/are.13808

WP 10 Nutrition – pikeperch

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

Objectives

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

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

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

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

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

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

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

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

Remaining analytical work that was delayed in period 2 has been performed, while some analytical work of the confirmatory exp. is still under analysis. The completion of deliverable 10.1 (due month 36) is awaiting the final experiment to be performed in month 50 - and subsequent analytical work. The completion of deliverable 10.2 (due month 36) is expected this month (month 48). Deliverable 10.3 (due month 48) is waiting results from on-going exp.to be started month 50.

Summary of progress towards objectives (49-60 Mo):

Remaining analytical work was finished. Two final experiments were conducted in the period, a first to complete work in Task 10.1. This was a confirmatory experiment to establish effects on larval/juvenile growth performance, enzymatic activity and deformities by inclusion of graded levels of dietary Ca/P ratio in weaning diets. The other experiment was conducted in task 10.2, (Deliverable 10.3). The experiment involved formulation of a test diet (based on information gathered by previous experiments in Task 10.1. and 10.2) and test of the performance of larvae/juveniles reared on this diet against a high quality commercial control diet until 52 days post hatch (DPH).

Deliverable 10.1 was uploaded month 58 and Deliverable 10.3 uploaded month 60. Both were delayed according to expected time schedule in DOW, but primarily as a result of additional work carried out within the WP, and the wish to include the work in the conclusive deliverables.

Details for each Task

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

The work in this task was finished and submitted as a Deliverable in month 58. (D10.1: Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch). Below are informative highlights, but please refer to the deliverable for full description of work and results.

The general objective of the research task 10.1.was to increase the knowledge on specific influence of essential nutrients in pike perch larvae. Work in this task therefore involved experiments for examination of optimal dietary levels of Ca/P, vitamins; FA and phospholipids to improve larval development and reduce skeleton alterations in pikeperch. In total 5 experiments were conducted.

The first experiment was carried out as a multifactorial exp. and investigated the effect of selected dietary nutrients (fatty acids, vitamins and minerals) on pikeperch larval development and performance. Two modalities (low and high levels) of 8 variables: Ca/P, EPA+DHA, ARA, Vit. E, D, C, A and Se were tested (**Table 10.1.1**) in order to identify the most influential nutrients as well as their interactions in pikeperch larvae

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

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

Several parameters were evaluated, including husbandry variables, biochemical assays, digestive enzymatic activities, organ development and tissue morphology, deformities and gene expression. At the end of the experiment, the highest survival was recorded in larvae fed high Ca/P, but final weight and specific growth rate (SGR) were significantly lower in larvae fed high Ca/P, associated with a higher incidence of kyphosis and pectoral deformities in these larvae. Ca/P, fatty acids and their interaction seem to be key nutritional factors influencing pikeperch larval development. However, only two levels of Ca/P and fatty acids were tested in the multifactorial experiment. Therefore, two confirmatory experiments testing 1) gradual levels of dietary EPA+DHA/ARA and 2) Ca/P ratios were conducted. The EPA+DHA/ARA exp. investigated combined effect of graded levels ARA with two DHA dietary levels (low and high, **Table 10.1.2**) in early weaning diets on larval performance, digestive capacity, biochemical composition, oxidative status, skeletal deformities and bone mineralization of pikeperch larvae.

Table 10.1.2. Dietary composition of the experimental diets

As feed	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Crude protein, % feed	54.2	54.2	54.2	54.2	54.2	54.2
Crude fat, % feed	20.2	20.2	20.2	20.2	20.2	20.2
Starch, % feed	9.7	9.7	9.7	9.7	9.7	9.7
Ash, % feed	9.0	9.0	9.0	9.0	9.0	9.0
Total P, % feed	1.67	1.67	1.67	1.62	1.62	1.62
Ca, % feed	1.52	1.52	1.52	1.52	1.52	1.52
Ca/P	0.91	0.91	0.91	0.93	0.93	0.93
LNA (C18:2n-6), % feed	0.53	0.40	0.33	0.50	0.37	0.30
ALA (C18:3n-3), % feed	0.13	0.13	0.13	0.10	0.10	0.10
ARA, % feed	1.20	0.59	0.30	1.19	0.59	0.30
EPA, % feed	1.19	1.19	1.19	1.22	1.22	1.22
DHA, % feed	0.61	0.61	0.61	2.49	2.49	2.49
EPA/ARA	0.99	2.00	3.95	1.02	2.07	4.12
DHA/EPA	0.52	0.52	0.52	2.04	2.05	2.05
Total phospholipids, % feed	7.76	7.76	7.76	6.22	6.22	6.22

Results and Discussion

Resulting growth differences were relatively small and non-significant. Enzymatic pepsin specific activity was influenced by the dietary DHA content, being higher at 22 dph in larvae fed diet 6 than in diets 1, 2 and 3. At 40 dph, the increase in dietary EPA/ARA levels enhanced the trypsin activity in fish fed low DHA level.

The dietary Ca/P experiment examined the effect not only by varying one of the two minerals, but also by varying both. As so six diets with three Ca/P levels (0.3, 0.6 and 1.2) were tested (**Table 10.1.3**).

Table 10.1.3. Proximate composition of experimental diets (%).

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

Results and discussion

Low growth was observed in larvae fed diet 6, containing the highest Ca/P ratio and the lowest P % (**Fig. 10.1.1**). In this regard, the need for a low Ca/P ratio in pikeperch larval diets has been suggested. No significant differences were observed in digestive enzymatic activities (**Table 10.1.4**).

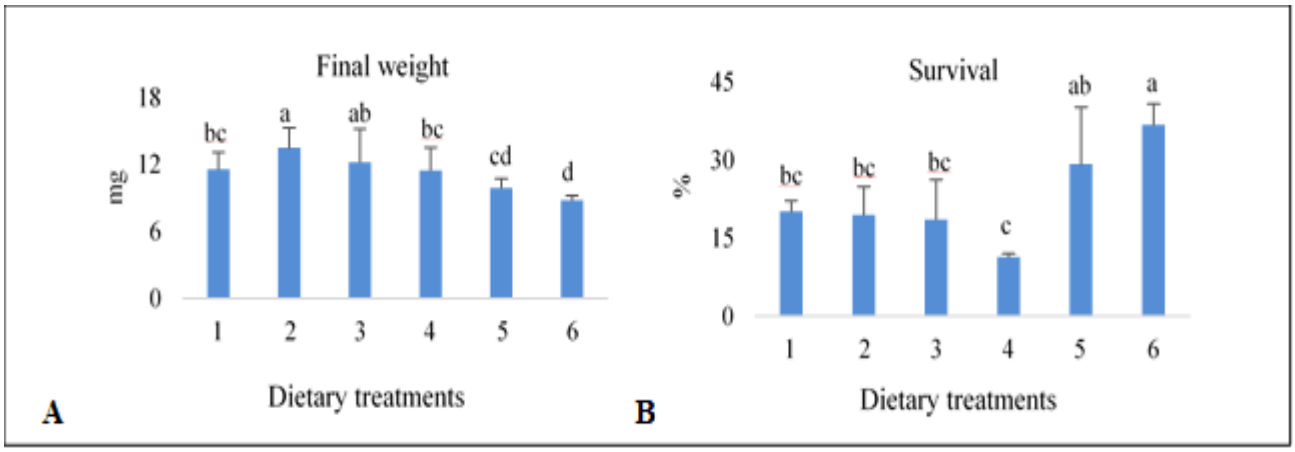


Figure 10.1.1 Effect of dietary Ca/P on husbandry variables, (A) final weight, (B) survival. Different letters denote significant differences ($p<0.05$)

Table 10.1.4. Larval specific enzymatic activities (mU/mg protein). Different letters within a row denote a significant (P<0.05) difference

mU/mg protein	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Trypsin	16.8±3.8	16.1±3.8	17.6±2.7	19.5±2.0	15.6±3.8	16.6±0.3
Alkaline phosphatase	48.9±8.1	48.4±10	58.5±27.1	57.1±6.7	48.8±22.4	46.6±18.5
Aminopeptidase	4.7±0.0	5.4±0.7	9.6±2.7	9.6±2.7	11.3±4.6	9.3±4.8

Regardless Ca/P dietary levels, growth and larval survival were significantly affected by the P dietary content (**Fig. 10.1.2**), suggesting the importance of a balanced Ca/P and P diet for pikeperch larvae.

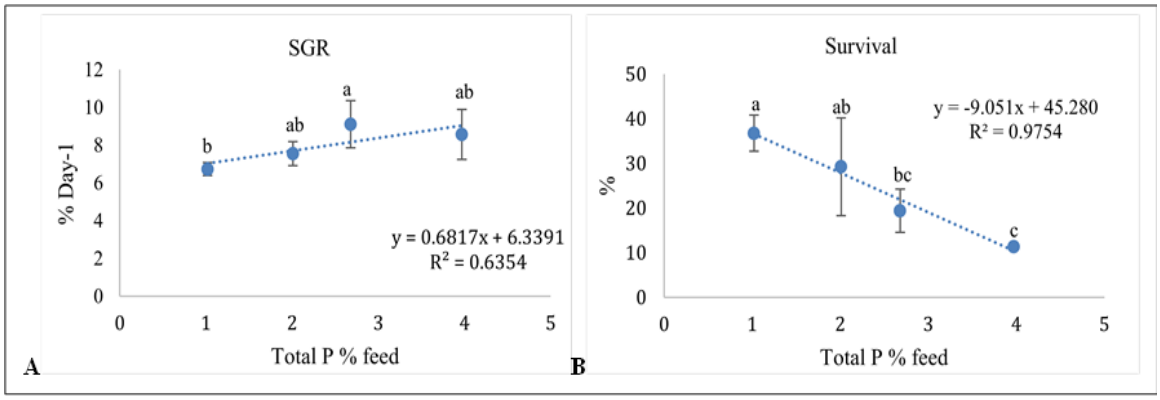


Figure 10.1.2: P effect on husbandry variables, (A) larval growth, (B) survival. Different letters denote a statistically significant difference between treatments.

Requirements of phospholipids and n-3 LC PUFAs in pikeperch larvae

The optimal requirements for phospholipids have so far not been determined, thus two experiments were carried out The main objectives of the first study (I) was to examine optimal levels of soy bean lecithin (SBL) derived PL in formulated extruded starter feeds for pikeperch larvae and determine additional effects of inclusion of TAG n-3 LC PUFAs on larval performance and larval development. A second supplementary experiment (II) was carried out to investigate the dietary effect on juvenile physiology and stress response.

Six diets were formulated to be isonitrogenous and isoenergetic (**Table 10.1.5**). The six diets contained 3 levels of PL (PL1, PL2, PL3), while three of the diets were further supplemented by 3 levels of DHA (PL1H1, PL2H2, PL3H3). Soybean lecithin powder was used as PL source and Algatrium DHA70 as supplemented DHA source (+EPA)

Table 10.1.5. Dietary composition and analytical content of the 6 experimental diets.

Diet Ingredients (%)	PL1	PL2	PL3	PL1H1	PL2H2	PL3H3
MicroNorse Fish Meal[a]	45	45	45	45	45	45
CPSP 90[b]	7	7	7	7	7	7
Squid meal[c]	13	13	13	13	13	13
Fish gelatin[d]	1	1	1	1	1	1
Wheat Gluten[e]	4.4	4.4	4.4	4.4	4.4	4.4
Wheat meal[f]	6.1	5.9	5.6	6.1	5.9	5.6
Algatrium DHA70[g]	0.0	0.0	0.0	0.55	2.0	3.4
Olive oil[h]	18.9	12.1	3.4	18.35	10.1	0.0
Vitamin & Mineral Premix PV01[i]	1.0	1.0	1.0	1.0	1.0	1.0
Soy lecithin powder[j]	3.0	10.0	19.0	3.0	10.0	19.0
Binder (guar gum)[k]	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant powder (Paramega)[l]	0.2	0.2	0.2	0.2	0.2	0.2
Antioxidant liquid (Naturox)[m]	0.2	0.2	0.2	0.2	0.2	0.2
Analysed content (% WW)						
Crude protein	54.1	54.7	55.6	54.1	55.8	55.3
Crude lipid	26.8	25.9	24.6	26.6	25.6	24.8
NFE + fibre (substracted)	3.0	3.0	2.8	2.8	3.1	3.2
Dry matter (DM)	93.0	93.0	93.1	93.6	92.8	93.5
Ash	9.1	9.4	10.0	9.0	9.3	10.2
Phosphorus	1.30	1.27	1.31	1.28	1.29	1.30
Calcium	1.84	1.85	1.85	1.83	1.84	1.85
(% of protein)						
Lysine	4.20	4.22	4.19	4.16	4.21	4.17
Methionine + Cysteine	1.90	1.93	1.90	1.91	1.87	1.89
Taurine	0.52	0.50	0.51	0.52	0.52	0.51
(% ww)						
Phosphatidylcholine (PC)	1.40	2.61	4.31	1.42	2.68	4.29
Phosphatidylethanolamine (PE)	0.43	1.22	2.20	0.40	1.14	1.87
Phosphatidylinositol (PI)	0.44	1.28	2.44	0.43	1.28	2.48
Total phospholipids(TPL)	3.73	8.19	14.38	3.70	8.32	14.51

Results and discussion

Growth

Figure **10.1.3** illustrates effect on growth (exp. I) by an increase in dietary inclusion of phospholipids and an additional positive effect by supplementation of DHA (+EPA) in the form of Algatrium DHA 70. Larval weights were statistically significantly different ($P<0.05$).

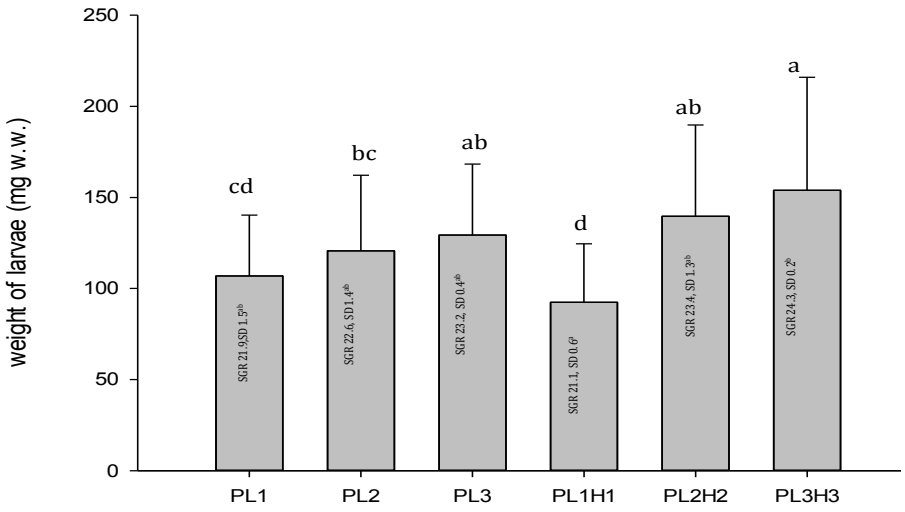


Figure 10.1.3. Mean larval weight (mg ww) (±SEM) at 30 dph and calculated SGR (±SEM (on each bar). Different letters denote statistically significant differences between treatments

Larval skeleton anomalies and gene expression

Overall there was a high incidence of severe anomalies, particularly those related with endochondral bones, such as cranium or dentary bones. The lowest incidence of severe anomalies was found in PL3H3 pikeperch, followed by PL3 (**Fig. 10.1.4**). An increase in dietary PL from PL1 to PL3 tended to reduce the incidence of severe anomalies

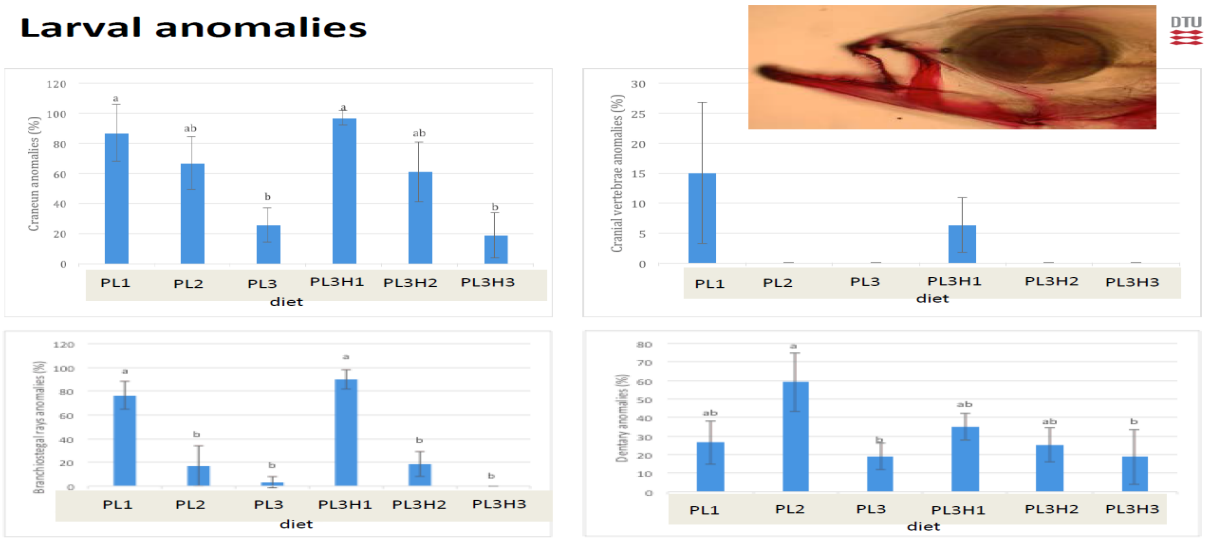


Figure 10.1.4 Craneum and Branchiostegal anomalies (%) in larvae fed one of 6 exp. diets. Different letters denote statistically significant differences between treatments.

Proteomics

Liver can be considered as the main metabolic reactor of the body, possessing a number of regulatory functions, including the storage of vitamins and minerals, as well as carbohydrate, fat, and protein metabolism and therefore chosen to access changes in protein expression profiles (proteomics)

The mean number of spots detected per gel was 1917 ± 498. The one-way analysis of variance among the six experimental groups revealed 27 spots with differential intensity at $p<0.05$ (**Fig 10.1.5**). 17 spots contained one protein identification per spot and 8 proteins displayed a differential intensity between treatments

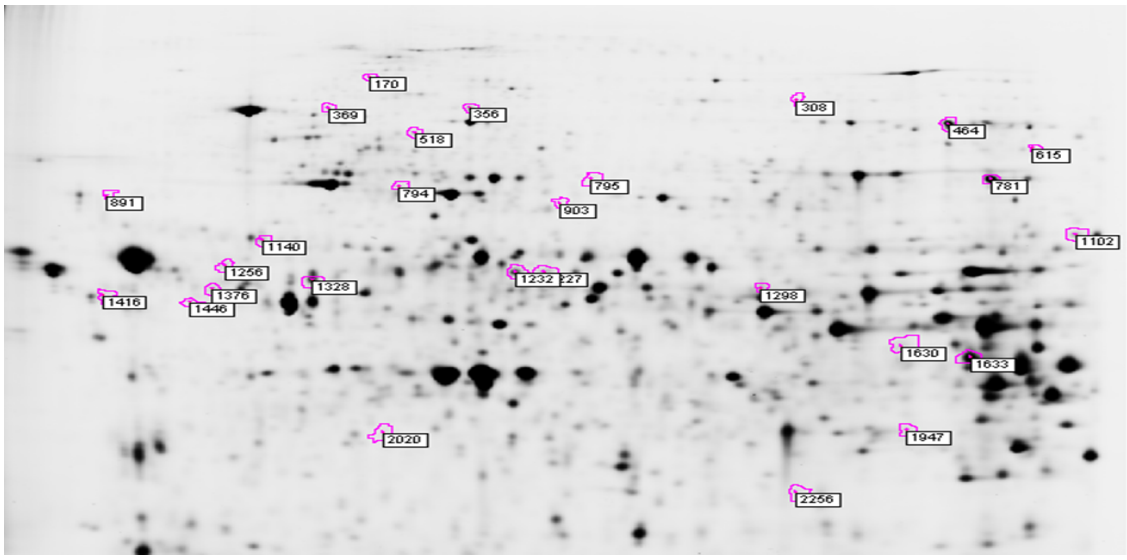


Figure 10.1.5. Representative two-dimensional gel electrophoresis of differential protein expression profile in pikeperch liver

Among the identified proteins, fatty acid synthase (FAS) (involved in lipid synthesis) was significantly under-expressed in PL3H3 (14.51/27.9) compared to PL1(3.73/6.2), PL2 (8.19/7.7), and PL1H1(3.70/7.9) (4.36, 3.65, and 3.50-fold respectively, $p<0.01$). FAS was under-expressed in larvae from PL3H3 compared to PL1 and PL1H1, suggesting a higher energy demand of the smallest larvae. Moreover, FAS seemed to be more regulated by LC PUFA content than by PL levels, which may indicate a positive effect of DHA supplementation. Indeed, regardless of PL levels, FAS expression changed between PL3H3 and PL3 (3.54-fold under-expressed in PL3H3), while no significant differences appeared between larvae fed the same n-3 LC- PUFA level (PL2 compared to PL1H1)

Metabolic rate; oxygen tolerance and chasing stress

In exp. II fish on diets low in phospholipids; i.e. PL1 and PL1H1 grew much slower and had a higher mortality until DPH 80 than the 4 other remaining diets. Therefore juveniles from these diets were not included in studies on fast escape response, metabolic rate and stress response to chasing.

For the fast escape response, there were no significantly differences in latency time between the tested diets (i.e. time to respond to a visual stimulus, velocity or in acceleration speed. As for the fast escape response, the results on metabolic rate likewise showed no significant differences between the diets tested. Results did not show any significant differences in levels of body cortisol, glucose or lactate between the experimental diets tested

Discussion and conclusion

We confirmed in these studies the importance of high dietary PL levels of approximately 8 % for optimal performance of pikeperch as well as a positive additional beneficiary effect of supplementation with DHA+ EPA in the form of concentrated TAG in otherwise identical formulated diets. Thus, combined supplementation of SBL up to 14.51% d.w. PL with n-3 LC-PUFA (1.00 % d.w. DHA and 0.16% d.w. EPA) in the form of triglycerides lead to the highest growth and lowest anomalies incidence, which improved digestive enzymes activities. . Data were supported by analyses of liver proteomics and revealed, that PL3H3 caused a down regulation of both FAS and ATP- citrate synthase involved in triglyceride and FA synthesis in comparison when fed PL3. We did not observe any significant effects on metabolic rate, oxygen tolerance or stress related tissue values of cortisol, glucose and lactate for juveniles from each diet following chasing stress (1.5 h), but fish reared on diets with lowest inclusion of PL were not included in these experiments as due to low survival and poor growth.

Task 10.2. Effects of pikeperch early fatty acid nutrition on long-term stress sensitivity (led by DTU, Ivar Lund).
This task involved two deliverables. ***Deliverable D10.2: Protocol for optimal early fatty acid enrichment to reduce stress sensitivity in pikeperch*** was finished and submitted in month 48. Deliverable ***D10.3: Formulation for a diet better adapted to pikeperch requirements*** was completed and submitted in month 60. Below is a brief description.

Based on gathered information obtained within the experimental framework of WP 10 the partners conducted a final larval experiment at commercial farm conditions. The experiment involved formulation of a test diet (D1) and test of this diet against a commercial control diet on performance of pike perch larvae from start of dry feed weaning at 14 days post hatch until 52 days post hatch (DPH). Emphasis was on optimal dietary lipid class composition; LC PUFA levels and ratios; levels and ratios of vitamins (Vit A, E, C, D) and minerals (Ca/P, Se).

Samplings and output variables

Several parameters were evaluated including husbandry variables; survival; growth performance and anomalies. Final survival was calculated by individually counting all the living larvae at the end of the experiment. Survival was calculated as the percentage between final and initial number of fish

Larvae from D1 sampled at 35 DPH and 52 DPH (**Fig.10.2.1)** were significantly larger ($P<0.001$) than larvae fed the commercial Otohime diet in terms of weight and length. Average growth calculated as SGR was 12.1 - and 10.8 % d^{-1} for D1 at 35 and 52 DPH, respectively, while 11.5 - and 10.1 % d^{-1} for Otohime, ($P<0.001$).

Cumulative recorded mortality in the tanks fed D1 was 57% and 55% whereas final survival was 22% and 23%. For the commercial diet this amounted to 51% and 53% for the recorded mortality and final survival was slightly higher around 28% and 26%.

Deformities

A few examined larvae in each tank showed scoliosis and lordosis, as well as some jaw deformities, but the occurrence was low and did not indicate an effect of dietary treatment, thus was not further examined.

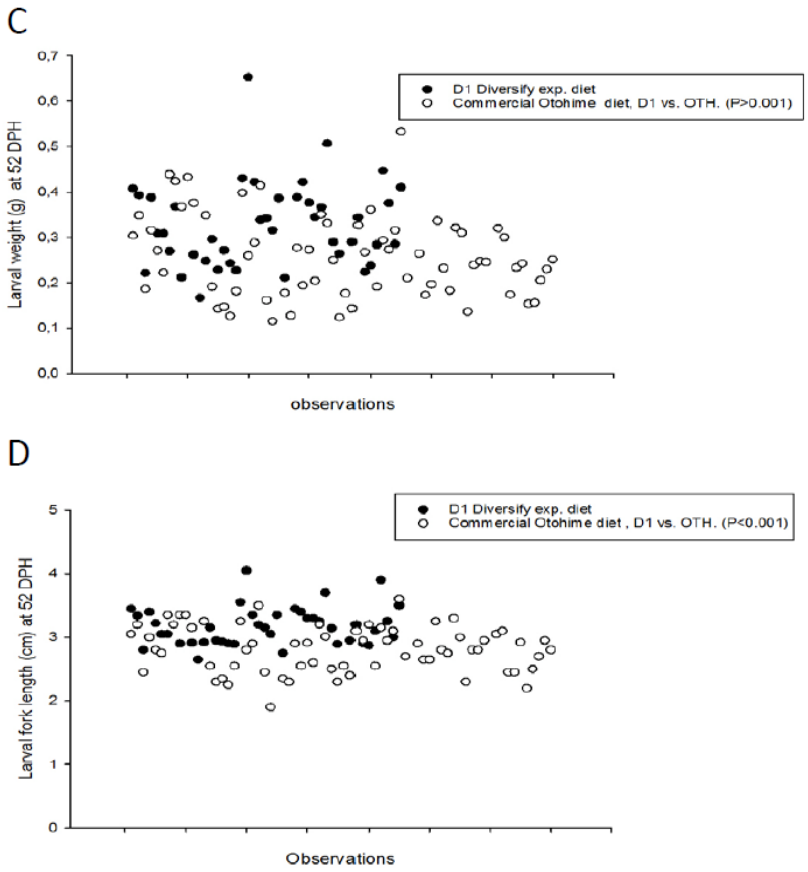


Figure 10.2.1: Individual weight (g) and fork length (cm) of sampled larvae at 35 (A, B) - and 52 DPH (C, D) for larvae fed exp. diet D1 or commercial Otohime control.

Discussion & conclusion

We succeeded to formulate a diet, which was superior in terms of larval growth at 35 DPH and 52 DPH to a commercial high quality performing diet, Otohime, when tested at commercial farm conditions. Estimated mortality for the experimental diet was slightly higher, than for the commercial diet tested, which may have affected the size distribution in the population of surviving larvae.

Deviations from Annex I and their impact:

No deviations.

Manuscripts that resulted from this WP (if not published, indicate Submitted, Accepted or In Preparation**)**

Lund, I., El Kertaoui, N., Izquierdo, M.S., Dominguez, D., Hansen, B.W., Kestemont, P., 2018. The importance of phospholipids combined with long-chain PUFA in formulated diets for pikeperch (*Sander lucioperca*) larvae. British Journal of Nutrition 120, 628-644.

Lund, I., Rodríguez, C., Izquierdo, M.S., El Kertaoui, N., Kestemont, P., Reis, D.B., Dominguez, D., Pérez, J.A., 2019. Influence of salinity and linoleic or α-linolenic acid based diets on ontogenetic

development and metabolism of unsaturated fatty acids in pike perch larvae (*Sander lucioperca*). Aquaculture 500, 550-561.

El Kertaoui, N., I. Lund, H. Assogba, D. Montero, D. Domínguez, S. Baekelandt, M.S. Izquierdo, V. Cornet, M. Schmitz, S.N.M. Mandiki and P. Kestemont. Key nutritional factors and interactions during larval development of pikeperch (*Sander lucioperca*), Aquaculture Research (Submitted)

El Kertaoui, N., I. Lund, P. Kestemont. Effect of DHA/EPA/ARA ratio on pikeperch larval development (In Preparation)

El Kertaoui, N., I. Lund, P. Kestemont. Evaluation of the optimal dietary Ca/P levels on pikeperch larvae (In Preparation)

Reis, D.B., I. Lund, J.A. Pérez, N.G. Acosta, A. Bolaños and C. Rodríguez. Effect of linoleic or α-linolenic acid-based diets and salinity on the *in vivo* metabolism of C14-unsaturated fatty acids and phospholipids in pikeperch (*Sanders lucioperca*) larvae (In Preparation)

WP 11 Nutrition – Atlantic halibut

WP No:	11	**WP Lead beneficiary:**		P17. NIFES (IMR)
WP Title (from DOW):	Nutrition – Atlantic halibut			
Other beneficiaries (from DOW):	P7. IMR	P15. ULL	P20. SARC	
Lead Scientist preparing the Report (WP leader):	Kristin Hamre			
Other Scientists participating:	Øystein Sæle (P17), Torstein Harboe (P7), Covadonga Rodriquez (P15), Ramon Fontanillas (P20)			

Objectives

1. Develop a protocol for early weaning,
2. Develop a production strategy for on-grown *Artemia,*
3. Improve growth in late larval stages, and juvenile quality, through feeding with on-grown *Artemia,*
4. Better understand the effects of RAS vs FTS on Atlantic halibut larval nutrient utilization,
5. Investigate how dietary phospholipids after weaning affects growth and lipid metabolism.

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

Although in the DOW it was indicated that the work should start from the beginning of the project, we only had planning activities in the period 1-12 Mo. The actual experiments started in Mo 13. This has to do with the relatively few activities in this WP and the need to organize the work in a practical way.

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

1. A protocol for weaning of Atlantic halibut at 28 days post first-feeding (dpff) has been developed and almost 100% of the larvae fed Ottohime diet (Japan) were filling up their guts with feed after a 5 d adaptation period.
2. A production strategy for ongrown *Artemia* has been established, which improves the nutritional value of *Artemia* with respect to protein, lipid and micronutrient contents.
3. Growth and juvenile quality was excellent in larvae fed *Artemia* nauplii in this experiment and was not improved by feeding ongrown *Artemia.*

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

Research under objective 4 found that RAS had a large positive effect on vitamin K (MK6) concentration in Atlantic halibut larvae. Most free amino acids, iodine, copper and zinc were also increased, while glycine concentration was decreased in larvae reared in RAS compared to FT.

Summary of progress towards objectives (49-60 Mo):

Inclusion of soy lecithin so that dietary phospholipid increased from 9 to 30% of total lipids had no effect on growth, but changed lipid metabolism in Atlantic halibut juveniles

Details for each Task

Task 11.1 Early Weaning of Atlantic halibut (led by IMR, Torstein Harboe) This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.2*** **Report on optimal characteristics of feed particles and feeding environment for early weaning of Atlantic halibut larvae.**

Task 11.2. Development of a production strategy for on-grown *Artemia* (led by IMR, Torstein Harboe). This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.1*** **Report on the nutrient profile of *Artemia* nauplii and on-grown *Artemia***.

Task 11.3. Nutrient retention and digestive physiology of Atlantic halibut juveniles fed *Artemia* nauplii or on-grown *Artemia* (led by NIFES, Kristin Hamre). This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.3*** **Report on the nutrient retention and digestive physiology in Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia.***

Task 11.4. Comparison of nutrient retention in Atlantic halibut larvae reared in RAS vs FTS (led by NIFES, Kristin Hamre). This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 11.4*** **Report on the nutrient retention and digestive physiology in Atlantic halibut larvae reared in RAS vs FTS.**

Task 11.5 Effect of dietary PL on digestion, absorption and metabolism of lipids in Atlantic halibut juveniles (led by NIFES, Kristin Hamre)

Introduction

The benefit, or even essential need for high inclusion of phospholipids (PLs) in marine larvae is well documented (Coutteau et al., 1997). But adding PLs to the feed of juvenile fish has also shown to be beneficial for a wide spectre of species (Atar et al., 2009, Niu et al., 2008, Sotoudeh et al., 2010). PLs are vital for lipid transfer from the intestinal tissue to the blood, probably due to limited capacity of de novo PL synthesis in the intestine. Limited PL synthesis will also inhibit membrane metabolism in the larval body, and thereby growth. Lipids are transported from enterocytes to other tissues in chylomicrons. Besides proteins, chylomicrons consist of a core of TAG and cholesterol esters and a monolayer of PL on the surface. Chylomicron production starts with the formation of PL rich particles, thus PL synthesis is a potential bottleneck for lipid transport.

We have shown that juvenile Ballan wrasse increase the growth rate by up to 40% when lipids are added as PL in stead of triacylglycerols (TAG, Sæle et al., unpublished), while requirements for PL in A. halibut juveniles are not known.

The main objective of this study is to investigate lipid composition in intestinal tissue as a function of dietary ratio of PL/TAG and postprandial time. We know that high dietary PL gives better growth in other marine fish juveniles, and we have also shown that dietary PL can affect intestinal transport of TAG. The hypothesis is therefor that the low PL/TAG diet will give fish with more total lipid and in particular TG in intestinal tissue. We are also curious as to how the PL/TAG ratio might affect liver and muscle lipid composition and are therefore including some samples from these tissues taken 24 h post feeding.

Methods

When juvenile fish reached 0.92 ± 0.42 g with a total length of 46 ± 7 mm, they were fed diets with increasing PL/TAG ratio. The experiment design was a regression with 3 replicates and 5 levels of PL, ranging from 9 to 31 % of total lipid (**Fig. 11.1**). The experiment lasted for two months. The larvae were sampled at a fixed time after the first meal in the morning. Sampled larvae were euthanized with an overdose metacaine (MS-222TM; Norsk medisinaldepot AS, Bergen Norway). Total length (TL) and weight were registered before intestine, liver and muscle tissues were snap frozen on liquid nitrogen for lipidomics analysis and RNA isolation for qPCR analysis. Lipids classes of the diets were analysed according to Bell et al. (1993) and Jordal et al. (2007) and samples for lipidomics were sent to Metabolon Inc. for analyses.

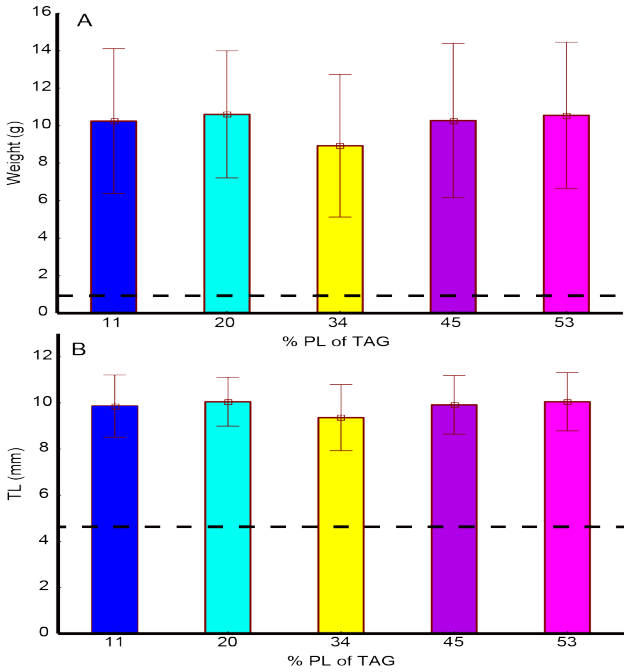


Figure 11.1. Final weight (A) and length (B) of halibut. Stippled line shows start weight and length

Summary and conclusions

Dietary PL varying from 9 to 31 % of dietary lipid did not affect growth of Atlantic halibut juveniles. Intestinal, muscle or liver lipid composition was not affected by the diets, but time after the meal influenced lipid level and composition in intestinal tissue. The relative concentration of neutral lipids such as TAG, DAG and FFA were high at 1 and 4 hours and decreased until 24 hours postprandial. In the same period, the relative concentration of CE, CER and some PL were lower at 1 and 4 hours and increased until 24 hours postprandial. qPCR showed increased expression at 4 compared to 1 and 24 hours, of some of the genes involved in absorption and remodelling of lipids in the enterocytes.

It appears that Atlantic halibut juveniles regulate their lipid species composition to be independent of the diet when a range of PL/TAG as in the present study is applied. Furthermore, absorption and metabolism of lipids in the enterocytes seems to be too fast for different dietary PL/TAG ratios to be detected by analyses of intestinal tissue.

However, the amount of lipids in the intestinal tissue does not show a significant increase between 1 and 4 hours. GPAT4 and CHPT1 are both involved in new synthesis of lipids and LPCAT1 remodels lyso-PL into PL. All these genes are expected to be up regulated when a lipid diet is being absorbed and metabolized in the enterocyte. However, genes associated with lipid transport from the enterocyte to periphery tissues, such as ApoA4 were not regulated as expected.

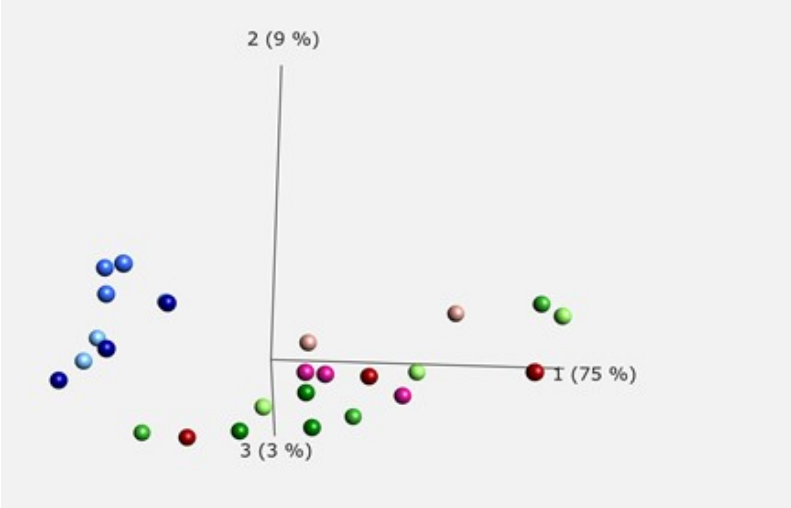


Figure 11.2. PCA plot based on all lipid species in intestinal tissue**.** The 24h (blue) sampling clusters away from 1(green) and 4h (red), but there is no separation between 1 and 4h. Nor does PL level in the diets have any effect. The same data are represented in a heat map below.

The full description of the work and results is provided in ***Deliverable 11.5 Report on the effect of dietary phospholipids on Atlantic halibut juveniles.***

Deviations: This task was reported six months later than planned and included lipidomics analyses that were financed by internal NIFES funds. Otherwise, there were no deviations from the approved DOW.

Manuscript that resulted from this Task

Sæle, Ø. et al., Increasing dietary phospholipid from 9 to 31 % of total lipid did not affect growth or lipid composition in Atlantic halibut (*Hippoglossus hippoglossus*, L.) juveniles. In preparation.

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):		Fátima Linares		
Other Scientists participating:	José Luis Rodríguez (P19), Blanca Álvarez-Blázquez (P8), Evaristo Pérez (P8), Gema Pazos (P19), Javier Roo (P2) and Marisol Izquierdo (P2)			

Objectives

1. Test the effectiveness of live prey and influence of enrichment on wreckfish larvae.
2. Determine the influence of broodstock feeds on fecundity and spawning quality.

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

During the first year of the project the work done in this WP was related to wild fish composition and the feeding of wreckfish broodstock to allow the formulation of the broodstock diets. Wild wreckfish were sampled from February to October 2014 and the stage of the reproductive development was evaluated. Fish dissection was performed and samples 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 (SAFA) and monounsaturated (MUFA) fatty acids were 36-46%, 28-30% and 25-33% respectively and n-3 PUFA content reached 32-40%. Liver fatty acid profiles showed a broader variability with a lesser content of EPA, DHA and ARA than muscle. Additionally, histological analysis of gonads were performed and showed that of 33 gonads examined, 15 were males and 18 were females and no evidence of hermaphroditism was obtained. With respect to the influence of the broodstock food composition on the reproductive development, some samples of semi-moist diet supplied to the P8. IEO broodstock were collected at different times of freezing to perform the biochemical analysis and no differences were found between samples taken at different times and with different freezing times.

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

During the second period of the project the highlights were:

1. Comparisons of wild and reared wreckfish composition showed that fish from intensive culture have more lipids in muscle (27.5% DW) and liver (62%) than those obtained in wild fish with 7% in muscle and 40% in liver. In contrast, protein content is higher in muscle of wild wreckfish than in reared fish and some differences were also observed in the fatty acid profile with higher values of PUFA and n-3 PUFA in wild than in reared wreckfish. DHA values represent 11% in reared fish and 26% in wild fish. Results from wild fish were very useful to formulate specific dry food for wreckfish broodstock.
2. First results of fatty acid profile of wreckfish larvae show that PUFA, SAFA and MUFA content (% of total fatty acids) have a little variation in the first 10 days of life.
3. Regarding wreckfish broodstock feeding regimes, results obtained from first experiments showed that most of commercial dry food has too much fat for wreckfish. First results with dry food 1

demonstrated that it should be increased the amount of proteins and decreased the level of fat. Furthermore, dry food for wreckfish must contain a big amount of n-3 PUFA and the EPA/ARA ratio must be around 1.5 similar to that obtained previously in wild wreckfish.

4. A clear relationship between fatty acid profile of oocytes and broodstock diet was found. Samples of oocytes were obtained from females fed with semimoist diet and dry food. Furthermore, some differences were observed in fatty acid profile of oocytes from females of different wreckfish broodstock showing that there is a relationship between fatty acid content and oocytes development.

Summary of work reported in the previous Reporting Period (31-48 Mo):

Summarizing the results of the period June 2016-December 2017 (3rd Period Report)

1. Enrichment products for living prey (rotifers and *Artemia*) were designed. Two levels of ARA content were used for enrichment product for rotifer and one level of ARA for *Artemia* and the effect of the enrichment of the new enrichment products on the biochemical composition of rotifers and *Artemia* was evaluated.
2. First data of fatty acid profile were obtained from 1dph until 26 dph larvae to complete the data obtained previously until 10dph.
3. A clear relationship between fatty acid profile of broodstock diet (semi-moisture, dry food and a mixture of hake and squid) and fatty acid profile of oocytes and eggs from females fed with the different diet was found.
4. Results obtained with dry food 2 demonstrated that the wreckfish diet must contain a big amount of proteins, low level of lipids, a high amount of n-3 PUFA and the EPA/ARA ratio must be similar to that obtained in wild females gonads (about 1-1.5). Nevertheless, the diet with a mixture of hake/squid (half and a half) seems to be a diet with good quality because of the protein content and the big amount of n-3 PUFA (EPA and DHA) although the EPA/ARA obtained in oocytes and eggs from females fed with this diet is high comparing with the one obtained in wild female gonads.
5. First data of fatty acid profile of sperm from wreckfish males of different broodstock were obtained.

Summary of progress toward objectives (49-60 Mo):

1. New enrichment products for live prey (rotifers and *Artemia* were developed with two levels of ARA being the enrichment less effective in *Artemia* than in rotifers.
2. Larvae of wreckfish exhibit, in general, a good acceptance of the enriched live prey tested.
3. The large amount of DHA, EPA and ARA found in wreckfish eggs from different spawnings reflect the composition of broodstock feed supplied.
4. No differences in fatty acid composition of wreckfish larvae fed with the prey enriched with the two enrichment products were found at 11, 18 and 21 dph.
5. Fatty acid profile of wreckfish larvae along the larval development was obtained, showing big amounts of PUFA specially DHA, EPA and ARA.
6. The first results of larval culture are promising for wreckfish, but it is necessary to continue with the research about nutritional requirements and their impact on the growth, survival and larval quality.
7. Gonads from females of wild wreckfish have a high level of ARA (7-10 %TFA) and EPA/ARA ratio nearly 1.
8. Dry food specifically formulated for wreckfish broodstock must contain a big amount of proteins, low level of lipids, a high amount of n-3 PUFA and the EPA/ARA ratio must be similar to the one obtained in wild females gonads. It is more appropriate to the needs of the broodstock and it is associated with greater success in reproduction.
9. A relationship was found between broodstock diets and fecundity, number of spawnings of the females, etc.
10. Relative fecundity (nº of eggs/Kg of female) and number of spawns per female have been increasing in females fed with dry feed over the years, from 2015 to 2018.

Details for each Task

Task 12.1. Live preys and enrichments for wreckfish larvae. led by Fátima Linares (P19), Javier Roo (P2) & Marisol Izquierdo (P2). Partners involved: José Luis Rodríguez (P19), Blanca Álvarez-Blázquez (P8), Evaristo Pérez (P8), Gema Pazos (P19)

In 2018 two new enrichment products were formulated (**Table 12.1.1**): Control enrichment product (CE) and ARA enrichment product (AE) with a higher level of proteins and lipids, 46.7 and 29.9% respectively in CE and 43 and 31.8% in AE, than the levels of proteins and lipids of the enrichment products formulated in 2017. Polyunsaturates fatty acids (PUFA) had values between 62-63%, being the n-3PUFA 41% in both enrichment products and n-6 PUFA 19 and 22% in CE and AE respectively. ARA content was 2.1% in CE and 5.4% in AE. DHA/EPA and EPA/ARA ratios were 8.3 and 2 in CE and 7.2 and 0.9 in AE. The analysis (proteins, lipids and fatty acids) of live prey (rotifer and *Artemia*) enriched with the enrichment products formulated in 2018 are shown in **Table 12.1.2.**

Table 12.1.1. Proteins, lipids and fatty acids of Enrichment products

	Control Enrichment Product (CE)	ARA Enrichment Product (AE)
Proximate analysis (%dry matter)		
Proteins	46,73±0,18	43,00±0,16
Lipids	29,94±1,74	31,79±6,51
Fatty acids content (%TFA)		
14:0	1,08±0,02	0,96±0,13
16:0	24,66±2,70	20,92±1,36
17:0	0,42±0,39	0,72±0,00
18:0	2,08±0,17	2,41±0,21
Saturated (SAFAs)	29,73±2,94	26,01±1,95
16:1 n-9	0,23±0,00	0,19±0,00
16:1 n-7	1,79±0,02	1,75±0,21
18:1 n-9	2,85±0,66	4,84±1,16
18:1 n-7	0,69±0,18	1,07±0,05
20:1 n-9	0,72±0,28	0,49±0,02
Monoenoics (MUFAs)	7,37±0,54	10,41±2,29
18:2 n-6	5,49±0,18	5,26±0,36
20:4 n-6 (ARA)	2,13±0,10	5,44±0,21
20:5 n-3 (EPA)	4,33±0,53	4,75±0,02
22:6 n-3 (DHA)	35,84±4,04	34,19±1,58
Polyunsaturated (PUFAs)	62,48±4,69	62,59±2,27
Σn-3	41,26±6,47	41,37±1,21
Σn-6	19,14±0,32	21,95±1,23
n-3/n-6	2,24±0,26	1,89±0,16
DHA/EPA	8,30±0,06	7,20±0,37
EPA/ARA	2,03±0,16	0,87±0,04

HUFA, highly unsaturated fatty acid; ARA arachidonic acid; DHA docohexaenoic acid; EPA eicosapentaenoic acid

Table 12.1.2. Proteins, lipids and fatty acids of live prey enriched with the enrichment products (2018)

	Without Enrichment Rotifer (NoERot)	Control Enriched Rotifer (CERot)	ARA Enriched Rotifer (AERot)	Without Enrichment Artemia (NoEart)	ARA Enriched Artemia (AEArt)
Proximate analysis (% dry matter)					
Proteins	62,33±1,11	60,45±0,58	59,41±2,79	61,64±0,41	69,48±1,96
Lipids	12,43±0,18	15,53±0,72	17,36±3,66	18,89±3,05	19,72±2,74
Fatty acids content (% TFA)					
14:0	1,96±0,04	1,17±0,01	1,17±0,06	1,15±0,00	0,71±0,02
16:0	7,89±0,10	10,78±0,03	10,41±0,10	12,08±0,04	11,41±0,00
17:0	0,87±0,03	0,66±0,01	0,67±0,01	1,51±0,03	1,61±0,04
18:0	6,34±0,08	3,44±0,02	3,82±0,22	4,54±0,00	6,82±0,05
Saturated (SAFAs)	17,88±0,26	16,83±0,05	16,80±0,31	19,93±0,00	21,06±0,07
16:1 n-9	1,16±0,01	0,68±0,02	0,64±0,02	1,04±0,01	0,70±0,01
16:1 n-7	20,64±0,15	10,14±0,04	10,30±0,10	11,00±0,03	8,09±0,12
18:1 n-9	28,48±0,21	14,14±0,22	15,68±0,05	18,18±0,03	17,64±0,03
18:1 n-7	4,34±0,02	2,42±0,03	2,47±0,04	10,73±0,04	11,54±0,00
20:1 n-9	3,97±0,03	2,15±0,00	2,20±0,02	0,65±0,05	0,83±0,05
Monoenoics (MUFAs)	64,91±0,25	32,94±0,28	34,77±0,33	45,58±0,09	42,31±0,03
18:2 n-6	6,80±0,01	6,36±0,05	6,26±0,03	8,23±0,01	7,66±0,06
20:4 n-6 (ARA)	1,08±0,04	1,79±0,02	4,04±0,03	2,48±0,01	4,82±0,02
20:5 n-3 (EPA)	1,08±0,04	4,06±0,02	3,74±0,01	9,88±0,00	10,93±0,02
22:6 n-3 (DHA)	0,71±0,01	26,71±0,16	23,97±0,23	0,06±0,00	3,38±0,04
Polyunsaturated (PUFAs)	17,49±0,14	50,60±0,24	48,81±0,35	34,56±0,09	36,78±0,06
Σn-3	5,40±0,09	33,78±0,20	30,52±0,25	22,48±0,10	22,11±0,02
Σn-6	8,87±0,04	14,83±0,05	16,23±0,11	11,22±0,01	13,99±0,01
n-3/n-6	0,61±0,01	2,28±0,01	1,88±0,00	2,00±0,01	1,58±0,00
DHA/EPA	0,66±0,02	6,57±0,04	6,42±0,05	0,01±0,00	0,31±0,00
EPA/ARA	1,00±0,03	2,27±0,03	0,92±0,01	3,99±0,02	2,27±0,01

HUFA, highly unsaturated fatty acid; ARA arachidonic acid; DHA docohexaenoic acid; EPA eicosapentaenoic acid

The level of proteins in rotifer vary between 62.3% (DW) in no enriched rotifers (NoERot), 60.4 % in rotifer enriched with Control enrichment (CERot) and 59.4% in rotifers enriched with ARA enrichment (AERot). The lipid content was 12.4% (DW) in NoERot, 15.5% in CERot and 17.4% in AERot.

The level of PUFA was much lower in the rotifer without enrichment (17.5%TFA) than in rotifer enriched with values of 50.6% when the rotifer was enriched with CE and 48.8% when the rotifer was enriched with AE; n-3 PUFA level increased from 5.4% in NoERot to 33.8 and 30.5% in CERot and AERot respectively and n-6 PUFA from 8.9% TFA in NoERot to 14.8% and 16.2% in CERot and AERot. ARA, EPA and DHA were 1.1, 1.1 and 0.7 % TFA respectively in NoERot; 1.8, 4.1 and 26.7% in CERot and 4, 3.7 and 24% in AERot. DHA/EPA was lower in NoERot (0.7) than in CERot (6.6) and AERot (6.4), EPA/ARA ratio was 1 in NoERot, 2.3 in CERot and 0.9 in AERot.

On the other hand, *Artemia* without enrichment (NoEArt) had 61.6 % (DW) of proteins and 18.9 % of lipids and *Artemia* enriched with ARA enrichment product (AEArt) had 69.5% of proteins and 19.7% of lipids. PUFA content reached the 34.6 and 36.8% of total fatty acids in NoEArt and AEArt. n-3 PUFA represent the 22% of TFA in both types of *Artemia*, no enriched and enriched and n-6 were 11.2 and 14% of TFA in NoEart and AEArt respectively. ARA, EPA and DHA levels were 2.5, 9.9 and 0.1 in NoEArt and 4.8, 10.9 and 3.4 % TFA in AEArt. DHA/EPA and EPA/ARA were 0.01 and 0.3 and 4 and 2.3 respectively in NoEArt and AEArt.

***Nutrition experiments of wreckfish larvae* (2018)**

Three different experiments of larval feeding were carried out in 2018:

1. *Effect of two different enriched live prey on larvae fatty acid composition*

The larval culture was performed at IEO facilities with larvae from one IEO broodstock spawning (24/18).

2. *Fatty acid composition of larvae from different spawnings.*

Larvae from five different spawnings, four from IEO broodstock (14/18, 15/18, 24/18, 30/18) and another one from AF were analysed. All of them were fed with the same feeding (rotifer and *Artemia* enriched with ARA enrichment product).

3. *Evolution of fatty acid profile of eggs and wreckfish larvae through the larval development (until 58 dph)*

Some samples of eggs and larvae from a spawning of IEO (30/18) and cultured at IGAFA facilities from 1 dph to 58 dph were taken out to be analysed at CIMA facilities.

The feeding sequence for each batch of larvae is shown in **Table 12.1.3.**

Table 12.1.3. Feeding sequence of larvae.

Spawns	0-5 dph	5-10 dph	10-15 dph	15-20 dph	20-25 dph	25-30 dph	30-40 dph	From 40- dph onwards
14/18 IEO	No feeding	No feeding	11 dph start feed Rot ARA	Rot ARA + Ao	Rot ARA+Ao End 24 dph			
15/18 IEO	No feeding	No feeding	13 dph start feed Rot ARA	Rot ARA + Ao	Rot ARA+Ao	Rot ARA+Ao End 28 dph		
24/18 IEO	No feeding	9 dph start feeding: Rot Control and Rot ARA	Rot Control and Rot ARA 12 dph Ao	Ao 17dph A_t enriched control and ARA	A_t enriched control and ARA	A_t enriched control and ARA End 21 dph		
30/18 IEO	No feeding	9 dph start feeding Rot ARA	Rot ARA	Rot ARA (15-17dph), Ao(15-18dph), A_t(18 dph)	A_t enriched ARA	A_t enriched ARA	A_t enriched ARA	A_t+ dry food 48dph
AF	No feeding	8 dph start feeding Rot ARA	Rot ARA	Rot ARA (15-19dph), Ao(18-20dph),	A_t(23 dph)	A_t	A_t	A_t+ dry food 48dph

Effect of different enriched live prey on larvae fatty acid composition

Results of larvae fatty acid profile (%TFA) of wreckfish larvae from one IEO spawning (24/18) at different days of life: 11, 18 and 21 dph fed with live prey enriched with two different enrichments are shown in **Figures 12.1.1 and 12.1.2.**

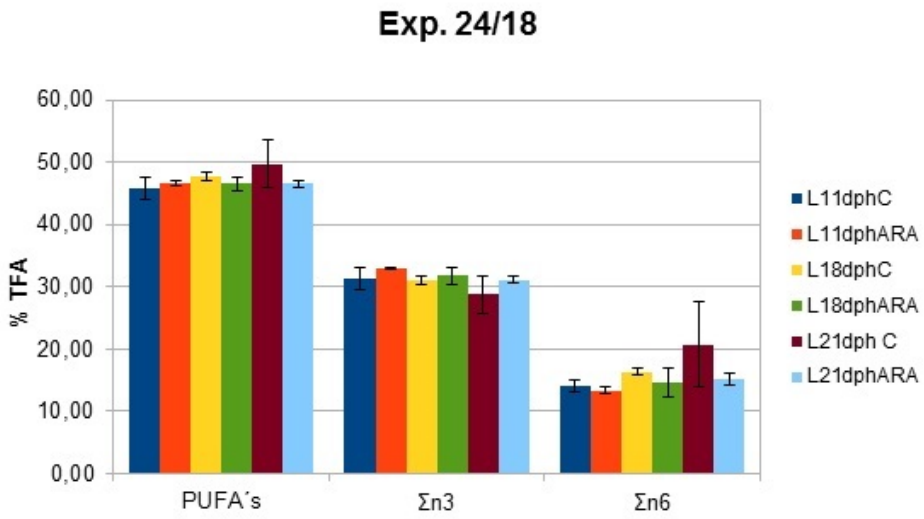


Figure 12.1.1. Fatty acid content (%TFA) in larvae at 11 dph, 18 dph and 21 dph fed with two different enriched live prey.

No differences were found between larvae fed with rotifer and *Artemia* enriched with the two different enrichments (Control and ARA) at 11dph, 18dph and 21dph. PUFA content varies between 46-50%, n-3 29-33%, n-6 14-17%. EPA, DHA and ARA content vary 5-5.5, 21-24 and 6-6.5%. The differences found in n-6 PUFA at 21 dph between larvae with ARA enrichment and Control are due to the high variability found in LNA (18:2n-6) content in larvae fed with ARA enriched *Artemia* and they are not significant.

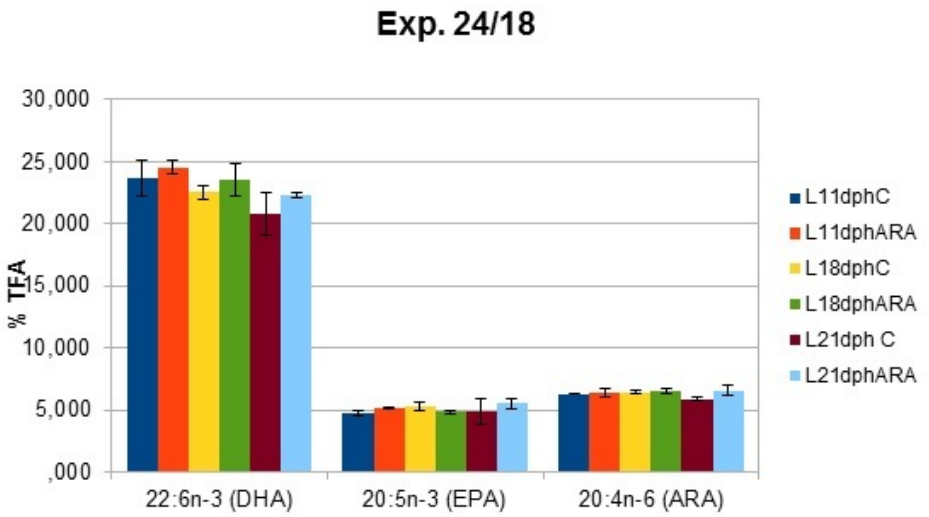


Figure 12.1.2. Fatty acid content (%TFA) in larvae at 11 dph, 18 dph and 21 dph fed with two different enriched live prey.

Fatty acid composition of larvae from different spawnings

Analysis of fatty acids from eggs and larvae from different spawnings at different days of life were performed. Live preys (rotifer and *Artemia*) enriched with the same enrichment product were used to feed the larvae.

Total lipids reached 15-19% (DW) in eggs without significant differences between the different spawnings. No significant differences were found in eggs PUFA content between spawnings with values of 43-45%TFA and similar values were found in 1 to 25 dph larvae. DHA vary between 20-25% TFA with only little differences between spawnings in eggs and 1-10, 10-15, 15-20 and 20-25 dph larvae, with the exception of AF larvae which present a higher variation. No differences were found in EPA and ARA in eggs and larvae from different spawnings varying between 4-7% and 6% respectively and only and increase in EPA value (10%) in 15-20 dph and a decrease of 3% in ARA in 10-15 dph larvae AF larvae were found. DHA/EPA and EPA/ARA ratios have similar values of 3-6 and 0.6-1.1 respectively in larvae from the different spawnings, only AF larvae show differences in both ratios at 10-15 and 15-20 dph that reflect the values found in EPA and ARA.

Evolution of fatty acid profile through larval development of wreckfish larvae

For this experiment, all the samples of eggs and larvae came from 30/18 spawning. The evolution of the size along the larval development from 5 to 65 days of life shows that larvae at 5 dph has a size of 6 mm and only a little increase was observed when reaching 22 dph (6.4 mm). Then, larvae show a higher increase in size having 7.75 mm at 39 dph, 11.16 mm at 50 dph and 12.56, 12.48 and 12.77 mm were reached at 50, 61 and 65 dph respectively.

The evolution of fatty acid profile along the larval development in wreckfish shows that SAFA were maintained in levels of 19-23%TFA up to 32 dph and an increase was found in larvae of 56-58dph (29%) and, on the contrary, MUFA was 37% in eggs and 1dph larvae, 31-35% in larvae from 12 to 32 dph and the lowest values (24-25%) were found at 56-58 dph. The level of PUFA was maintained between 43-47% TFA along the culture (1-58 dph), ARA represents around 6% up to 32 dph and a slight increase (7.5-8) was observed at 56-58 dph. The highest values of EPA were obtained from 32 dph larvae (8.9%TFA) and DHA is higher in larvae from 1 to 12 dph (24-25%). DHA/EPA ratio is higher at 12 and 18 dph larvae (6) than in eggs and 1dph larvae (4) and the lowest values were obtained at 32-58 dph (2-3). EPA/ARA ratio has the lowest values (0.7) at 12-18 dph and 1 in the rest of larvae

The full description of the work and results is provided in Deliverable ***D12.1*** "***Effect of live prey enrichment products on wreckfish larval performance"***.

Task 12.2. Influence of broodstock feeding regimes for fecundity and spawn quality. [led by Fátima Linares (P19), Blanca Álvarez (P8) & Marisol Izquierdo (P2)].

Partners involved: Fátima Linares (P19), Blanca Álvarez-Blázquez (P8), José Luis Rodríguez (P19), Evaristo Pérez (P8), Gema Pazos (P19), Antonio Vilar (P32) & Marisol Izquierdo (P2)

Regarding wreckfish broodstock, the results obtained so far demonstrated that most of commercial dry food has too much fat for wreckfish broodstock, and a new dry food (Dry food 2) was specifically formulated for wreckfish with a level of fat much lower than in commercial food and containing a large amount of n-3 PUFA and the EPA/ARA ratio must be around 1.5, similar to the one obtained previously in wild fish.

The different diets supplied to wreckfish broodstock from the beginning of the project are shown in **Table 12.2.1.** Based on the results obtained with Dry food 2, some little modifications were made in the dry food by Sparos to get a more commercial feed, Dry food 3 (**Table 12.2.2**) which was supplied to IEO broodstock in September 2018 and at the end of this year to the rest of wreckfish broodstock being very well accepted by wreckfish.

Table 12.2.1. Type of food used for wreckfish broodstocks

	2014	2015	2016	2017	2018
Stock IEO Tank S1 (n = 5)	Semimoist diet	Semimoist diet	Semimoist diet	Semimoist diet	Semimoist diet and Dry food 3
Stock IEO Tank S2 (n = 6)	Semimoist diet	Dry food 1	Dry food 2	Dry food 2	Dry food 2 and 3
Stock IGAFA (n = 10)	Vitalis Repro/Vitalis Cal	Squid	Squid	Hake/Squid	Hake/Squid and Dry food 3
Stock AF (n =17)	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M and Dry food 2 and 3

Table 12.2.2. Composition of Dry food 3

Dry Food 3	
Composition	
Crude protein (%)	60
Crude fat (%)	13
Crude ash (%)	9
Total phosphorus (%)	1.2
Calcium (%)	1.7
Sodium (%)	0.6
Tau (%feed)	0,9
Se (mg/Kg)	2,5
Vit A (IU/Kg)	40248
Vit C (mg/Kg)	2000
Vit E (mg/kg)	650
Vit D (IU/Kg)	2902
Astaxanthin (mg/Kg)	50
Composition (% feed)	
C14	0.55
C16	2.10
C18	0.44
C18:1n9	1.65
LNA (C18:2n6)	1.08
ALA (C18:3n3)	0.18
ARA	0.51
EPA	0.72
DHA	1.66
EPA+DHA	2.38
ARA/EPA	0.70
Total phospholipids	2.40

Data were supplied by Sparos

The composition of the different diets and their effect on the composition of oocytes and eggs from females fed with different diets has been described in the previous reporting periods of the work.

Fecundity of females and diet

Relative fecundity of females (nºeggs/Kg female) fed with semi-moist diet, dry food and hake/squid were recorded from 2015 to 2018.

The relative fecundity (nºeggs/Kg female) from nine females were recorded, one from tank S1 (♀9703-IEO) in 2015 and 2018 fed with semimoist diet, four from tank S2 (♀7938, 9821, 9837, 9931-IEO) in 2016, 2017 and 2018 fed with dry food and four from IGAFA broodstock (♀3FF2, 7B19, 6D01, 499A-IGAFA) during 2016, 2017 and 2018 fed with hake/squid (**Fig.12.2.1**)

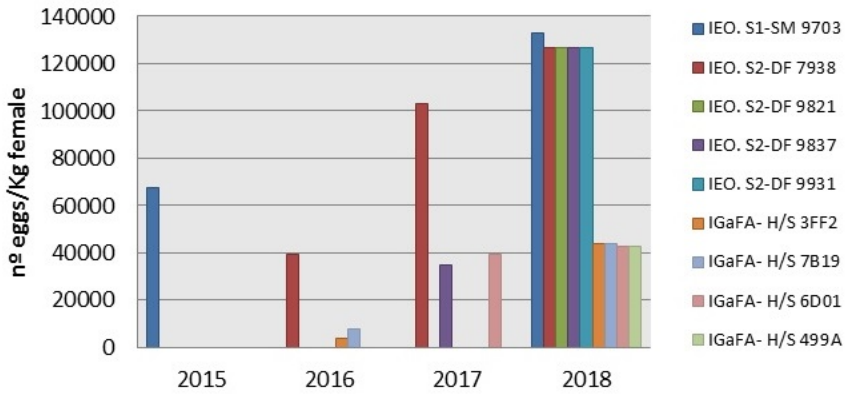


Figure 12.2.1. Relative fecundity (nº of eggs/kg of female) from 2015 to 2018

The number of spawns was also recorded from 2015-2018 in females from the IEO and IGAFA broodstock and fed with different diets (**Fig.12.2.2**).

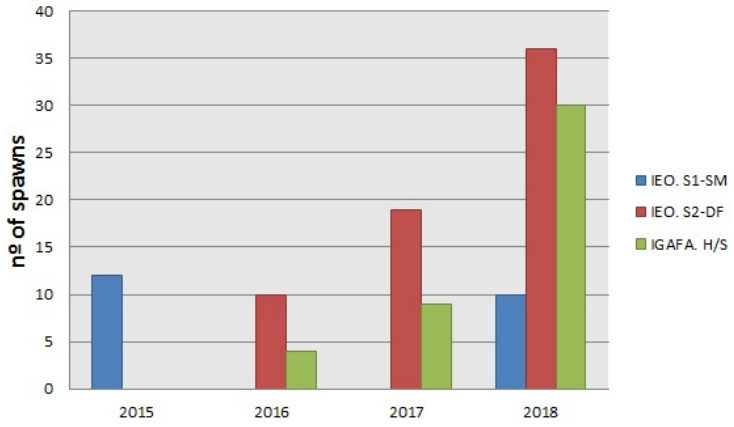


Figure 12.2.2. Number of spawns obtained from 2015 to 2018

The highest relative fecundity was observed in females fed with dry food (tank S2-IEO) with an average value of 126627.36 eggs/kg of female in 2018 with the exception of only one female from tank S1of EO broodstock fed with semi-moist diet with a value of relative fecundity of 133178.57 eggs/kg of female. Females from IGAFA broodstock had and average fecundity of 43530,06 eggs/kg of female in 2018 fed with hake/squid (half and a half). In all the females from IEO and IGAFA broodstocks the fecundity have been increasing for over the years.

With respect to the number of spawns, they were increasing too for over the years in both, IEO and IGAFA broodstocks. With respect to IEO broodstock, females fed with dry food had 10 spawns in 2016 from only one female, 19 spawns in 2017 from two females and 36 in 2018 from 4 females. In the case of females from this broodstock fed with semi-moist diet, only one female spawns in 2015, no spawns were obtained in 2016 and 2017 and 10 spawns were obtained in 2018. Regarding females from IGAFA broodstock fed with hake/squid, the spawns started in 2016 from only one female (4 spawns) and they were increasing in 2017 (9 spawns) and 2018 with 30 spawns from 4 females.

Even though these results about female fecundity and number of spawns are preliminary and it is necessary to check them with data from more females, the results obtained in 2017 and 2018 suggest an influence of the broodstock diet on fecundity of females.

The full description of the work and results is provided in Deliverable ***D.12.2 "Recommendations for wreckfish broodstocks feeds".***

Deviations from Annex I and their impact:

Task 12.1. The influence of different enrichment products for live food on wreckfish larvae was tested in this period because the batches of wreckfish larvae obtained were sufficient to perform the experiments.

There were no deviations in Task 12.2.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Pérez Rial, E., Linares, F., Rodríguez Villanueva, J.L., Vilar, A., Mylonas, C.C., Fakriadis, Y., Papadaky, M., Papandroulakis, N., Duncan, N., Robles, R., Lluch, N., Pazos, G., Méndez, B., Sigelaki, I., Gómez, C., Pérez, M. and Álvarez-Blázquez, B. 2019. Wreckfish (*Polyprion americanus*). New knowledge about reproduction, larval culture and nutrition. Promise as a new species for aquaculture. Fishes IP. (Submitted))

WP 13 Nutrition – grey mullet

WP No:	13	**WP Lead beneficiary:**		P4. 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):	Bill Koven			
Other Scientists participating:	Hanna Rosenfeld (P4), Iris Meiri-Ashkenazi, Aldo Corriero (P13), Rocio Robles (P18)			

Objectives

1. Improve enrichment products, weaning, grow out and broodstock diets,
2. Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.

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

Studies on the taurine requirement at different stages of development during the larval rearing of grey mullet showed a significant ($P<0.05$) effect of dietary taurine on larval growth and survival. This effect is strongest during rotifer feeding compared to *Artemia* feeding which also significantly ($P<0.05$) influences growth in later stages of larval development. Nevertheless, the results indicated that larvae fed both high taurine enriched rotifers and *Artemia* survived and grew (length) significantly better and these protocols have been recommended for larval rearing.

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

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. As un-enriched *Artemia* have considerable levels of taurine, there appears to be no added benefit of feeding taurine enriched *Artemia* on larval weight. However, fish that had fed on the high taurine rotifers or both high taurine rotifers and Artemia were markedly ($P<0.05$) longer than fish in the rest of the treatments. In fact, larvae that were fed the high taurine diets from 2-19 dph survived significantly ($P<0.05$) better than the rest of the treatments.

Dietary taurine during rotifer feeding (4.4 and 6.4 mg taurine g -1 DW) had a prolonged effect at 44 dph, which was weeks after rotifer feeding had ceased. Consequently, 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 m3 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 ($P<0.05$).

Summary of work reported in the previous Reporting Period (31-48 Mo):

In **Sub-task 13.1.1** the effect of dietary DHA on larval performance was investigated. This study found no dietary DHA effect on larval performance, in terms of wet weight gain and rotifer consumption rate. This suggests that 5.5% DHA in the commercial enrichment "Red Pepper" is sufficient for the growing of this species, although DHA levels below this weren't tested. Interestingly, the 5.5% DHA DW diet level resulted in significantly improved larval survival over the other higher DHA treatments at 40 dph or 15 days after the rotifer treatments had ceased. This emphasizes the importance of feeding an effective level of DHA at the rotifer stage on survival in later development stages. In **Sub-task 13.2.1** preliminary results suggested that the hepatic CSD pathway for taurine synthesis, in the absence of dietary taurine, is still active but that the expression of this key gene increases with increased levels of dietary taurine until 1% where its expression drops at the high dietary taurine level of 2%. Similarly, the gene for ADO, which is a key enzyme in another less dominant pathway in taurine synthesis, was highly expressed in the 1 % taurine fish livers and then dropped in fish feeding on the 2% taurine diet. Preliminary findings in **Sub-task 13.2.2** also show a taurine dose dependent response on the gene expression of hepatic CYP7A1 in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of this enzyme in fish consuming the highest dietary taurine level (2% DW diet). In **Sub-task 13.3.1** the effect of dietary DHA on juvenile mullet wet weight gain found no treatment effect on growth and that variability in weight gain was mostly attributed to age (dph). In addition, all treatment fish exhibited generally similar size distribution in their respective populations and as well as excellent survival (92.4, 88.8 and 97.6% in the 0.4, 0.8 and 1.2% DHA DW diets, respectively). In **Sub-task 13.3.2** the effect of four levels of Tau supplementation on best performing DHA treatment using non-fish meal grow out diet from 13.3.1. The results showed that grey mullet juveniles have a minimum 0.5% taurine requirement**,** which is within the range of taurine requirements measured in a variety of marine teleosts. However, there was no taurine effect on size distribution. In **Sub-task 13.4.1** Notable differences in the fatty acid profiles when comparing the gonads from wild and domestic adult grey mullet broodstock were found in saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids of the n-3 and n-6 groups. Differences were particularly marked in EPA in both sexes and DHA in wild males compared to captive cohorts. This was primary rational to investigate the effect of replacing soybean oil (VO) with fish oil (FO) in mullet broodstock diets. The percent hatching of eggs from the FO broodstock was significantly ($P<0.05$) higher than eggs from the VO broodstock, while survival in larvae in the two treatments at the end of 0 dph were not significantly different ($P>0.05$) from each other, although the FO was higher. Larvae from the FO broodstock, regardless of salinity exposure, demonstrated 100% swim bladder inflation by 5 dph, where there was no swim bladder inflation in fish from the VO broodstock during the course of the food deprivation study. In **Sub-task 13.4.2** adult wild grey mullet showed large ripe ovaries with late vitellogenic (Vgs) oocytes as the most advanced oocyte stage while hatchery-produced grey mullets showed extensive alpha and beta atresia of vitellogenic follicles, a sign of cessation of the reproductive activity. The highest relative levels of Vgs mRNA were observed in adult wild specimens, which are indicative of a residual Vtg transcription activity in specimens at the end of vitellogenesis. The domestic mullet brooders showed very low Vgs expression levels corresponding to a negligible Vgs transcription activity agreeing with spent condition (extensive atresia of vitellogenic follicles).

Summary of progress towards objectives (49-60 Mo):

The **sub-task 13.1.1** was reported in the 3rd periodic report and was submitted during the 4th reporting period as Deliverable **D13.1.** *Determine changes in the essential fatty acid requirement (DHA) as a function of developmental stage and ambient salinity in grey mullet.* This deliverable suggested that dietary levels of DHA can be decreased when feeding older juvenile mullet grown in captivity, provided that the salinity is reduced to levels found in estuarine waters. This would translate as a significant savings for farmers as the purchase of feed for the grow-out of fish to market weight can represent more than 60% of production expenses and DHA is costly as a feed additive. The **sub-tasks 13.2.1 and 13.2.2** were reported in the 3rd periodic report and were submitted during the 4th reporting period as deliverable **D13.2** *Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet.* The results suggest that the CSD pathway is active as the main taurine synthesizing pathway and that the expression of this key gene increases with increased levels of dietary taurine until 1% where CSD expression drops at the 2% dietary taurine level. On the other hand, there was no clear effect of dietary taurine on CYP7α1 gene expression and bile salt synthesis. The **subtasks 13.4.1 and 13.4.2** were reported in the 3rd periodic report and submitted during the 4th reporting period as deliverable **D13.3** *Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression accumulation.* This deliverable found, in terms of fatty acids and fatty acid groups, no conspicuous differences, independent of age, between the fish oil (FO) fed wild and captive brood stock and soybean oil (SO) fed female brood stock gonads. Nevertheless, when comparing the fatty acid and lipid class profiles between female and male gonads, there were highly marked differences. Higher contents of total lipids, triacylglycerols and wax and sterol esters in female gonads compared to male gonads while there were higher quantities of phospholipids in male gonads compared to female gonads. The results suggest that another FO component, possibly carotenoids, are responsible for the observed benefit of the broodstock fish oil diet on larval performance. The **subtasks 13.3.1 and 13.3.5** were partially reported in the 3rd periodic report and were submitted during the 4th reporting period as the deliverable **D13.4 4** *Determine the effects of essential fatty acids and Tau in non-fish meal feeds on flesh and bottarga quality in grey mullet.* This study concluded that (a) the rearing conditions established at **P4.IOLR** allowed a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea and (b) hatchery-produced grey mullet have a good potential to develop ovaries spontaneously up to a condition useful for bottarga production (advanced vitellogenesis). The supplementation of carotenoids to brood stock diets not only appears to improve larval performance (**D13.3**) but also contributes to bottarga coloration and a platform for further dietary and bottarga product development. The **task 13.3.5** *Comparison of vegetable oil-no fish meal grow out diet with a n-HUFA rich fish meal finishing diet on the nutritional and organoleptic values of fish flesh and bottarga quality.* In this task a commercial carp diet and the Irida (**P4.IOLR**) were compared in terms of fish performance and organoleptic values. Poor survival and consumption of both diets were affected by the poor adaptability to captive conditions and the RAS system leading to similar growth in fish fed the two treatments. Fish feeding on the commercial carp diet exhibited green dark spots on the surface of the liver where coloration of the liver in mullet fed the Irida diet was normal. On the other hand, filleting yield of all the reared fish from both diets was very good and indicated the potential of this species for processed products such as smoked fish fillet or filet preserved in olive oil. There were no differences in sensory parameters such as odour, flavour and texture between the commercial carp feed and the Irida diet. The **subtasks 13.3.3** are reported in the 4th periodic report as deliverable **D13.5** *Comparing the effect of two types of potential soybean meals to be used in the IOLR grow out diet for grey mullet on growth, intestinal morphology and inflammation, peroxidation and antioxidant mechanisms and intestinal pathology.* This deliverable found a significant improvement in the performance of fish fed the control-poultry meal diet, in terms of growth, digestive tract length and weight. However, this change was not large nor due to triggering an inflammation response in the all-plant based diets, which did not exhibit shortened mucosal folds or thickening of the lamina propria and sub-epithelial mucosa. In fact, digestive tract samples from all fish examined in all treatments exhibited healthy tissue with no signs of disease and presumably no oxidation stress. Further studies should determine if the performance of all-plant based meal diets can be further improved to match the poultry meal diet by the supplementation of essential amino acids such as methionine and the sulfonic amino acid, taurine.

Details for each Task

Sub-task 13.1.1 Improvement of larval performance through adequate first feeding regimes. This subtask was reported in the 3rd periodic report and was submitted during the 4th reporting period as the Deliverable D13.1 *Determine changes in the essential fatty acid requirement (DHA) as a function of developmental stage and ambient salinity in grey mullet* **and is presented here in brief.**

The aims of this study were (1) to determine the effect of salinity and DHA on grey mullet juvenile growth. (2) To characterize sodium potassium ATPase pump activity in gill epithelium. (3) To quantify expression of genes related to DHA synthesis. (4) To establish the relationship between the expression of these genes and environmental salinity.

Grey mullet eggs were stocked (100 eggs l^{-1}) in eighteen 1.5 m^3 tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25 ^oC entered the bottom of the tanks and exited near the top through a 500 µm filter. The salinity was incrementally reduced to 25 ‰ and the larvae reared according to the IOLR protocol until 35 dph. At this age, the tanks were divided into two groups where the salinity in each group of 9 tanks was gradually adjusted to 15 or 40 ‰. In each salinity group low, medium and high *Artemia* DHA treatment levels (1.7, 6.6 and 12.2 mg DHA g^{-1} DW) were tested on 35-59 dph fish in replicates of three tanks per treatment. On 59 dph, samples of fish were dried and weighed, gills removed for Na^+/K^+ATPase determination and RNA extracted for gene expression of selected genes. The fish, in each salinity group, were then grown from 60 to 89 dph and continued to be fed pellets that tested low, medium and high dietary DHA levels (7.1, 9.8, 13.5 mg DHA/g DW). At 89 dph, samples of fish were dried and weighed, gills removed for Na^+/K^+ATPase determination and RNA extracted from the liver for gene expression of selected genes.

In **Fig. 13.1a** 59 dph fish demonstrated that whole body DHA correlated with prey DHA level in a dose dependent manner independent of salinity. On the other hand in older 89 dph fish (**Fig. 13.1b**), whole body DHA was dependent on rearing salinity. Fish reared at 15 ‰ showed no correlation between whole body and prey DHA level while fish grown in 40 ‰ seawater continued to show tissue DHA levels that corresponded with levels of this long chain polyunsaturated fatty acid (LCPUFA) in the diet. These results are consistent with those in **Fig. 13.2**, which demonstrated that there was no dietary DHA dose dependent effect on growth in 89 dph fish grown in the lower salinity while there was a dietary DHA dose dependent effect on growth in fish grown in 40 ‰. There was a marked ($P<0.05$) salinity effect on Δ6 desaturase gene expression, which was highest in fish reared at 15 ‰ fed the lowest DHA (**Fig. 13.3**). Conversely, there was no dietary DHA effect on the expression of this gene independent of salinity. On the other hand, in **Fig. 13.4**, there was a significant ($P<0.05$) inverse effect of dietary DHA on the relative gene expression of elongase in the low salinity fish. In fact, the highest ($P<0.05$) expression of this gene in both salinity treatments was in fish fed the low dietary DHA treatment reared in 15 ‰. Fish reared in 40‰ seawater demonstrated no clear effect of dietary DHA on elongase gene expression.

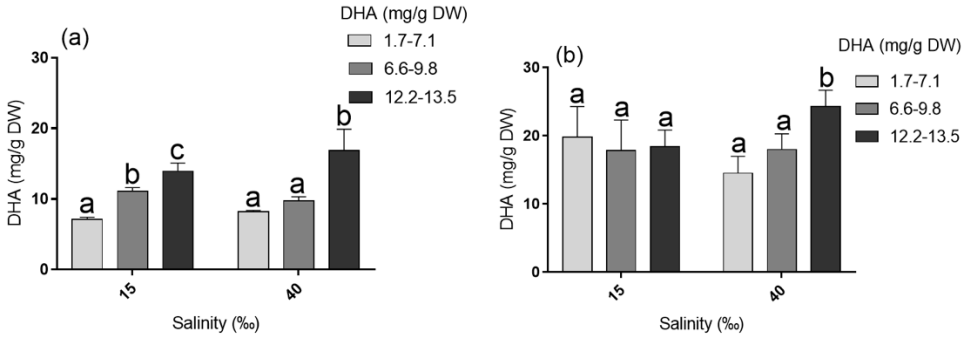


Figure 13.1. Effect of (a) *Artemia* DHA (1.7, 6.6 and 12.2 mg DHA g^{-1} DW) on 58 dph fish DHA levels (mg DHA g^{-1} DW) reared at 15 and 40 ‰ and (b) pelleted diet DHA (7.1, 9.8, 13.5 mg DHA g^{-1} DW) on 89 dph fish DHA levels (mg DHA g^{-1} DW) reared at 15 and 40 ‰. DHA values within a salinity group having different letters were significantly (P<0.05) different.

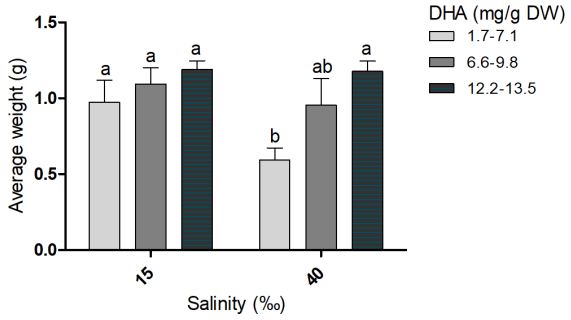


Figure 13.2. The effect of *Artemia* and pelleted diet different DHA levels on average weight in 89 dph fish reared at 15 and 40 ‰. Average weight values having different letters within a salinity group were significantly (P<0.05) different.

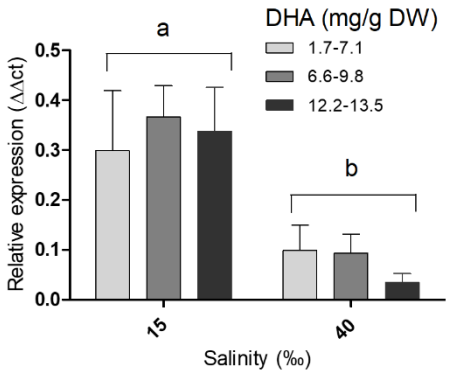


Figure 13.3. The combined effect of dietary treatments in each of the salinity treatments on the relative gene expression of Δ6 desaturase. Combined values of each of the salinity treatments having different letters were significantly (P<0.05) different.

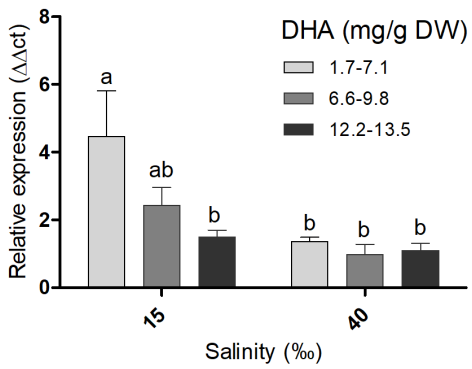


Figure 13.4. The effect of *Artemia* and pelleted diet different DHA levels on relative gene expression of elongase in 89 dph fish reared at 15 and 40 ‰. Average weight values having different letters within a salinity group were significantly (P<0.05) different.

Discussion

There was a dietary DHA dose dependent effect on fish whole body DHA in 59 dph fish reared in low and high salinity. However, this changed in older 89 dph fish, where there was no dietary DHA dose dependent effect on tissue DHA and growth in fish reared at 15 ‰ while the DHA dietary level did significantly (P<0.05) modulate fish body DHA and weight gain in cohorts grown at the higher salinity of 40 ‰. This broadly hints that older (89 dph) mullet grown in low salinity are adopting the fresh water model of LCPUFA biosynthesis and have the capability to produce DHA from shorter carbon chain precursors. This suggests that the DHA requirement would be less in fish exposed to 15 ‰ and/or that these individuals also have the ability to satisfy their requirement for this LCPUFA through biosynthesis. This is suggested by the fact that both biosynthetic enzymes; Δ6 desaturase and elongase were markedly (P<0.05) activated by the low salinity treatment. This follows as mullet individuals in nature would be moving to the lower salinity waters of river mouths and estuaries, which are characterized by an environment less rich in LCPUFA and more abundant in smaller chain PUFA precursors. Low salinity upregulated the gene expression of the rate limiting enzyme of LCPUFA biosynthesis; Δ6 desaturase but was independent of DHA dietary level. On the other hand, both low salinity and DHA level upregulated the gene expression of elongase.

Broadly speaking, these results suggest that dietary levels of DHA can be decreased when feeding older juvenile mullet grown in captivity, provided that the salinity is reduced to levels found in estuarine waters. This would translate as a significant savings for farmers as the purchase of feed for the grow-out of fish to market weight can represent more than 60% of production expenses and DHA is costly as a feed additive.

Sub-task 13.2.1 (IOLR) Determine expression of Tau rate limiting enzyme; cysteine sulfinate decarboxylase (CSD) at various stages (larval and grow out) and **Sub-task 13.2.2 (IOLR) Determine expression of rate limiting enzyme of bile salt synthesis, cholesterol 7α-hydroxylase (CYP7A1) at various stages (larval and grow-out)**

These subtasks were reported in the 3rd periodic report and were submitted during the 4th reporting period as the ***Deliverable D13.2 Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet*** and is presented here in brief.

The β-amino sulfonic acid taurine, which is not incorporated into proteins, plays an array of critical roles in its free form. These include involvement in bile salt synthesis, anti-oxidative defence, cellular osmoregulation, as well as contributing to visual, neural and muscular function. Taurine cannot be synthesized in carnivorous teleosts and therefore must be provided in the diet. However, it is unclear if herbivorous and omnivorous fish, where vegetation is a major component of their diet and characteristically deficient in taurine or contains low levels of this nutrient, have taurine synthesis capability. Taurine is mainly synthesized either from the oxidation of cysteine via cysteine dioxygenase (CDO), which generates cysteine sulfinate that is decarboxylated by cysteine sulfinic acid decarboxylase (CSD), or less commonly from the oxidation of cysteamine by cysteamine (2-aminoethanethiol) dioxygenase (ADO). Both pathways generate hypotaurine, which is oxidized to taurine. Studies carried out on juvenile grey mullet at **P4.IOLR** demonstrated a significant dietary taurine requirement at the 0.5% DW dietary level. Nevertheless, it remains unclear if this species is able to synthesize taurine through the CSD and/or ADO pathways. One of the major roles for taurine is to conjugate with bile acids such as cholic acid or chenodeoxycholic acid in the liver, which is then stored in the gall bladder. Moreover, 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in bile salt synthesis and has been associated with dietary taurine and its growth promoting properties. Fish liver samples taken in **Sub-task 13.3.2** for analysis of the gene expression of CSD and ADO, were also analysed for CYP7A1 in order to determine if dietary taurine up regulated bile salt synthesis.

Methods and Materials

Total RNA was extracted from *Mugil Cephalus* liver tissue and was used for the synthesis of cDNA. Degenerate primers were designed in order to sequence the desired genes. Gene identity was confirmed by comparing the obtained sequences with those available at the Genebank (http://www.ncbi.nlm.nih.gov/Genbank/). Real time PCR was performed and analysed using a 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = 2-ΔΔCt, Ct – threshold cycle.

Results

In **Fig. 13.5a** the results show a taurine dose-dependent and significant (P<0.05) response in the gene expression of cysteine sulfinic acid decarboxylase (CSD) in fish fed the control (0% taurine) to 1% taurine treatments and then a decrease of the gene expression of these enzymes in fish fed the highest dietary taurine level (2% DW diet). Fish fed the 0.5% taurine diet demonstrated almost 8 times the expression level of this gene compared to the control. The general level of gene expression of cysteamine (2-aminoethanethiol) dioxygenase (ADO) in the less common taurine synthesis pathway was much lower, where 0.5% dietary taurine stimulated the highest but non-significant (P>0.05) ADO response (**Fig. 13.5b**). Moreover, the results show considerable CSD synthesis when the fish are not consuming any taurine although the ADO pathway is not stimulated until fish are ingesting 1%. In **Fig. 13.6** there was no clear, significant (P>0.05) response by dietary taurine on the expression of fish liver CYP7A1.

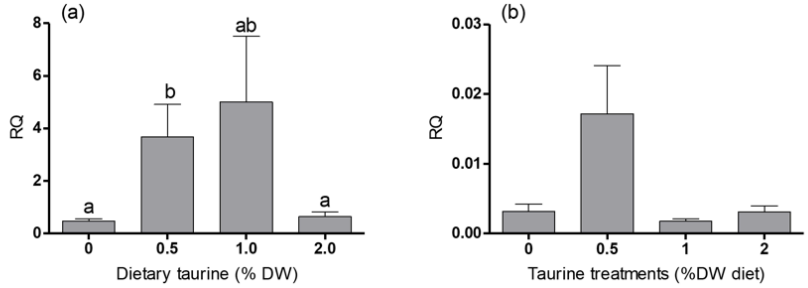


Figure 13.5 The effect of dietary taurine on the gene expression of (a) cysteine sulfinic acid decarboxylase (CSD) and (b) cysteamine (2-aminoethanethiol) dioxygenase (ADO) in juvenile fish. Bars represent average values ± SEM (N=15). Values having different letter(s) were significantly (P<0.05) different.

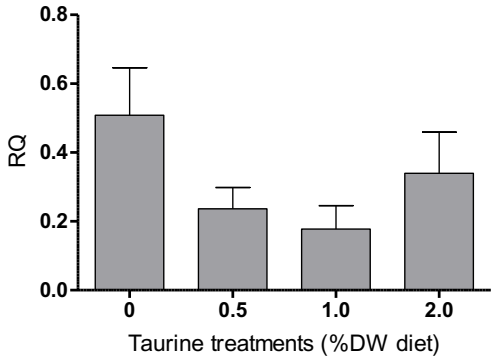


Figure 13.6 The effect of dietary taurine (0, 0.5, 1.0, 2.0% DW diet) on the gene expression of CYP7α1 in juvenile fish. Bars represent average values ± SEM (N=15).

Discussion

The results suggest not only that the CSD pathway is active in the main taurine synthesizing pathway in the absence of dietary taurine but that the expression of this key gene increases with increased levels of dietary taurine until 1% where CSD expression drops at the high dietary taurine level. The synthesis of taurine in the liver when levels of this nutrient are increasing in the diet seems counter intuitive. However, taurine can function as an osmolyte to maintain cell volume. Conceivably, increased taurine in the blood circulation of the liver, due to higher dietary taurine, may stimulate increased synthesis within liver cells to reduce osmotic pressure across the membrane in order to prevent cell shrinkage and changes in intracellular hydro-mineral balance. Although there was a clear but non-significant ($P>0.05$) increase of ADO gene expression in fish feeding on the 1% taurine diet, the RQ values were generally very low, suggesting that this pathway represented a minor contribution to endogenous taurine synthesis. The sharp decrease in CSD in the 2% taurine diet may indicate a negative feedback mechanism and/or a strategy to increase taurine synthesis and increased transport of taurine into the cell as more effective than intracellular biosynthesis at this dietary concentration.

In our study, there was no clear effect of dietary taurine on CYP7α1 and bile salt synthesis. In fact, the highest expression of bile salt synthesis was in the control where there was no dietary taurine suggesting that CSD endogenous taurine synthesis was supplying sufficient levels of this nutrient. On the other hand, **Sub-task 13.3.2** found that a diet containing at least 0.5% taurine resulted in significantly ($P<0.05$) improved weight gain in juvenile fish. This suggests that endogenous taurine synthesis, although possibly sufficient in the synthesis of bile salt, may not be adequate in contributing taurine to muscle function and growth. Taken altogether, it appears that grey mullet juveniles have the capacity for endogenous taurine synthesis that may be sufficient for cell volume homeostasis and bile salt production but may fall short in optimizing skeletal muscle function and growth, thereby requiring a minimum of 0.5% of taurine in the diet.

Sub-task 13.4.1 (IOLR) Broodstock dietary effects on mullet reproduction (e.g., natural pigments, DHA/EPA/ARA ratio, Tau) on egg quality, in terms of fecundity, hatching success, and larval first feeding and **Sub-task 13.4.2 (UNIBA) Definition of specific requirements of protein, TAU, ARA, DHA and carotenoid sources to optimize spawn quality in mullet. Analysis of liver Vtg gene expresssion, oocyte Vtg receptor gene expression and yolk accumulation under different dietary conditions.**

These subtasks were reported in the 3rd periodic report and were submitted during the 4th reporting period as the ***Deliverable D13.3 Determine the effects of pigments, essential fatty acids and Tau in grey mullet***

broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression accumulation and is presented here in brief.

Fish reared in captivity may exhibit reproductive dysfunction, such as the inhibition of final oocyte maturation and spawning for females and the production of poor quality sperm by males. These dysfunctions can be overcome by hormonal therapies and modulation of environmental parameters as well as providing an effective broodstock diet, which should mimic body composition of brooders in the wild.

During vitellogenesis significant quantities of proteins must be made available for transfer to the developing oocytes as well as lipids and carotenoids. Lipids that are mobilized are particularly rich in both saturated and monounsaturated fatty acids for energy provision as well as long chain polyunsaturated fatty acids (LCPUFA), which are primarily represented by the essential fatty acids (EFA) eicosapentaenoic (EPA; 20:5n-3), arachidonic (ARA; 20:4n-6) and docosahexaenoic (DHA; 22:6n-3) acids. The supplying of proper levels and ratios of EFA in broodstock diets is vital not only to produce eggs with the suitable contents of these fatty acids to ensure embryo and larvae development, but also in the regulation of reproductive physiology. N−3 PUFA derived directly from the dietary input of broodstock as well as from body reserves in the period of gonadogenesis are crucial to female fecundity and to embryo and early larval development, growth and survival. In contrast to the carnivorous gilthead sea bream and European sea bass (*Dicentrarchus labrax*), the grey mullet is omnivorous following metamorphosis, which suggests a diet relatively low in n-3 PUFA. However, grey mullet bottarga or intact roe is a highly prized delicacy in Japan and around the Mediterranean and is a rich source of n-3 LCPUFA. This suggests that grow out diets may not be suitable and that there is a dietary requirement for n-3 LCPUFA in the brood stock feed.

Materials and methods

Sampling

Eight hatchery-produced 6-year old grey mullet stock were sampled by **P4. IOLR** during early October (3 females) and November (3 females and 2 males), 2016. In parallel, 16 wild specimens (10 females and 6 males), caught by traditional trap nets (lavoriera) in the Schiapparo Channel (Apulia, Italy) during their migration from the Lesina Lagoon to the spawning grounds of the Adriatic Sea, were sampled by **P13. UNIBA** in early September, 2016. The age of wild grey mullets sampled by UNIBA was estimated through the analysis of the scales (Meunier, 2002). Gonads from wild and captive male and female individuals were taken, immediately frozen and kept at -80ºC, until analysis. Proximal and main fatty acids composition analyses of diets and gonads from mature grey mullet were carried out by **P15. ULL**.

Fish holding and experimental design

Based on the results of proximal analysis and fatty acid (FA) composition in gonads from wild and domesticated mullets, the present study attempted to improve the brood stock diet for grey mullet by increasing the n-3 LCPUFA through the addition of fish oil to the diet. During the onset of the reproductive season, 6-year old captive grey mullet brood stock were divided into two groups, that were fed either a mullet grow out diet containing soybean oil (SO), that was previously developed by **P4. IOLR**, or a diet containing fish oil (FO), which is relatively rich in n-3 LCPUFA. The experiment was conducted in triplicate tanks per treatment. Fish were maintained in 4 m^3 tanks supplied with ambient seawater at 40 ‰ and subjected to natural fluctuations of light and temperature. Food was provided at the rate of 1-1.5% of their body weight. Fish were conditioned for spawning using protocols developed by **P4. IOLR** with some modifications elaborated in WP7.

Larval rearing trials

Fertilized and spawned eggs from each of the brood stock groups (SO and FO) were stocked in eight 400 l V-tanks (200 eggs l^{-1}) or four tanks per treatment for the food deprivation experiment. Hatching rate was determined 24 h after stocking and survival of the hatched larvae at the end of 0 dph. Tanks in the food deprivation experiment were supplied with temperature controlled (Gavish, Israel; 24-25 ^{o}C), filtered (10 µm) and UV treated sea water (40 ‰) that entered the tanks from the bottom and exited through a 500 µm filter at an exchange rate of 300% per day. In two tanks from each brood stock treatment, the salinity was decreased to 25 ‰ at 2 dph over the course of one day so that 3-7 dph larvae were exposed to only 25 ‰ in these tanks. This means duplicate tanks were used for each of the four treatments (SO-25 ‰, SO-40 ‰, FO-25 ‰, and FO-40 ‰).

Results

Table 13.1 summarizes the reproductive performance of the FO and VO broodstocks during the natural spawning season (September-October 2017). All tested parameters including the percentages of fully mature specimens (spermiating males and post-vitellogenic females), successful spawns and fecundity showed no significant variations between the treatment groups.

Table 13.1 Summary of grey mullet broodstock body weight (BW) and reproductive performance in two different diets distinguished by their oil source: fish oil (FO) and soybean oil (SO).

	Treatment groups	
	FO	**SO**
Av. BW females (g)	1660.36±67.8	1753.67±75.5
Av. BW males (g)	987.86±46.06	905.77±48.11
Post vitellogenic females (%)	71.86±5.9	69.86±9.4
Spermiating males (%)	28.97±16.8	13.09 ±7.2
Fertilized spawns (%)	50	66
Fecundity	2.12±0.1	2.89±0.9

The FO diet was higher in monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) than the SO diet. On the other hand, the SO diet was considerably higher in n-6 PUFA than the FO diet. The FO diet had higher total phospholipids, triacylglycerols as well as wax and sterol esters than the SO diet. In contrast, there were no marked differences between the lipid classes and fatty acid composition in 6 year old captive female gonads fed the FO or SO diets.

In 3 year old wild and F2 females fed the FO or SO diets, there are no distinct differences in their gonadal total lipids or phospholipids while both FO wild and F2 females were considerably higher in the phospholipids compared to the SO females, particularly phosphatidylcholine. In the neutral lipids, there was a drop in cholesterol in the FO wild and captive females when compared to the SO females. Conversely, there was a marked increase in waxes and cholesterol esters in the FO wild F2 females compared to the SO females. However, the most dramatic difference was in total lipid between the FO wild and captive females and male gonads in both the FO and SO groups. In contrast, PL classes increased markedly in the male gonads compared to the female gonads in both the FO and the SO groups as well as cholesterol. The drop in total lipid is mostly explained from the marked reduction in waxes and cholesterol esters comparing female to male gonads in the FO and SO groups.

Although female gonads had less saturated fatty acids (SFA) than male gonads in both FO and SO groups, they had more monounsaturated fatty acids (MUFA) than male gonads in the FO and SO groups. However, FO captive female gonads did not differ markedly in their n-3 PUFA content from FO wild female gonads but contained considerably less n-3 PUFA than male gonads in both FO and SO, which was mainly due to the difference in DHA.

In **Fig. 13.7** the larval rates of decline of weight with age (dph) during food deprivation in the four treatments were not significant (P>0.05) from each other. However, there was an observed pattern where the highest rate of dry weight (DW) decline was in the SO larvae where all fish were dead at 5 dph or 3 days after the capability of exogenous feeding. In contrast, larvae from the FO brood stock demonstrated a slower DW decline, particularly in the 25 ‰ treatment, and were still alive at 7 dph, which was 5 days after the onset of the ability to consume live prey. The percent hatching of eggs from the FO brood stock was significantly (P<0.05) higher than eggs from the SO brood stock (**Fig. 13.8**), while survival in larvae in the two treatments at the end of 0 dph were not significantly different (P>0.05) from each other, although the FO was higher (**Fig. 13.8**). There was a very significant effect (P<0.0001) of brood stock treatment on swim bladder inflation in the food deprivation experiment (**Fig. 13.9**). Larvae from the FO brood stock, regardless of salinity exposure, demonstrated 100% swim bladder inflation by 5 dph. In stark contrast, there was no swim bladder inflation in fish from the SO brood stock during the course of the food deprivation study.

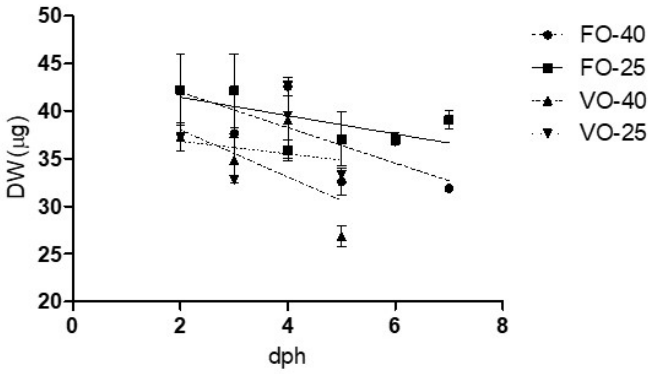


Figure 13.7 The rate of decline in 2-7 dph larval dry weight (DW) during food deprivation. Slopes of lines were not significantly different from each other (P=0.5754)

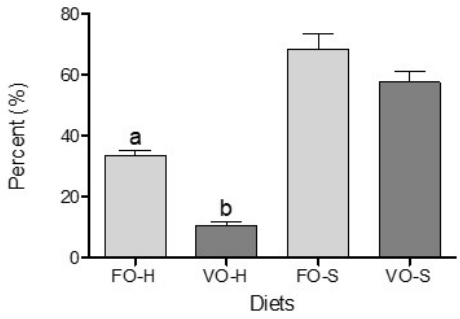


Figure 13.8 The effect of brood stock diets FO (Fish oil) and VO (vegetable oil) on percent hatching (H) and survival (S) at the end of the day of hatching (T0). Percent values having different letters were significantly different (P<0.05).

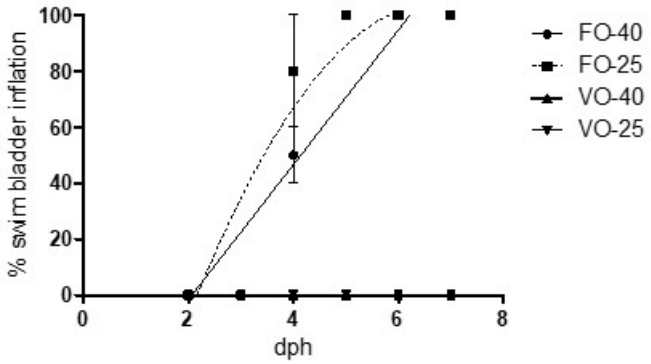


Figure 13.9 The rate of % swim bladder increase in 2-6 dph larvae during food deprivation. There was no swim bladder inflation in larvae from the VO brood stock diet, irrespective of rearing salinity.

Discussion

In this study the mobilization of energy reserves in terms of lipids and proteins is quite similar between wild and captive mature females. Moreover, in fatty acids and fatty acid groups, there were no conspicuous differences, independent of age, between FO wild and captive FO brood stock and SO female brood stock gonads. Nevertheless, when comparing the fatty acid and lipid class profiles between female and male gonads, there were highly marked differences. Researchers have reported higher contents of total lipids, triacylglycerols and wax and sterol esters in female gonads compared to male gonads and higher quantities of phospholipids PC, PS and PE as well as cholesterol in male gonads compared to female gonads These trends agree with the results in this study and are independent of dietary regime (FO or SO). This suggests that another FO component, possibly carotenoids, is the responsible for the observed benefit of the fish oil diet on larval performance. Carotenoids such as β-carotene or astaxanthin act as antioxidants and are involved in various physiological processes. Importantly, they are precursors for retinal and vitamin A synthesis that are critical in development. The use of fish oil in brood stock diets of mullet leading to better hatchability, tolerance of food deprivation and improved swim bladder inflation, which was found in this study, is largely supported in the literature.

Task 13.3.5 Comparison of vegetable oil-no fish meal grow out diet with a n-HUFA rich fish meal finishing diet on the nutritional and organoleptic values of fish flesh and bottarga quality (CTAQUA, Rocio Robles).

In order to evaluate the difference between two feeds with a complete different formulation, a lab trial has been conducted with adult specimens of grey mullet. The fish were harvested from a farm located in the South Spain, (not part of the Diversify consortium) producing seabass in earthen ponds and having also few ponds with grey mullet in monoculture. A batch of adult grey mullet (*Mugil cephalus*) of 550-600 g average body weight was received at the facilities of Ctaqua.

The fish remain in the RAS system, during 6 months. The two feed planned to be test were: experimental diet (specifically formulated diet for the species; formula from **P4. IOLR**) and a feed without fish meal, which was the commercial diet in use at the farm (commercial carp diet).

Materials and Methods

Farmed grey mullet adult specimens were obtained at the farm and transported to the premises of CTAQUA facilities. The transport of adult fish is always a challenge but the grey mullet arrived in fairly

good conditions at Ctaqua. Upon arrival, they were evenly distributed among the tanks of the recirculation aquaculture system (RAS) and left undisturbed during 24 h. Only after that period, they received a preventive formalin treatment.

The fish arrived to the facilities of P18.Ctaqua the 25th of January 2018. A total of 130 adult grey mullet, were distributed in a RAS with 6 tanks of 1200 l each. The RAS is comprised of units for mechanical filtration (drum filter), biofiltration, protein skimmer and UV treatment (**Fig. 13.10)**.

Figure 13.10. RAS system used for the trial with the adult grey mullet at **CTAQUA** facilities.

Water quality parameters were controlled twice per week, except temperature and dissolved oxygen that have been checked daily, as well as mortality and fish welfare.

Grey mullet were coming from the farm conditions of earthen ponds (natural temperature, photoperiod and lower salinity than the culture water of Ctaqua). They arrived to the facility in good condition but their adaptation to the captive conditions in the RAS was not easy.

During the first week, progressive water change was applied to the RAS in order to adapt the fish from the 12 psu salinity of the ponds to the 36 psu of the culture water in the RAS.

The two diets were tested in triplicate tanks:

- Experimental diet: tanks 1, 3 and 5. This diet was specifically formulated for the species by P4.IOLR. Total protein content of this diet is 35% and total fat content is 15%. The experimental diet was prepared by Sparos (Portugal).
- Commercial diet: tanks 2, 4 and 6. This diet was the commercial diet used in the farm for the species. It is a commercially available diet for carp. According to the label information, total protein content is 32% and total fat content is 9%.

Feed samples have been sent to **P.16 ULL** for further analyses of proximate composition and fatty acid profile.

Experimental fish were hand fed twice per day to apparent satiation. However, during the first weeks of acclimation, were not consuming the feeds very well. In order to test the influence of the people around the tank on the feeding behavior, automatic feeders were also used.

Stocking of the fish with the initial number of fish per tank was done on February 21, 2018. The trial was carried out from February to August. Fish were individually weighed and measured. Fish from the initial batch that presented small wounds or any abnormality were not used for the stocking.

Since grey mullet are known to be good "jumpers", tanks had to be covered with rigid plastic nets to avoid losing fish during the hours that the staff was not around the tanks (**Fig. 13.11**). Fish were stocked at a ratio of 15 individuals per tank, at an initial density of 7.01±0.38 kg/m^3. Natural photoperiod was established for the trial; the artificial lights of the system were used only during staff working hours, keeping the fish in low light conditions to avoid any source of stress. Working hours for the daily maintenance of the system were reduced to the minimum (1 hour per day) until the fish started to eat normally. Then lights were on during the standard working hours from 8.00 h to 15.00 h. Temperature was kept at 19ºC during the first two weeks of the trial and then gradually increased during a 5 day period to 22 ±1 ºC. The temperature of 22±1 ºC was maintained for the rest of the experimental period. Trial duration was 180 days of feeding the two diets.

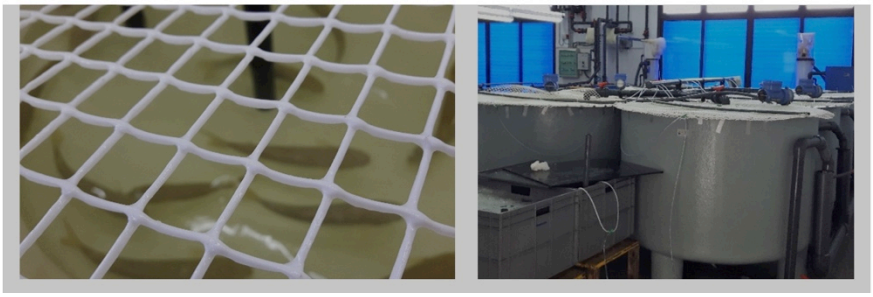


Figure 13.11. Close-up of the net and view of the tanks covered with plastic nets to avoid the jumping of the grey mullet.

Six samplings have been performed: at stocking (February 2018), four intermediate samplings and the final sampling in August 2018, where all the fish were individually weighed and measured and dissected to collect the data for the somatometric measurements and to collect the different tissues for further analyses. From 4 individuals per tank, samples of filet and gonads have been taken to be analyzed for proximate composition and fatty acid profile (following the protocol developed with **P16. ULL**).

For all the samplings, fish were fasted during 24 h. On the sampling day, the fish were harvested from the tanks and transfer immediately to a tank with 2-phenoxyethanol in order to tranquilize them and then proceed to the weighing and measurement sampling (**Fig. 13.12).**

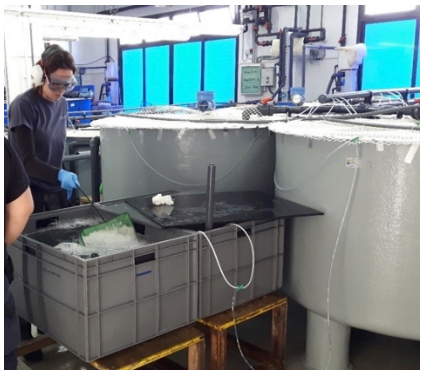


Figure 13.12. Sampling moment at the facilities of Ctaqua. It can be noticed that the nets had to be strongly fixed to the tanks to avoid that the powerful jump of the grey mullet could open the nets.

Somatometric measurements and production parameters

Grey mullet have been weighed and measured individually at each sampling time. At the end of the trial, four fish per tank (8 fish for Irida Diet and 12 fish for Commercial diet) had their viscera, liver and gonads removed from the carcass and all separately weighed. It has not been possible to separate the visceral fat since the high temperatures of that period of the year (August) did not allow to collect the perivisceral fat in a proper (solid) state to be weighed. 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) and
- Viscerosomatic index (VSI) = 100 × (total viscera weight / body weight).

During the sampling, a piece of approx. 15 g. was taken from the right side filet of each sampled fish, labelled and stored at -80ºC, to be sent to **P18. ULL** for proximate analysis and fatty acid profile determination. When the fish had gonads, they were weighed, labelled and immediately frozen at -80ºC to be sent also to **P18. ULL** for proximate analysis and fatty acid profile analyses. The rest of the right side filet of each sampled fish, was packed and stored at -80 °C to be further processed for sensory analysis **(P3.IRTA)**, while the second fillet was stored also at -80ºC as back up sample.

The following production parameters were calculated per tank for the 180 days of the trial:

- Survival= 100×final number of fish per tank/ initial number of fish per tank; (%)
- Specific growth rate (SGR)= 100×ln (final weight (g)/initial weight (g))/days of feeding); (%/day)
- Total feed per individual = total feed intake per tank (g)/number of fish per tank; (g)
- Feed intake expressed as percentage of the initial and final average body weight per tank per day (Feed intake %ABW/d) = 100×feed intake per tank in the period (g) ×number of days of the period/ (final weight (g)+initial weight (g))/2); (%ABW/d)
- Food conversion ratio (FCR)= (average feed intake (g)/average wet weight gain (g))

Methodology for sensory analyses

This task has been done by **P3.IRTA** (Luis Guerrero). The fillets from grey mullet were sensory characterized in four modalities: odour, appearance, flavour and texture, as described in Deliverable **28.3**. To make this characterization, a list of sensory attributes previously generated was used. Panellists were specifically trained to be familiarized with the descriptors and their intensity scales. Eight panellists with previous experience in the sensory profiling of food products were recruited for this training before evaluating the samples. Sample analyses were performed in three sessions of five samples each. Samples from the different diets and tanks were tasted in each session. Panellists assessed samples in the subsequent order: odour modality first then appearance followed by flavour and finally texture.

In each tasting session, the order of sample presentation and the first order and carry-over effects (Macfie et al., 1989) were blocked. In all cases, the evaluation was carried out in isolated sensory testing booths (ISO, 2007). All assessors were provided with mineral water to cleanse their palates between samples. Samples were cooked in a convection oven at 115ºC for 20 minutes in individual transparent glass jars designed to make samples easy to visualize. Jar lids were used to keep the samples' odour from disappearing (Model B-250, Juvasa, Spain). Jars were then placed inside electrical heaters at 60ºC to keep them warm while being tasted.

Data were analysed by means of an ANOVA. The selected model included the diet, the growing tank, the interaction diet x growing tank, the taster and the tasting session as fixed effects.

Results

With regard to feeding, fish did not show any interest for the feed at any moment during the first 5 weeks after arrival. After this period and once they started to show some interest for the feed, both diets (experimental and commercial diet) were provided to the corresponding tanks

Experimental diet (formulated by **P4. IOLR** and prepared by Sparos) was not accepted by the fish at any moment (tanks 1, 3 and 5). The experimental diet was provided during 5 consecutives days without success. Since the fish could not continue without eating, it was decided to provide the also formulated by **P4. IOLR** and prepared by **P.31 IRIDA**. This diet was used in WP23, for the farm trial performed by Ctaqua in grow out of grey mullet in earthen ponds (Task 23.4, Deliverable 23.3).

Grey mullet did eat the IRIDA diet so it was decided to use this diet as Experimental diet for the rest of the trial. IRIDA diet is a complete diet including *Ulva lactuca* in its formulation. The details of the IRIDA formulation are protected by the IPR of **P4. IOLR**.

After the diet change, the two diets used for the trial have been:

- Irida diet: tanks 1, 3 and 5.
- Commercial diet: tanks 2, 4 and 6.

Commercial diet (carp) was well accepted by the grey mullet specimens from the first moment they started to eat actively. This diet is currently used by the farm for the ponds where they culture grey mullet. No problem was encountered with this diet in the RAS at CTAQUA facilities.

Grey mullet have proven to be powerful jumpers against the nets during the whole trial duration. It seems the interactions among the individuals within the tanks provoked that some specimens jumped and in several occasions, were found dead on the floor or even in the tank besides the tank where they belonged. Some of these individuals could be recuperated but others were just found dead.

After the stocking of the fish to start the trial, the nets were fastened more strongly to each tank to avoid losing fish. Probably as a consequence of the poor adaptation and as result of the attempted jumping out of the tank against the netting and damaging their skin, several specimens were found in the tanks with heavy wounds. Peroxide treatments were applied locally and some specimens recovered. Four specimens (2 from tank 5 and 2 from tank 2) were too damaged and were euthanized (**Fig**. **13.13)**. In the month of May, during one of the peroxide treatments, all the fish from tank 3, (IRIDA diet) died accidentally due to a technical failure.

Although the results from tank 3 are included in **Table 13.2**, they have not been taken into account for the average calculation of productive parameters per dietary treatment. The growth and productive results from this tank correspond to a trial period of only 84 days.

Figure 13.13. Grey mullet specimens heavily wounded that were euthanized during the trial.

The growth results obtained during the trial reflect the difficulty of the adult grey mullet to cope with the captivity conditions. The fact that they did not eat during 5 weeks and the problem of diet acceptance has heavily affected the trial results. Only at the end of the trial the fish started to be more adapted to the RAS tank conditions. Thus survival during the trial has been influenced by the inadaptability of adult grey mullet (the batch used for this trial coming from earthen ponds) to the captive conditions. As it is presented in **Fig. 13.14,** survival ranged from 100% in tank 6 to 53% in tank 2. Tank 3 was completely lost accidentally.

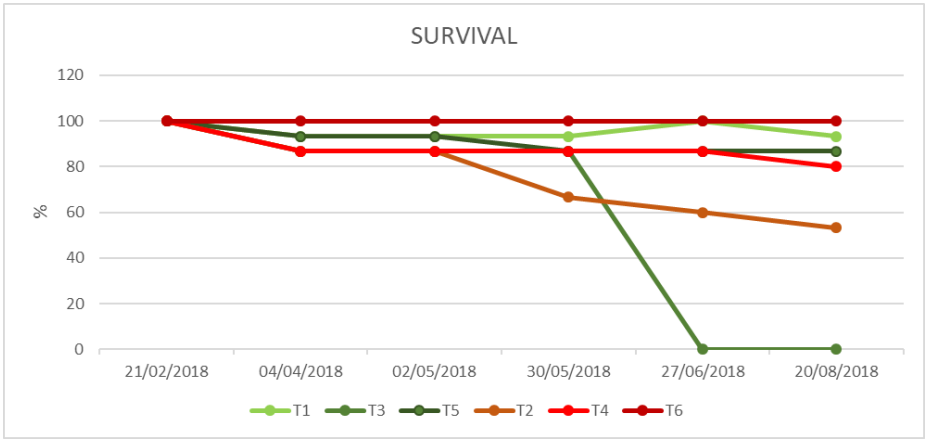


Figure 13.14. Survival (expressed as percentage per tank) of grey mullet during the trial.

With regard to the growth curve, the data are very different from tank to tank, indicating a strong tank effect. This could be observed also during the time of feeding. Grey mullet from tanks 1 and 2 were always reluctant to eat and it took longer time to feed those fish. As it can be observed in the growth curve (**Fig. 13.15**) fish of tank 2 hardly increased the individual average body weight along the 6 months trial. On the contrary, the fish from tank 5 had the best growth of all. Average data of the productive parameters have not been calculated as the mean of the corresponding replicates per dietary treatment due to the high standard deviation values and the out of range variation coefficient values. Data are presented per tank.

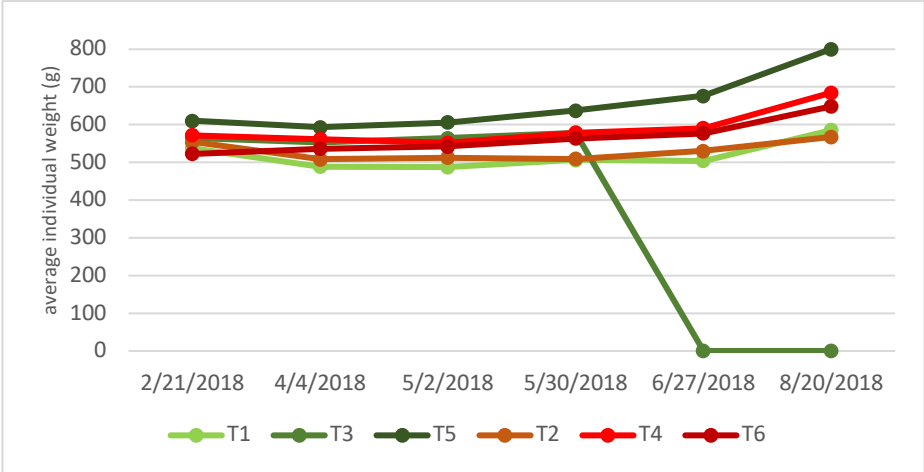


Figure 13.15. Growth curve of the grey mullet (per tank) during the trial (fish from tank 3 were accidentally lost).

Growth within each tank fish group was very irregular with a high variation in individual weight increased during each period of the trial (**Fig. 13.16**). Specific growth rate (SGR) was rather low and food conversion

ratio (FCR) values demonstrate that the feed were not well used by the fish. In **Tables 15.3.5.2** and **15.3.5.3** productive results per tank are shown. SGR data from the five tanks after 180 days of trial range from 0.01 %/day in tank 2 to 0.15 %/day in tank 5. In the case of tank 3, data shown correspond to the results of 84 days of trial. FCR data are very different from tank to tank, also due to the strong tank effect observed in this trial and reflecting the growth data. FCR for tank 2 was 30.81 and for tank 5, FCR was 2.63, clearly reflecting the strong tank effect.

Results of somatic indexes, CI, HSI and VSI are presented in **Fig. 15.3.5.8.** One-way ANOVA analyses of the CI, HSI and VSI data did not show significant difference ($p>0.05$) for any of the indexes. However, the CI index is quite low which is just the reflection of the poor feeding during the trial.

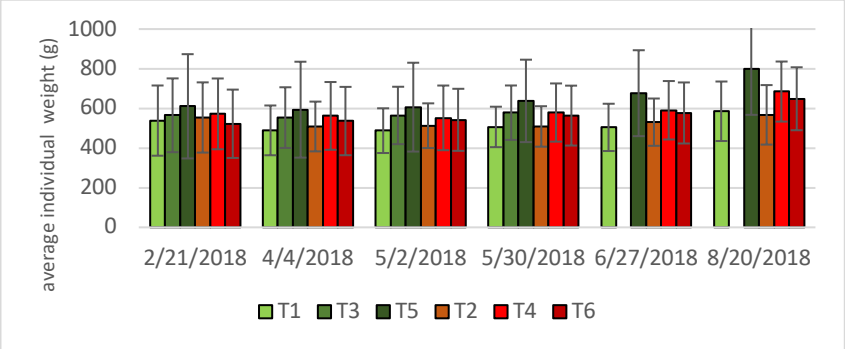


Figure 13.16. Evolution of the average individual weight per tank during the trial. Tanks 1 and 5 were fed with Irida diet; tanks 2,4 and 6 were fed with Commercial diet for carp.

Feeds provided during the trial have been analyzed by **P16.ULL** and the results are shown in **Table 13.3**. Irida diet is an improved extruded diet. The Commercial diet is formulated for carp and it is commercially available. Proximate composition of the two diets used in the trial differs mainly in total fat content, Irida diet 15.09 ± 0.68 % compared with the 8.79 ± 0.42% of the Commercial diet. The proximate analyses of the diets show that regarding total protein content, both dietary treatments are very similar (35.10 ± 2.46% for Irida and 35.80 ± 1.95% for Commercial diet). Another remarkable difference is the total n-3 PUFA value of both diets, 15.02 ± 0.16 % total fatty acids for IRIDA diet compared with 8.37 ± 0.37% total fatty acids for the Commercial diet. It is expected that this difference fatty profile will be reflected in the fatty acid profile of the muscle (results pending)

Table 13.3. Proximate composition (% wet matter) and main fatty acid profile (% total fatty acids) of IRIDA diet and Commercial diet used in the trial.

	Irida	Commercial
Moisture	6.47 ± 0.02	6.67 ± 0.06
Fat	15.09 ± 0.68	8.79 ± 0.42
Protein	35.10 ± 2.46	35.80 ± 1.95
Ash	7.01 ± 0.07	9.02 ± 0.20
16:0	11.99 ± 0.05	18.56 ± 0.05
18:0	2.50 ± 0.04	5.38 ± 0.09
Total SFA	19.16 ± 0.22	27.05 ± 0.01
16:1[1]	4.19 ± 0.05	3.75 ± 0.17
18:1[2]	32.89 ± 0.32	32.61 ± 0.16

Total MUFA	47.02 ± 0.29	39.94 ± 0.12
18:2n-6	15.57 ± 0.00	22.50 ± 0.02
20:4n-6	0.37 ± 0.01	0.54 ± 0.01
Total n-6 PUFA	16.62 ± 0.01	23.50 ± 0.18
18:3n-3	3.35 ± 0.02	2.47 ± 0.13
20:5n-3	3.45 ± 0.06	1.52 ± 0.03
22:6n-3	4.98 ± 0.09	3.06 ± 0.10
Total n-3 PUFA	15.02 ± 0.16	8.37 ± 0.37
DHA/EPA	1.45 ± 0.00	2.02 ± 0.03
ARA/EPA	0.11 ± 0.00	0.35 ± 0.01
n-3/n-6	0.90 ± 0.01	0.36 ± 0.01

Data correspond to average ± standard deviation (n=2)

Table 13.2. Productive data from tanks 1, 3 and 5 (fish fed Irida diet during 180 days). In the case of tank 3, data correspond to only 84 days of trial.

Diet	Irida Tank 1	Irida Tank 3(†)	Irida Tank 5
Survival (%)(*)	93.33	86.67	86.67
Average initial weight (g)	538.41±177.08	565.19±186.09	610.49±262.95
Average final weight (g)	585.46±150.06	578.28±137.99	800.68±233.327
Weight gain (g)	47,04	26.42	190.19
SGR (%/d)/ind	0.05	0.06	0.15
Total feed/ind (g)	402.12	146.83	500.41
Feed intake (%ABW/d)	0.40	0.31	0.39
FCR	8.55	5.56	2.63
CI (**)	1.16±0.11	1.16±0.09	1.20±0.07
HIS(**)	0.98±0.14	1.32±0.28	1,03±0,21
VSI(**)	5.36±0.88	6.34±1.24	6.08±0.96
GSI(***)	0.61±0.14	0.31±0.21	0.64±0.18

*180 days trial period; (†) all data from tank 3 correspond to 84 days of trial (fish lost accidentally)

** data correspond to average and standard deviation of 14, 14 and 13 individual for tanks 1, 3 and 5 respectively.

*** data correspond to average and standard deviation of 6, 9 and 9 individuals for tanks 1, 3 and 5.

Table 13.2. Productive data from tanks 2, 4 and 6 (fish fed Commercial diet during 180 days).

Diet	Commercial Tank 2	Commercial Tank 4	Commercial Tank 6
Survival (%)(*)	53.33	80	100
Initial weight (g)	554.14±137.89	572.35±178.51	522.41±172.33
Final weight (g)	567.46±148.75	684.85±151.49	648.53±158.78
Weight gain (g)	13.31	112.50	81.32
SGR (%/d)/ind	0.01	0.10	0.07
Total feed/ind (g)	410.17	496.54	460.58
Feed intake (%ABW/d)	0.41	0.44	0.42
FCR	30.81	4.41	5.66
CI (**)	1.20±0.10	1.13±0.07	1.18±0.09
HIS(**)	1.18±0.21	0.98±0.20	1,02±0,19
VSI(**)	5.22±1.35	5.02±0.59	5.36±0.96
GSI (***)	0.48±0.15	0.63±0.21	0.52±0.19

*180 days trial period

** data correspond to average and standard deviation of 8, 12 and 15 individual for tanks 2, 4 and 6 respectively.

*** data correspond to average and standard deviation of 6, 7 and 11 individual for tanks 2, 4 and 6 respectively

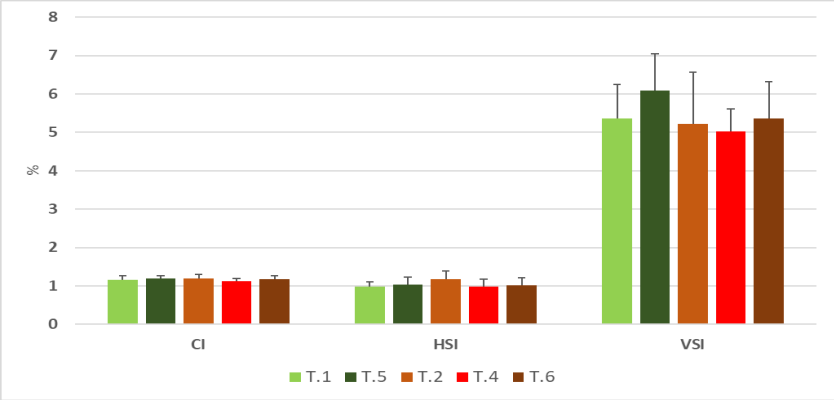


Figure 13.17. Average values of condition index (CI), hepatosomatic index (HSI) and viscerosomatic index (VSI) per tank. Tanks 1 and 5 were fed with Irida diet; tanks 2,4 and 6 were fed with Commercial diet for carp.

Concerning the gonadal development, as it could be expected due to the poor adaptation of the fish, only some of the fish had visible gonads, and in any case the GSI was in all cases lower than 1% (**Fig. 13.18)** . One-way ANOVA analyses of the GSI data for both diets, did not show significant difference ($p<0.05$). Probably these fish would need a much longer growing period to be able to have adequate gonad development.

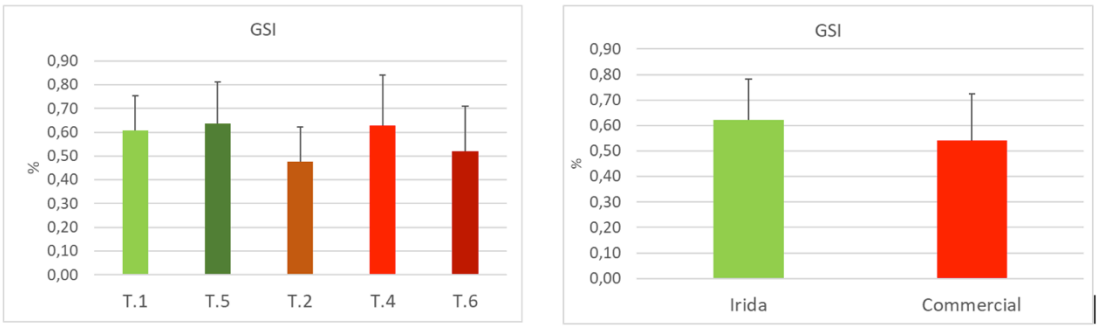


Figure 13.18. GSI values per tank (left) and per diet (right) of the grey mullet that presented a incipient gonadal development.

At the moment of the final sampling and after 180 days of culture, the grey mullet did not display any wound or lesion on the skin nor in the fins (**Fig. 13.19)**. However, when dissecting the fish for somatic indexes calculation, abnormal pigmentation of the liver was observed in some specimens fed the Commercial diet. Although no statistically significant difference was found between the HSI of the grey mullet fed the Irida diet and the Commercial diet for carp, there was a marked effect on the liver appearance of fish fed the Commercial carp diet. As it can be observed in **Fig. 13.20**, the livers presented dark green spots on the external surface of the organ. This problem might be caused by a deficiency in taurine, which would provoke an imbalance on the release of bile acids from the liver and they would accumulate in the liver causing the green coloration. Grey mullet fed the Irida diet did not present any green spot or abnormal coloration on the liver of any of 27 dissected specimens. In the case of the grey mullet fed the Commercial diet, 46% of the livers had green spots, indicating that such a diet is causing a hepatic disorder. Further analyses of the liver could help to elucidate these findings. Unfortunately no liver samples were taken at the moment of the end of trial.

Figure 13.19. Healthy grey mullet specimens during final sampling showing the typical blueish spot on the base of the pectoral fin of *Mugil cephalus*.

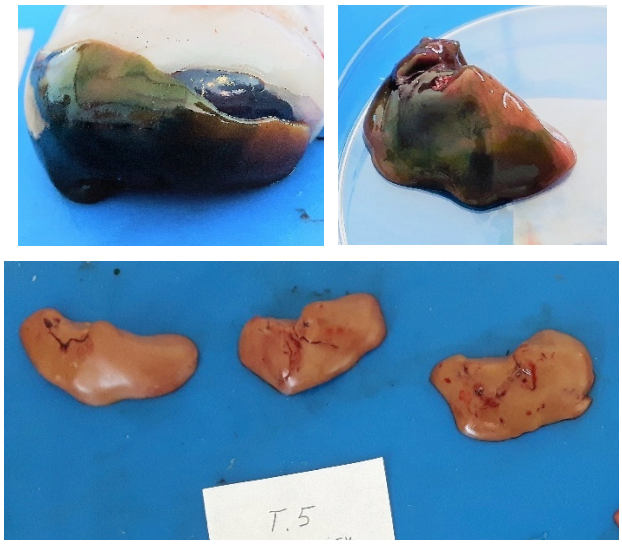


Figure 13.20. External aspect of the livers from fish fed the Commercial diet with evident green dark spots on the surface of the organ (top images) compared with the liver coloration of grey mullet fed IRIDA diet.

Proximate composition and fatty acid profile of the filet

Data on fileting yield, dressing yield and proximate composition of the samples taken at the end of the trial are included in **Table 13.3.** Filleting yield of the reared fish was very good and, as it was concluded in the **D 23.3**, indicate the potential of using the species for elaborating processed fish products such as smoked fish fillet or filet preserved in olive oil.

Table 13.3. Average technical yields and proximate composition of grey mullet reared in the RAS at Ctaqua facilities and fed extruded diets for the trial: IRIDA diet and Commercial diet.

	Irida(*)	Commercial
Body weight (g)	575.51 ± 139.66	687.28 ± 113.23
Dressing yield (%)	93.68 ± 0.68	93.32 ± 1.05
Filleting yield (%)	53.82 ± 1.77	54.08 ± 1.10

(*) Fish from tank 3 have not been taken into account for these calculations.

Data are means ± SD (n=4).

Considering that the fat content of the extruded diet (Irida) was 15.09%, it is surprising that the grey mullet flesh reflects exactly the opposite trend, however this is consistent with the low values of HSI and VSI

Sensorial analysis

Tables 13.4 and **13.5** show respectively the mean values obtained for each diet and growing tank. No significant differences were detected in any case. The absence of differences can be explained by the

high variability observed within each treatment. This high variability has affected the trial in all the measured parameters.

Table 13.4. Means values and standard deviation (SD) for the sensory parameters (O, Odour; F, Flavour; T, Texture) from each diet.

Sensory descriptor	Irida		Commercial	
	Mean	SD	Mean	SD
O_Sardine	1.8	1.617	1.7	1.435
O_Ammonia	0.9	1.328	0.9	1.500
O_Earthy	1.9	1.574	1.2	1.554
O_Butter	3.3	2.322	2.1	2.201
O_Sea food	0.9	0.984	0.9	1.125
O_Acid	0.5	1.015	0.4	0.770
O_Boiled vegetables	0.6	1.391	0.7	1.290
Colour white to brown	2.3	1.079	2.9	1.370
Colour uniformity	8.2	1.001	7.8	1.160
White spots	3.4	1.248	2.3	1.218
Laminar structure	2.1	1.298	2.8	1.322
Exudates quantity	4.4	1.716	2.9	1.770
Exudates turbidity	5.5	2.116	3.6	2.057
Fat droplets	2.4	1.614	0.9	1.681
Exudate particles	4.3	2.567	4.2	2.605
Exudate proteins	1.2	1.743	2.1	1.693
Black lines in the flesh	0.1	0.373	0.1	0.405
Brightness	6.0	2.919	5.0	2.740
F_Sweet	4.0	2.119	2.9	2.068
F_Acid	2.8	2.165	1.0	2.129
F_Bitter	1.7	2.283	3.9	2.416
F_Earthy	1.4	2.205	2.2	1.980
F_Sardine	1.4	2.066	1.9	1.792
F_Butter	1.7	1.492	1.8	1.354
F_Sea food	1.0	1.436	0.9	1.286
F_Boiled vegetables	1.1	1.263	1.2	1.381
T_Firmness	5.1	1.599	5.0	1.595
T_Crumbliness	4.5	1.987	5.8	1.889
T_Juiciness	5.0	1.326	4.4	1.312
T_Chewiness	5.9	1.963	5.4	1.718
T_Pastiness	3.4	1.628	4.0	1.713
T_Teeth adherence	4.2	1.860	5.2	1.499

Table 13.5. Means values and standard deviation (SD) for the sensory parameters (O, Odour; F, Flavour; T, Texture) from each tank. Irida diet: tanks 1 and 5; Commercial diet: tanks 2, 4 and 6.

Sensory descriptor	T1		T2		T4		T5		T6	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
O_Sardine	2.1	1.873	1.4	1.132	2.3	1.833	1.7	1.363	1.4	1.096
O_Ammonia	1.0	1.225	0.7	1.565	1.0	1.572	1.1	1.504	0.8	1.384
O_Earthy	0.6	1.056	1.9	1.602	1.8	1.654	1.5	1.863	1.7	1.517
O_Butter	1.9	2.266	3.5	2.253	2.8	2.432	2.1	2.376	3.3	2.029
O_Sea food	0.8	0.926	0.9	0.988	0.8	1.260	1.0	1.056	1.1	1.161
O_Acid	0.5	1.007	0.5	0.922	0.4	0.698	0.5	1.029	0.4	0.733
O_Boiled vegetables	0.8	1.487	0.7	1.296	0.7	1.309	0.6	1.294	0.6	1.363
Colour white to brown	2.9	1.199	1.9	1.127	2.9	1.495	2.5	0.927	2.7	1.372
Colour uniformity	7.8	0.970	8.4	1.191	7.9	1.228	8.1	1.059	7.8	0.979
White spots	1.8	1.138	3.0	1.141	3.5	1.123	2.0	1.425	4.0	1.145
Laminar structure	2.7	1.132	2.3	1.372	2.4	1.375	2.7	1.461	2.3	1.301
Exudates quantity	1.6	1.073	4.3	1.917	4.2	1.753	3.5	1.730	4.5	1.776
Exudates turbidity	2.9	1.937	5.4	2.148	5.1	2.057	3.9	2.211	5.2	2.109
Fat droplets	0.1	1.031	2.4	1.549	2.2	2.097	1.5	1.802	2.1	1.423
Exudate particles	3.7	2.636	4.5	2.756	4.2	2.383	4.2	2.653	4.5	2.733
Exudate proteins	2.0	1.761	1.2	1.535	1.3	1.784	2.0	1.726	1.5	1.878
Black lines in the flesh	0.1	0.471	0.2	0.518	0.1	0.471	0.0	0.236	0.1	0.118
Brightness	4.2	3.010	6.1	2.793	6.3	2.935	5.1	2.850	6.0	2.694
F_Sweet	2.6	2.222	3.6	2.182	4.0	1.912	2.9	2.011	4.3	2.211
F_Acid	0.9	2.321	2.5	2.036	2.7	2.218	0.8	2.012	2.6	2.283
F_Bitter	4.9	2.503	1.7	2.432	2.2	2.396	3.8	2.228	1.3	2.210
F_Earthy	2.4	2.157	1.8	2.480	1.2	1.713	2.3	2.265	1.3	1.767
F_Sardine	1.9	2.184	1.6	1.857	1.6	2.013	1.8	1.984	1.2	1.549
F_Butter	1.4	1.437	1.7	1.574	1.9	1.342	2.1	1.491	1.5	1.163
F_Sea food	0.6	1.165	1.0	1.396	0.9	0.987	1.1	1.650	1.1	1.505
F_Boiled vegetables	1.1	1.156	1.2	1.468	1.2	1.507	1.0	1.366	1.1	1.263
T_Firmness	4.7	1.437	5.1	1.638	5.2	1.634	4.7	1.775	5.6	1.604
T_Crumbliness	6.3	1.871	4.8	1.886	4.6	1.971	6.2	2.099	3.8	1.815
T_Juiciness	4.2	1.419	5.1	1.226	4.9	1.312	4.5	1.215	4.9	1.478
T_Chewiness	4.8	1.838	6.0	1.646	6.2	1.517	5.4	2.042	5.9	2.079
T_Pastiness	4.7	1.445	3.1	1.841	3.5	1.350	3.7	1.730	3.6	1.908
T_Teeth adherence	5.3	1.699	4.4	1.625	4.2	1.396	5.3	2.079	4.4	1.504

References

Macfie, H., Bratchell, N., Greenhoff, K. & Valiis, L.V. (1989) Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. *Journal of Sensory Studies*, **4**, 129-148.

ISO (2007) ISO Standard 8589 Sensory analysis -- General guidance for the design of test rooms.

Sub-task 13.3.1 Effect of DHA/EPA/ARA ratio in non-fish meal grow-out diets on fish performance.

The subtask was reported in the 3rd periodic report and was submitted during the 4th reporting period as the Deliverable ***D13.4 Determine the effects of essential fatty acids and Tau in non-fish meal feeds on flesh and bottarga quality in grey mullet*** and is presented here in brief.

Carotenoids in fish, such as astaxanthin and β carotene have a range of functions that include protection against ultraviolet rays, production of provitamin A, tolerance against hypoxia, better growth performance, enhancement of the immune system, and as antioxidants. Grey mullet can ingest carotenoids through the consumption of micro- and macroalgae, epiphytes, small invertebrates and detritus. During female gonad development, carotenoid is released into the circulatory system from liver and muscle tissues before transferring to the ovaries. This experiment tested if dietary carotenoid supplementation enhances egg roe size and coloration as an initial stage for controlled bottarga (karasumi) production.

Materials and methods

Grey mullet captive brood stocks consisting of 3-year old (G2 domesticated) females and males, were maintained in outdoor 4 m^3 tanks supplied with ambient (Red sea) seawater at 40 ‰ salinity which were exposed to natural fluctuations of light and temperature. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. During early June, concomitant with the onset of gametogenesis, the fish were divided into two groups and fed over 3 months with the IOLR pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid (see D13.3). However, the FO pelleted diet was also supplemented with Marigold petal meal (MgM; 3 mg kg^{-1} feed) as another carotenoid source, apart from the fish oil and 3% dry Ulva (produced at the IOLR). This meant that the total carotenoid level in the FO+MgM diet was *ca.*138 mg kg^{-1} while the SO diet was *ca.* 99 mg kg^{-1}. During mid-September, coinciding with advanced stages of gametogenesis, 10 to 12 fish were sampled from each dietary group and the following parameters were measured; total length (cm), body weight (BW;g), gonadal weight [GW;g), liver weight [LW;g] and viscera weight (g). The gonadosomatic and hepatosomatic indices were calculated as GSI = 100xGWxBW^{-1} and HSI = 100xLWxBW^{-1}, respectively. In addition, histological examination of the gonadal reproductive status was assessed and analyses of gonadal proximate composition, lipid classes, fatty acid profiles as well as water and lipid soluble vitamins were carried out. Finally, the gene expression of vitellogenin, as a function of diet, was determined.

Results

The FO+MgM dietary treatment demonstrated significantly ($P<0.05$) higher carotenoids than the SO diet (**Fig. 13.21**). **Fig. 13.22** clearly shows that fish receiving the (A) FO+MgM had distinctly yellow gonads compared to (B) the pale, colourless gonads from females fed the SO diet and this is supported by the significantly ($P<0.05$) higher carotenoid level in the FO+MgM female gonads compared to those feeding on the FO diet (**Fig. 13.23**). Mean values of Vgs mRNA expression levels relative to β-actin of grey mullet individuals showing vitellogenic oocytes are reported in **Fig. 13.24**. Liver VgA and VgB transcript levels of hatchery-produced individuals were significantly lower than wild breeders, whereas no difference in VgC transcript levels was found between the two groups. Wild grey mullet sampled during their migration from the Lesina Lagoon to the open sea spawning grounds showed significantly larger oocytes at late

vitellogenesis stage than hatchery-produced individuals (487.4 ± 10.0 *vs* 428.5 ± 7.9; P<0.05). However, the yolk content of oocytes from hatchery-produced grey mullet did not differ from that of oocytes having similar diameter from wild adults (**Fig. 13.25**).

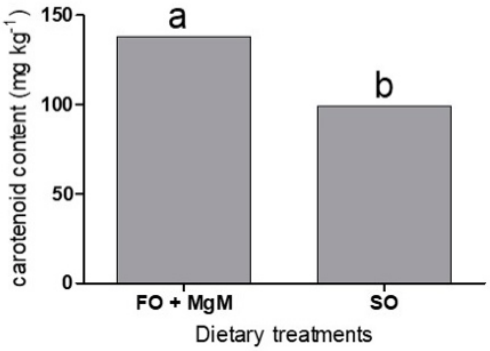


Figure 13.21. Carotenoid content of the different diets. The fish oil (FO) + marigold petal meal (MgM) and soybean oil (SO) treatments were based on the IOLR formula that were pelleted in a California pelleting mill.

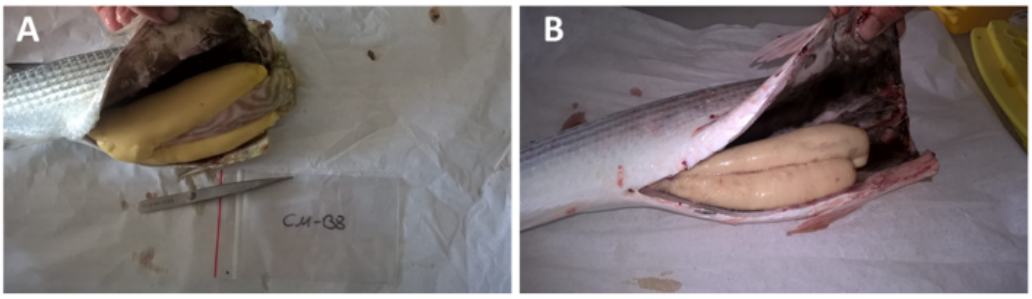


Figure 13.22. Roe derived of 3-year old captive mullet fed (A) a fish oil based diet (FO) demonstrating a yellow colour and (B) a vegetable oil based diet (SO) showing a lack of colour.

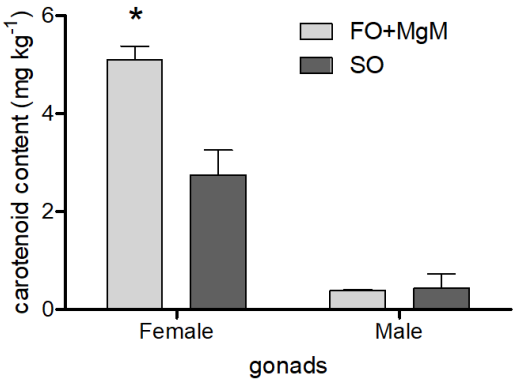


Figure 13.23. The effect of the fish oil +marigold petal meal (FO+MgM) and soybean oil (SO) on carotenoid (mg kg^{-1}) levels in female and male gonads. The asterisk (*) represents a significant (P<0.05) difference between bar values in female gonads.

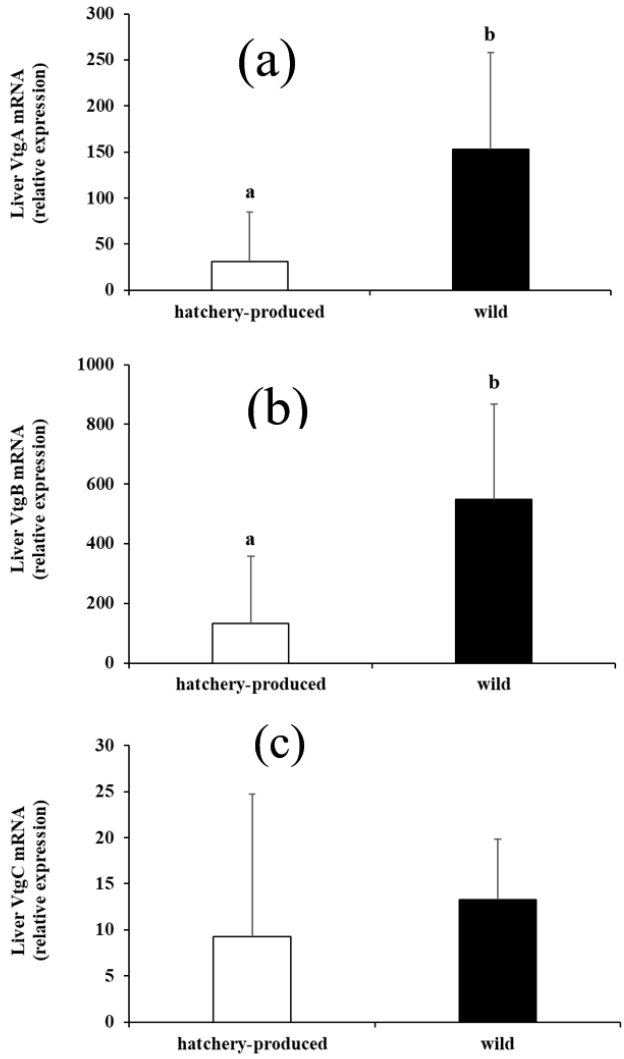


Figure 13.24. Mean vitellogenin A (VgA), vitellogenin B (VgB) and vitellogenin C (VgC) expression levels relative to β-actin in wild and hatchery-produced grey mullet.

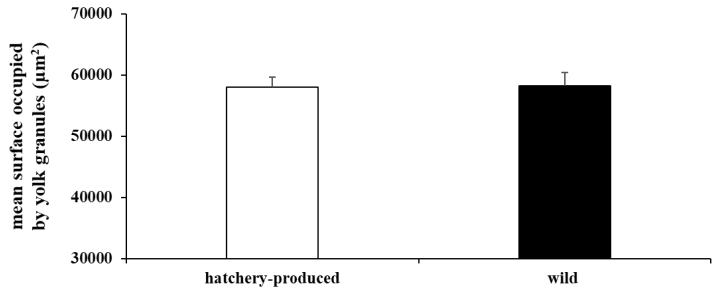


Figure 13.25. Mean surface occupied by yolk granules in late vitellogenic oocytes having similar diameter from hatchery-produced and wild grey mullet.

Discussion

In the Mediterranean, captive reared grey mullet brooders fail to proceed with gametogenesis. Males with running milt are rarely observed, and female dysfunctions were confined to two critical phases, i.e., the early stages of vitellogenesis, and final oocyte maturation and ovulation. It was suggested that low percentages of fully mature females, surrounded by undeveloped females (of the same age and size), typify a state of social hierarchy, in which the dominant female(s) suppresses sexual maturation of conspecifics. However, there may be potential effects of domestication such as in oogenesis when comparing captive-reared vs. hatchery-produced grey mullet (D7.5). In captive reared females, analysis of GSI and oocyte diameter clearly confirmed that age 3 hatchery-produced specimens attained a more advanced ovarian development than fish caught from the wild and reared in captivity. Nonetheless, the 3-year old hatchery-produced grey mullets had less advanced oocytes than 4-6-year-old wild breeders. About 50% of the hatchery-produced fish had late vitellogenic oocytes whose mean diameter was lower than that of late vitellogenic oocytes from wild individuals. VgA and Vg B liver gene expression was significantly lower in hatchery-produced compared to wild fish, whereas VgC expression was similar. Oocytes from hatchery-produced individuals had the same capacity to uptake vitellogenin and accumulate yolk proteins compared with oocytes of similar size from wild breeders. It was concluded that (i) the rearing condition established at IOLR allows a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea, and (ii) hatchery-produced grey mullet have a good potential to develop ovaries spontaneously up to a condition useful for bottarga production (advanced vitellogenesis).

Grey mullet ovaries described a predominance of neutral lipids (mainly TAG and WE-SE) over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs. This pattern can be related to the role of neutral lipids as metabolic energy resources for oocyte formation and embryo development. In fact, wax esters have been described as a particularly monounsaturated fatty acid (MUFA)-rich lipid fraction. It can be seen in the fatty acid profiles of all female gonads that the MUFA group was the most abundant one (45-50%).

In spite of 18:2n-6 being higher in the SO diet, homeostatic mechanisms seem to allow the brood stock to maintain a balance and similar profile of polyunsaturated fatty acids (PUFA) in the gonads, independently of the sex or diet, displaying also a similar pattern to that of the wild counterparts. Higher levels of 18:2n-6 and lower of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. Finally, it is also worthwhile to highlight that independently of the diet the testes always displayed lower contents of total fat and lower proportions of 18:2n-6 but much higher ones of DHA and ARA than the ovaries.

Taken altogether, the supplementation of carotenoids to brood stock diets not only appears to improve larval performance (D13.3) but also contributes to bottarga coloration and a platform for further dietary and bottarga product development.

Sub-task 13.3.3 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.

Deliverable 13.5 ***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*** has been submitted during the 4th reporting period and is presented here in brief.

Due to its high protein content and digestibility as well as a balanced amino acid profile, soybean meal (SBM) is widely used as a protein meal replacement in the growing of carnivorous and herbivorous

species. Importantly, the use of significant levels of soybean meal in fish diets can cause an inflammatory response in the distal intestinal epithelium resulting in enteritis, which has been reported in salmonid and other carnivorous fish as well as omnivores such as carp. Inflammation is generally associated with oxidative stress, which affects the innate antioxidant system. As a result, the aim of this study was to evaluate the effect of further increasing the level of soybean meal in the diet, which contains other plant-based proteins, such as wheat and rapeseed, on juvenile grey mullet performance and the presence of inflammation. In addition, the effect of dietary supplementation of powdered *Haematococcus* algae (containing *ca.* 3% astaxanthin), as an effective antioxidant to reduce oxidative stress and the upregulation of the innate antioxidant system, was tested.

Materials and Methods

Juvenile F2 grey mullet (50 dph) spawned from **P4.IOLR** brood stock were stocked (100 fish tank^{-1}) in twelve 400 l V-tanks where UV treated, filtered (10 µm), ambient seawater (diluted to 25 ‰) at 25±0.5 ^{o}C, entered the tank from the bottom and exited through 500 µm mesh filters near the top with an exchange rate of 9.5 exchanges day^{-1}. The experimental set up allowed the testing of three dietary treatments (A, B, C) with 4 replicate tanks treatment^{-1}. Treatment A was the IOLR formulated grey mullet control diet and contained *ca.* 13% DW poultry meal, which was the only source of animal protein. Treatment B replaced the poultry meal fraction with soybean meal, so that the diet was isonitrogenous with the control. Treatment C was identical to Treatment B but was supplemented with dried microalgae (*Haematococcus* sp.), which contains *ca.* 3% astaxanthin, resulting in 25 ppm of this carotenoid being added to the diet.

The fish were fed the 1.4 mm pelleted experimental diets from 50 to 90 dph at a ration size of 6% wet body weight day^{-1} equally distributed over 4 meals. The experimental system was illuminated under a 12L:12D photoperiod with a light intensity of 500 lx. At the end of the study, 4 fish from each experimental tank were taken, sacrificed in excess MS-222, weighed individually and standard length measured. All sampled fish at the end of the study were treated identically in the following manner. The digestive tracts (DT) from the fish were dissected out and measured. The midgut was considered to be between 4-6 cm posteriorly from the junction of the stomach and foregut while the hindgut was considered to be between 10-12 cm from the end of the midgut posteriorly to the anus. The DT sections from two sampled fish from each tank were placed in Eppendorf tubes containing 10% buffered neutral formalin and stored at -32 ^{o}C for later histological analysis. In parallel, the DT sections of two fish from each tank were placed in Eppendorf tubes containing RNAlater and stored at -32 ^{o}C for later RNA extraction and gene expression of the innate antioxidant system. For histology, DT samples were dehydrated and embedded in paraffin blocks where 5 µm sections were stained with hematoxylin and eosin and the slides examined under a microscope.

Results

In **Fig. 13.26a** juvenile grey mullet fed the control diet A, which contained poultry meal, gained significantly more weight ($P<0.05$) than fish fed the diets B and C, respectively. Diet A fish also exhibited markedly ($P<0.05$) longer and heavier digestive tracts (**Fig. 13.26b,c**) than fish fed diets B and C which, in general, differed little from each other. In addition, fish fed Diet A produced higher numbers of larger fish (>5 g) than fish fed Diets B and C, which was significant ($P<0.05$) when comparing diets A and C (**Fig. 13.27**). Conversely, there was a tendency for Diet C to produce larger numbers of smaller fish, particularly in the <3-5 g range. There was no dietary effect on general digestive tract health, mucosa cells length and the presence of inflammation nor was there any mortality found among all tanks in the experiment during the course of the experiment.

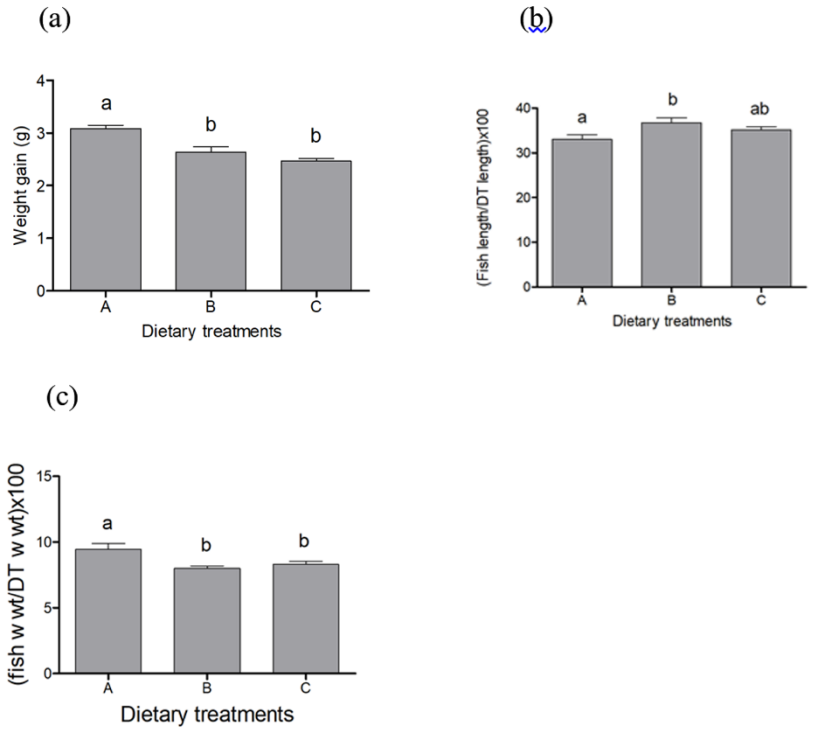


Figure 13.26. The effect of dietary treatments (A, B, C) on (a) average fish wet weight gain (g), (b) the ratio of the average fish length/digestive tract (DT) length x 100, (c) the ratio of the average fish wet weight (wwt)/digestive tract (DT) wwt x 100. Bars within a graph having different letters were significantly ($P<0.05$) different.

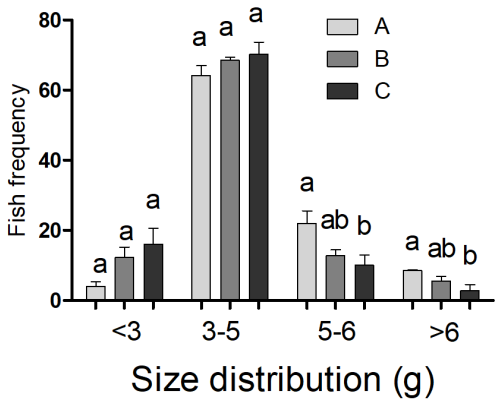


Figure 13.27. The effect of dietary treatments (A, B, C) on the size distribution among replicate populations in each of the treatments. Bars having different letters, within a size group, were significantly ($P<0.05$) different.

Discussion

In this study, there was no indication of inflammation, in terms of shortened mucosal folds or thickening of the lamina propria and sub-epithelial mucosa. In fact, DT samples from all fish exhibited healthy tissue with no signs of disease and presumably oxidation stress. Although there was a significant improvement in the performance of fish fed the control-poultry meal diet, the change was not large nor due to triggering an

inflammation response. There are differences in nutrient composition between soybean and poultry meal which may suggest an alternative hypothesis to the moderate effect of animal based meals. Plant based meals do not contain taurine, while it is found in considerable levels in animal protein. Taurine is an amino sulfonic acid that plays an array of critical roles in bile salt synthesis, anti-oxidative defense, cellular osmoregulation, as well as contributing to visual, neural and muscular function and development. Consequently, its absence in all plant based protein in diets B and C might explain the benefit of poultry meal. Another potential deficiency is the sulphur containing essential amino acid methionine, which is often the first limiting nutrient and is generally at lower levels in plant based proteins such as soybean meal while considerably represented in the protein fraction in poultry meal. Taken together, the results suggest that there is a significant improvement in grey mullet juvenile performance when using animal based proteins, such as poultry meal, at about 13% DW diet in the IOLR formula. On the other hand, this advantage may be modulated by the supplementation of essential amino acids such as methionine and the sulfonic amino acid, taurine.

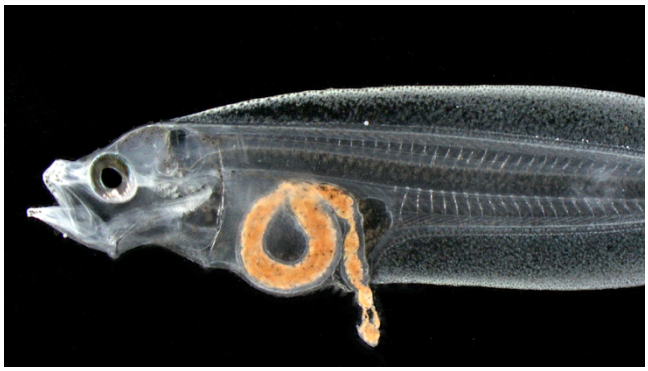
Deviations from Annex I and their impact:

There were no deviations

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Group Work Packages

Larval husbandry



In greater amberjack an industrial protocol based on the results of previous reports was tested in two commercial hatcheries in Greece, which produced 15,000 and 48,300 juveniles, respectively, for on growing.

In pikeperch, the overall aim was to develop an industrial protocol based on an optimal combination of rearing factors. The protocol was not applied fully due to technical limitations and problems with the pikeperch broodstock of the SME. However, six recommendations have been proposed to promote an industrial protocol for pikeperch larval rearing.

In Atlantic halibut, all objectives, tasks and deliverables have been achieved and reported on. In wreckfish, no final industrial protocol was developed. On the other hand, a significant step forward was achieved as juvenile wreckfish were produced and weaned onto prepared diets. At the writing of this report, there are 25 juveniles of more than 150 dph.

In grey mullet, semi-commercial production runs testing the P4.IOLR protocol in 6 m^3 tanks produced 78,704 juveniles while the entire production in 2017 was ca. 200,000 fish. When comparing the "greening" of larval mullet rearing tanks with effective levels of lyophilized or live *Nannochloropsis oculata,* the results showed very similar larval performances, in terms of rotifer ingestion rate, swim bladder inflation, growth and survival as well as larval digestive tract enzyme activity. The protocols for the production of *Tisbe* spp. copepods and their ozone disinfectant treatment were developed at P4.IOLR. However, testing the effect of co-feeding rotifers and disinfected copepods did not succeed due to the complete mortality of eggs, pre-larvae and end of rotifer feeding larvae, when transferred to the experimental aquarium system. These results suggest that mullet larvae are extremely sensitive and all handling should be avoided before 25 dph.

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

1. Group **A:** Weaning on dry feed started from 20 dph and completed on 30 dph, (control group)
2. Group **B:** Weaning started from 20 dph and completed on 30 dph (same as the control but using half the amount of *Artemia* metanauplii)
3. Group **C:** Weaning started from 15 and completed on 25 dph
4. Group **D:** Weaning started from 12 dph and completed on 23 dph, with three replicates each.

Samples were taken periodically for biochemical analyses (lipids and fatty acid composition of larvae and live prey), digestive and antioxidant enzyme analyses, growth (length and weight) and skeletal deformation analysis.

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

The task was finished in 2015 and the deliverable already delivered in May 2016. Two trials were carried out, one in 2014 (already described in the first periodic report) and another in 2015. In both trials different feeding regimes using the standard protocol for larval rearing and weaning at day 20 or early weaning at day 12 or day 15 post hatch, using half of the amount of the Artemia provided in the standard protocol. Although early weaning in meagre can be carried out, care should be taken to avoid cannibalism (very high especially if weaning is carried out very early -12 dph- and using low quantity of Artemia). Thus, it was recommended to use low light intensity (200 lux), increase the number of feeding doses along the day, and grade the fish separating big larvae (cannibals) from the tank to allow small larvae to continue growing. In the trials a commercial feeding (Gemma micro, Skretting) was used, the formulation of this type of microdiets has been improved in the last years and probably in the future the big differences in microdiet acceptance and larval growth will be improved.

The results obtained in the study also showed that the larvae were able to digest the microdiets in an effective way and no severe skeletal deformations could be detected, early weaning had not any significant effect on the incidence of total skeletal deformities in the juveniles of meagre.

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

During the 3rd Reporting Period the results reported in the deliverable ***D14.1 Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better-quality juveniles*** (2nd periodic report) were published in Aquaculture Research

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A. 2017. Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research, 48: 5289-5299

Summary of progress towards objectives (49-60 Mo):

No work was undertaken during this reporting period, as all work was completed in previous reporting periods.

Details for each Task

Task 14.1 Determining the earliest and most cost effective weaning period (led by IRTA, Alicia Estevez and Enric Gisbert and ULL, Covadonga Rodriguez and Jose Perez).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D14.1 Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles***.

Deviations from Annex I and their impact:

There were no deviations from the DOW.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estévez, A. 2017. Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research, 48: 5289-5299.

WP 15 Larval husbandry – greater amberjack

WP No:	15	**WP Lead beneficiary:**		P2. FCPCT
WP Title (from DOW):	Larval husbandry – greater amberjack			
Other beneficiaries (from DOW):	P1. HCMR	P8. IEO	P15. ULL	P27. FORKYS
Lead Scientist preparing the Report (WP leader):	Carmen María Hernández Cruz (P2)			
Other Scientists participating:	Nikos Papandroulakis (P1), Jerez Salvador (P8), Covadonga Rodríguez (P15), Popi Tsakoniti (P40)			

Objectives

1. Effects of different feeding strategies on larval performance in intensive systems,
2. Development of feeding protocol and rearing system in mesocosm semi-intensive systems,
3. Development of industrial protocol for larval rearing.

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

Task 15.1 Effect of feeding regime and probiotics. To achieve the objectives proposed in this task a first trial of rotifer enrichment was performed. Different proportions of *Echium* oil were used to enrich the rotifers considering 4 different enrichment periods (3, 6, 10 and 24 hours). The best results obtained, density and frequency supply of enriched prey will be assayed on amberjack larval rearing. **Task 15.2 Comparison of semi-intensive and intensive rearing.** During the reporting period preliminary trials were performed in order to establish the larval rearing methodologies in the two rearing systems at the P2. FCPCT. Three different larval rearing densities will be evaluated: 25, 50 and 75 eggs l^{-1} in triplicate tanks for a period of 30 days in two experiments. In all experiments, severe cannibalism and dispersion of total length was observed.

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

The main objective of the studies during this period was to improve the survival, growth and performance of greater amberjack larvae by improving the feeding regime, culture density and larval culture conditions. 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. Following the first trial with the very low overall survival in the two systems, a second experiment was organised in 2016. The survival in the case of the mesocosm was 18.7±0.8% while it was 8.2±3.1% for the intensive tanks. The gene expression of IGF-I and some of the IGFBPs were affected by the rearing method while the GHRH, GH, IGF-II were not.

Task 15.3 Effect of environmental parameters during rearing. The hydrodynamic field was estimated in tanks of 2,000 and 40,000 l, and the results showed higher survival at the end of the experiment in 2,000 l tanks, independent of egg stocking density, compared to the 40,000 l mesocosm tanks. The results of deformity evaluation showed a markedly appearance of different types of skeletal anomalies in all treatments throughout the larval stages The photoperiod study of (24L:00D vs 18L:6D), according to the results, photoperiod did not affect the mRNA expression of any of the IGF binding proteins studied except IGF-BP1, which was higher in fish reared under the condition of 24L:00D. The study of the effect of tank color showed no differences in larval growth in terms of total length and body weight between the different tank colors, but differences were observed in the survival rates among the different groups. The gene expression analysis revealed significant differences among the treatments. Tank colour appeared to have an effect at the mRNA expression levels of GH at 17 dph with fish reared with a white background exhibiting the highest levels of expression while the lowest was in fish reared in the green tanks. The work done is fully described in deliverable 15.3

Task 15.4. Development of industrial protocol. During this period a preliminary assay of semi-intensive mesocosm larval rearing was performed in preparation for experiments in the following years. Samples of larvae from hatching to end of metamorphosis were collected to evaluate ossification pattern and staining protocols.

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

The main objective of the present studies was the development of an industrial protocol for the larval rearing of greater amberjack based on the results of the previous tasks. To achieve this objective, the trials carried out were based on the information collected in the previous reports. Survival obtained differed widely between the different trials, ranging from < 1% to ~ 10%. With all the available information, general guidelines for larval culture were established. To evaluate ossification pattern and staining protocols, samples of larvae, from 3.38 ± 0.15 mm to 18.52 ± 0.73 mm standard length, were studied. Staining protocols were made point. It was observed that the ossification begins in the skull when the larva has a size of 3.38 ± 0.15 mm and the larva is completely ossified when it has a size of 13.03 mm ± 0.09. For the evaluation of the developed protocols some trials were tested in two Greek hatcheries: Galaxidi Marine and NIREUS Aquaculture hatchery in collaboration with HCMR. Following the hatchery phase, individuals were transferred for weaning and selected in size. The result of the adaptations was a significant improvement in the performance of the larvae and in particular their survival rate. A total of 63.300 juveniles were finally transferred to cages.

Task 15.1. Effect of feeding regime and probiotics. In this study different rotifer enrichment treatments were tested. They included commercial enrichments; LC60/20:4n-6/10ppm carotenoids, LC60/20:4n-6/10ppm carotenoids and 20% *Echium* oil, and T4 LC60/20:4n-6/10ppm carotenoids+ 20% Black cumin oil. The study showed that the rotifers enriched for a short period (3 h) with marine lecithin supplemented with 20% *Echium* oil showed the best results compared to the other commercial treatments although it was not significant. The results of the present trial suggest the positive effect of experimental live prey enriching emulsions supplemented with immune modulators such as *Echium* oil and black cumin oil compared to commercial emulsions on larval performance of *Seriola dumerili*.

Task 15.2. Comparison of semi-intensive and intensive rearing. Following the first trial with the very low overall survival in the two systems, a second experiment was organised in 2016. The survival in the case of the mesocosm was 18.7±0.8% while it was 8.2±3.1% for the intensive tanks. The gene expression of IGF-I and some of the IGFBPs were affected by the rearing method while the GHRH, GH, IGF-II were not.

Task 15.3 Effect of environmental parameters during rearing. The hydrodynamic field was estimated in tanks of 2,000 and 40,000 l, and the results showed higher survival at the end of the experiment in 2,000 l tanks, independent of egg stocking density, compared to the 40,000 l mesocosm tanks. The results of deformity evaluation showed a marked appearance of different types of skeletal anomalies in all treatments throughout the larval stages The photoperiod study of (24L:00D vs 18L:6D), according to the results, photoperiod did not affect the mRNA expression of any of the IGF binding proteins studied except IGF-BP1, which was higher in fish reared under the condition of 24L:00D. The study of the effect of tank color showed no differences in larval growth in terms of total length and body weight between the different tank colors, but differences were observed in the survival rates among the different groups. The gene expression analysis revealed significant differences among the treatments. Tank colour appeared to have an effect at the mRNA expression levels of GH at 17 dph with fish reared with a white background exhibiting the highest levels of expression while the lowest was in fish reared in the green tanks. The work done is fully described in deliverable 15.3.

Task 15.4. Development of industrial protocol. During this period a preliminary assay of semi-intensive mesocosm larval rearing was performed in preparation for experiments in the following years. Samples of larvae from hatching to end of metamorphosis were collected to evaluate ossification pattern and staining protocols.

Summary of progress towards objectives (49-60 Mo):

The main objective of the present studies was the development of an industrial protocol for the larval rearing of greater amberjack based on the results of the previous reports. The results obtained were validated and tested in two commercial hatcheries in Greece. In the first hatchery, incubation was directly in the larval tanks at a density of around 120 eggs l^{-1}. Following hatching the density of the larvae was about 75 individuals l^{-1}, indicating a survival rate of 62%. Feeding was based on enriched rotifers and subsequently with Artemia and dry feeds. Frozen eggs were also added in the tanks after 20 dph. Following the hatchery phase, individuals were transferred for weaning and selected in size. The final number of juveniles transferred for pre-growing was around 15.000 that were classified in 4 size-classes between 0.3 and 2.5 g. The trials in the second hatchery were implemented again with direct incubation of eggs in the larval rearing tanks. According to the standard protocol of the hatchery, eggs after transport are incubated and transferred only after mouth opening to larval rearing tanks, a procedure proved to be lethal for the larvae. The hatchery received 4 batches of eggs of 1.0, 1.2, 0.65 and 0.5Ms respectively. Larval rearing was performed following the standard protocol and the feeding was based on enriched rotifers, instar I and enriched instar II Artemia nauplii followed by artificial diets. Following 20 dph, fish were selected in size and grouped accordingly, improving thus significantly the performance of the larvae and in particular their survival rate. The hatchery finally transferred to cages 48.300 juveniles of 25-50 g.

Details for each Task

Task 15.1. Effect of feeding regime and probiotics (led by IEO, Salvador Jerez, Virginia Martín, ULL, Covadonga Rodríguez, José Pérez).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.2 Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing.***

Task 15.2 Comparison of semi-intensive and intensive rearing (led by HCMR).

Sub-task 15.2.1 Comparison between intensive and semi-intensive larval rearing (lead by HCMR, Nikos Papandroulakis).

Sub-task 15.2.2 effect of stocking density on larval performance (lead by FCPCT, Carmen Mª Hernández-Cruz).

Sub-task 15.2.3 Ontogeny of greater amberjack larval digestive system focusing on proteases, amylases and ATPase (lead by ULL, Covadonga Rodríguez).

This task (and its 3 subtasks) has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.1. Effective greater amberjack larval stocking densities***, and ***Deliverable 15.2. Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing*** and in ***Deliverable 15.4 Ontogeny of greater amberjack larval vision and digestive system***.

Task 15.3 Effect of environmental parameters during rearing (led by FCPCT, Carmen Maria Hernández Cruz and HCMR, Nikos Papandroulakis).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing.***

Task 15.4 Development of industrial protocol (led by IEO, Jerez Salvador).

This task (and its 3 subtasks) has been completed and the full description of the work and results is provided in ***Deliverable 15.5 "An industrial protocol for greater amberjack larval rearing".***

Sub-task 15.4.1 Development of an industrial protocol for larval rearing based on the results of the previous tasks (by IEO, Salvador Jerez, Virginia Martín, Eduardo Almansa).

A trial to evaluate the effects of a feeding protocol on growth, survival and development of larvae of greater amberjack reared in two different tank volumes and light intensities was carried out and assessed in terms of the development of organs involved in swimming, food detection, prey intake and health status (oxidative stress and immune system).

The newly hatched larvae obtained from F1 *Seriola dumerili* broodstock at the IEO-COC facilities were stocked (3 larvae l^{-1}) in circular rearing tanks of 32 and 40 m^3 with different surface-volume ratios (0.5 and 0.7 m^{-1}, respectively), and surface light conditions (2,500 and 1,000 lux, respectively).

The larvae were reared under natural photoperiod (14L:10D) at ambient seawater temperature (23-25ºC) with a rate of renewal during the larva rearing as follows: 15-40% day^{-1} at 1 dph, 30-40% at 10 dph, 100-120% at 20 dph, and 200-240% at 30 dph, achieving an oxygen saturation of 89-95% (6.3 to 6.6 mg l^{-1}).

Phytoplankton (*Chlorella sp.*) was added daily to the larvae tanks from 1 to 25 dph. Rotifers (*Brachionus plicatilis*), enriched with DHA Protein Selco (INVE S.A., Belgium), were added twice a day (8:00 and 16:00) from 3 to 25 dph at densities between 3 and 10 rot ml^{-1}. During the rotifer feeding, copepods were introduced to the rearing tanks due to the natural productivity in the rotifer culture. At 12 dph, Artemia AF nauplii were added during 5-7 days, and Artemia EG 1-day enriched with A1 DHA Selco (INVE S.A., Belgium) were offered twice a day at 14-18 dph. The weaning diet began at 18-20 dph, and were added progressively according to fish size (NRD 2/4 size of 200-300 µm, and NRD 3/5 size of 300-500 µm, INVE S.A., Belgium).

Larvae were sampled periodically to evaluate growth parameters (total length, eye diameter, percentage of larvae with inflated swim bladder and swim bladder large and height). Prey intake was determined by analysis of stomach contents. At the end of the trial (30 dph) larvae of each tank were counted and the percentage of survival calculated.

Larvae samples were collected from 32 and 40 m^3 tanks at 12 and 25 dph, and were examined for oxidative stress in terms antioxidant enzymes catalase (CAT), Glutathione S-transferase (GST), Superoxide

dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) and humoral parameters of the immune system (activity of peroxidase, proteases and anti-proteases, anti-bactericidal).

The newly hatched greater amberjack larvae (3.50±0.16 mm TL) started to feed at 4 dph (3.63±0.32 mm TL). The TL increased exponentially during the larval rearing (**Fig. 15.4.1.1a**). The growth of larva reared in 32 m^3 tank was faster and the survival higher than those reared in 40 m^3, reaching 13.6±1.9 and 10.1±0.7 mm TL, and 2.5 and 1.8 %, respectively, at 30 dph. The percentage of larvae with inflated swim bladder (**Fig. 15.4.1.1b**) was higher and its volume greater in 32 m^3 tank than 40 m^3 tank. In 32 m^3 tank, all larvae showed the swim bladder inflation at 7 dph (3.84±0.30 mm TL), and at 14 dph in 40 m^3 tank (4.59±0.29 mm TL).

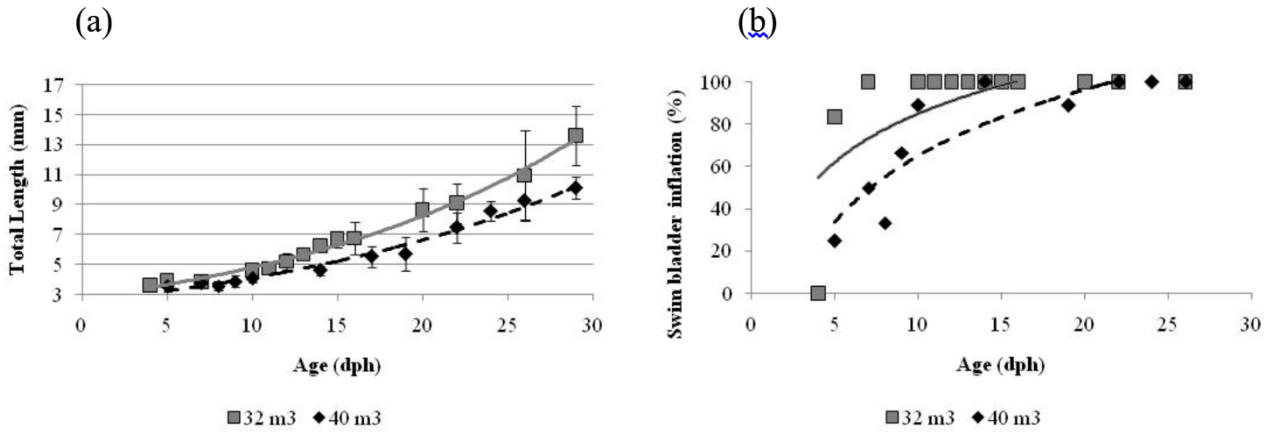


Figure 15.4.1.1, a, b. Total length (mm) **(a)** and swim bladder inflation (%) **(b)** of greater amberjack larvae reared in 32 and 40 m^3 tanks. Values are mean ± SD (n=10).

Larval growth and survival are affected by the successful capture of the prey by the larvae and the proper development of the organs (eyes) involved. The ability to detect the prey in the first days of feeding is linked to the positive allometric increase of organs. In this study, allometric growth pattern of the ratio of eye diameter to TL was positive and higher in 32 m^3 than 40 m^3 tanks in larvae up to 6.0 mm TL, and it became isometric and similar after this larva size. The different location of the larva rearing tanks (indoor or outdoor) as well as the different tank depth (volume) leads to a higher light intensity (surface and depth) in the 32 m^3 than 40 m^3 tank. This could explain the earlier inflexion point in eye diameter-TL ratio at younger age and higher growth in larvae reared in 32 m^3 compared to 40 m^3 tank.

The prey items in the stomach of greater amberjack were related to age (dph) and TL of larvae. The larvae showed prey items at 4 dph. The intake remained low over the first days, and increased considerably with age in both tanks. Larvae ingested a higher number of rotifers at 7-10 dph (between 3.7 and 4.2 mm TL) and they did not show rotifers in their stomach after 16-19 dph (about 6.4 and 4.6 mm TL in larvae reared in 32 and 40 m^3 tank volume, respectively) (**Fig. 15.4.1.2a**). Copepods were present in the stomach of larva from first feeding to more than 20 dph. This prey item supposed from 10% to more than 50% of total prey intake in 32 and 40 m^3 tanks (**Fig. 15.4.1.2b**). Artemia nauplii were found in the larvae stomach immediately after being offered (12 dph), in larvae from 5.2 and 4.3 mm TL in 32 and 40 m^3 tanks, respectively (**Fig. 15.4.1.2c**).

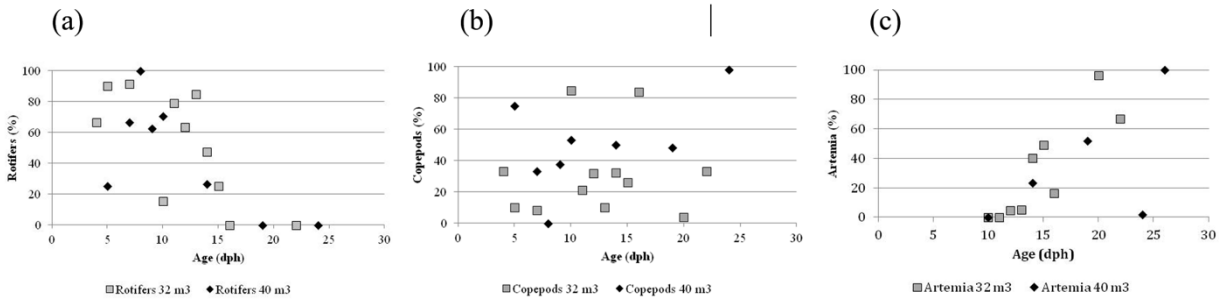


Figure 15.4.1.2 a, b, c. Relationship between rotifers (**a**), copepods (**b**) and *Artemia* (**c**) intake and Age (dph) in greater amberjack larvae reared in 32 and 40 m^3 tanks.

The levels of antioxidant enzymatic activities and lipid peroxidation measured in larvae at 12 and 25 dph tended to decrease with age in both tanks. In addition, larvae from 40 m^3 tanks seemed to show lower Catalase and SOD activities than 32 m^3 tank larvae. Lipid peroxidation levels (based on TBARS presence) also seemed to be higher in larvae from tank 32 m^3 compared to 40 m^3 at 12 dph (**Fig. 15.4.1.3)**.

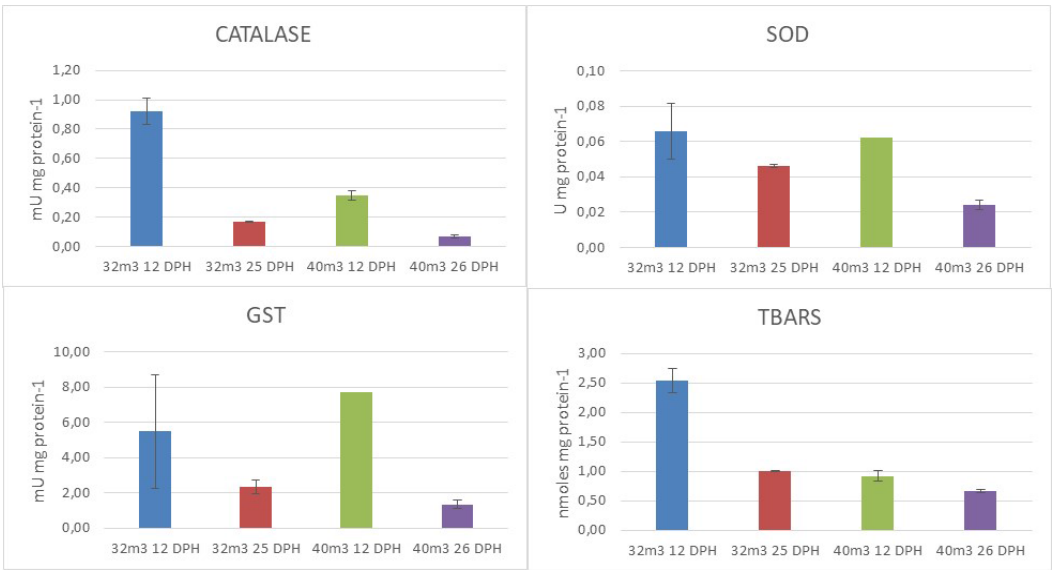


Figure 15.4.1.3. Antioxidant enzymes Catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) of 12 and 25 dph greater amberjack larvae from 32 and 40 m^3 tanks. Values are mean ± SD (n=2).

The age was a crucial factor to consider determining the biochemical responses to oxidative stress. A decrease in some of the antioxidant activities was observed from 12 to 25 dph larvae independent of tank volume. The catalase activity was affected by rearing conditions differently depending on the age of the larvae. The activity of this enzyme seemed reduced in 40 m^3 tank compared to 32 m^3 at 12 dph. The effects of tank volume on the peroxidation status of the larvae were evident at 12 dph.

Higher values of TBARS were observed in larvae from 32 m^3 tank. In general, an amelioration of enzymes antioxidant activities was observed in 25 dph larvae compared to 12 dph, and in 40 m^3 tank compared to 32 m^3.

Sub-task 15.4.2 Ossification pattern and incidence of skeletal deformities for amberjack larvae (by FCPCT, Carmen Maria Hernández Cruz)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing*** and are provided briefly here**.**

Different trials (3.1 Density) were used to study bone ossification and skeletal anomalies. Samples of amberjack larvae from hatching to the end of metamorphosis were collected at regular intervals, to evaluate ossification pattern and skeletal anomalies. The bone mineralization of the vertebral column was completed at 30 dph as illustrated in **Fig. 15.4.2.1.** The cranial structures began to ossify by 3.38 ± 0.15 mm (**Fig. 15.4.2.2)**, with the calcification of upper jaw (premaxilla) and cleithrum. The last bones to calcify were registered in the otic region at 10.15 ± 1.86 mm

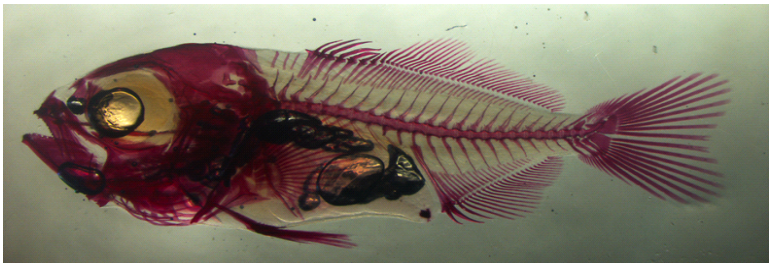


Figure 15.4.2.1. Image of alizarin red staining of greater amberjack *Seriola dumerili* (30 dph).

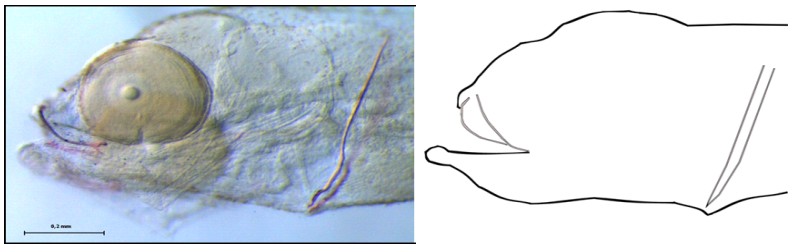


Figure 15.4.2.2. Cranial development to a size of TL (mm): 3.38 ± 0.15

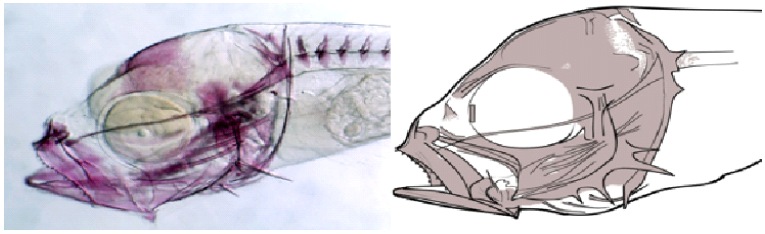


Figure 15.4.2.3. Cranial development to a size of TL (mm): 10.15 ± 1.86

There was a high percentage of severe anomalies found in all larvae of greater amberjack *Seriola dumerili* (30 dph)**,** where the highest percentages corresponded to the cranium and the haemal area. The results showed a marked appearance of different types of severe skeletal anomalies in all treatments throughout the larval stages such as lordosis, vertebral body fusion, and anomalous dentary that could lead to a lower survival (**Fig. 15.4.2.4).**

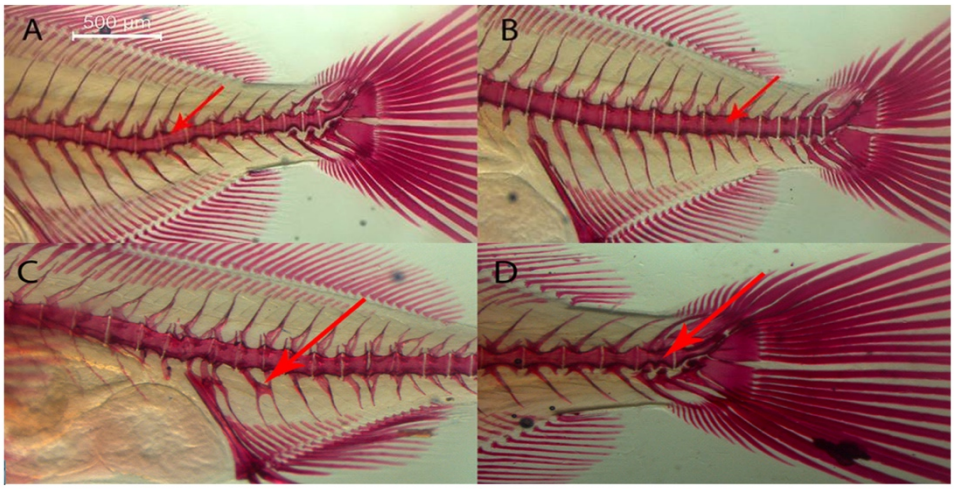


Figure 15.4.2.4. Different anomalies in greater amberjack *Seriola dumerili* (30 dph) **A**: Lordosis, **B**: Vertebral fusion, **C**: Fusion of neural arch and spines, **D**: Fusion of caudal vertebrae

Sub-task 15.4.3 Validation of the developed protocol initially at FCPTC and over two successive years in an SME hatchery (by HCMR, Nikos Papandroulakis and GMF, Popi Tsakoniti)

The evaluation of the developed protocols was tested in two hatcheries: *Galaxidi Marine Farms* and *NIREUS Aquaculture*

Galaxidi Marine Farms (GMF) larval rearing trials

The trials in GMF were performed in the hatchery of the farm at Galaxidi. The hatchery had already performed larval rearing of greater amberjack during 2015 and 2016 unsuccessfully while the trials in 2017 resulted in several thousands of individuals that were introduced in cages. Therefore, the personnel had experience with this species.

The eggs from induced spawning of breeders kept in GMF and Argosaronikos SA farm were used for the rearing. Incubation was directly in the larval tanks at a density of around 40 larvae l^{-1}. Phytoplankton was added in the larvae tanks from 2-15 dph. Light intensity was 800 lux on 3 dph, and increased to 1200 lux on 6 dph until 12 dph when it was decreased to 1000 lux and gradually to 500 lux until 20 dph. The photophase was continue (24L:00D) from mouth opening to 20 dph when it was decreased to 18L:06D until 30 dph where it was set to natural conditions.

Feeding was based on enriched rotifers and subsequently with Artemia and dry feeds.

An indicative growth curve of the larvae during 2018 is presented in **Fig. 15.4.3.1**.

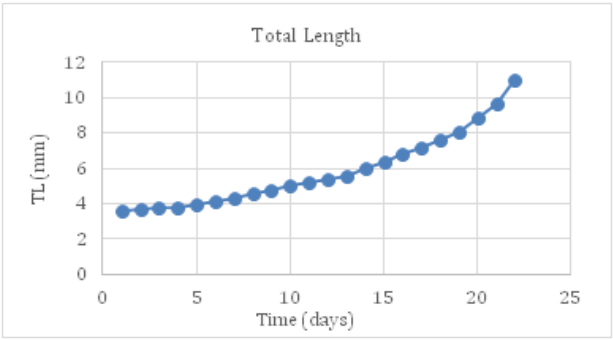


Figure 15.4.3.1. Total length (mm) of greater amberjack larvae reared in *Galaxidi Marine Farms.*

Until 20 dph no significant problems were observed and the estimated survival was about 10%. Following this day, significant mortality was observed due to cannibalism. The result of absence of any sorting in less than 10 days was the loss of more than 65% of the population. On day 30 dph 15.000 individuals were transferred to nursery.

NIREUS Larval rearing trials

The trials were implemented in the hatchery of the company at Nafpaktos, where some first trials for the larval rearing of greater amberjack had been performed the previous years.

The trials were implemented in the hatchery of the company at Nafpaktos, where some first trials for the larval rearing of greater amberjack had been performed the previous years.

During 2018 the hatchery repeated a larval rearing trial starting on June with eggs from the Argosaronikos SA. Eggs were delivered at a temperature of ~24.5 ^{o}C, and pH 7.54-7.8. Eggs were incubated directly in larval rearing tanks (T 24,5 ^{o}C και pH 7.8) without any previous treatments. Since mouth opening light intensity was high and diffused. First feeding was performed with rotifer with live algae supplementation. Artemia AF was delivered when larvae reached TL >5mm (10 dph), and Artemia EG when TL >6mm (14 dph). Since 18 dph artificial diet was provided (Skretting). Swim bladder inflation was >85%.

Density was reduced at 20 dph and larvae were sorted at 35 dph. It is estimated that presently there are several tens of thousands of individuals that are in the on growing phase

Deviations from Annex I and their impact:

There were no deviations from the DOW

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Tsalafouta, M., Pavlidis, N. Mitrizakis, N. Papandroulakis. Effect of background color and expression of genes related to the GH/IGF axis at early development of greater amberjack (*Seriola dumerili*). Article in preparation

Djellata, A. La Barbera, S. Sarih, A. Mesa-Rodríguez, R. Saleh, M. Izquierdo, H. Fernández-Palacios and C. M. Hernández-Cruz. Effect of different eggs stocking densities on growth, larval survival and skeletal deformities of greater amberjack (*Seriola dumerili*). Article in preparation

WP 16 Larval husbandry – pikeperch

WP No:	16	**WP Lead beneficiary:**		P9. UL
WP Title (from DOW):	Larval husbandry – pikeperch			
Other beneficiaries (from DOW):	P3. IRTA	P21. DTU	P29. F2B	
Lead Scientist preparing the Report (WP leader):	Pascal Fontaine			
Other Scientists participating:	Enric Gisbert (P3), Ivar Lund (P21), Jiri Bossuyt (P39)			

Objectives

1. Improvement of pikeperch larval rearing protocols by using a multifactorial approach,
2. Reduction of cannibalism rate to increase survival,
3. Development of industrial protocol to improve larval performance during rearing.

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

An experiment was planned in April 2014 to study the effects of four environmental parameters on the 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 work reported in the previous Reporting Period (13-30 Mo):

1 – The first experiment (exp. 1), initially planned in 2014 was repeated in January-March 2015. 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.

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

In the Task 16.1 optimal combinations of factors to improve pikeperch larval rearing were tested. The results related to the experiments 2 (effects of feeding-related factors) and 3 (effects of population parameters) are presented. In experiment 2 it was demonstrated that weaned juveniles of 1.0-1.5 g mean body weight can be produced in 53 days, with relatively good survival (3.6-13.1%). A longer weaning

duration increased mean swim bladder inflation (18% vs 67%) and final biomass increase. In addition, discontinuous feeding increased the final biomass produced in tanks while co-feeding (6 days) and the onset of the weaning period (10 or 16 days dph) had no significant effect on the final biomass and the percent of inflated swim bladders, while the method of food distribution only affected the rate of swim bladder inflation. During the course of the experiment, the mean specific growth rate (SGR) was 15.6% day^{-1}. There was a strong effect of the interaction between the onset of weaning (10 vs 16 dph) and its duration (3 vs 9 days) on the mean larval size and weight measured at 25 and 53 dph, which were higher when fish were weaned later with a longer weaning duration. Pikeperch larvae growth was also influenced by the interaction between the method of food distribution and whether or not co-feeding was implemented. In fact, when co-feeding was applied, no effect of the method of food distribution was observed, whereas in the absence of co-feeding, the larvae were heavier and larger with continuous feeding. On the other hand, this effect was no longer observed after 25 dph suggesting that this interaction is effective only during the weaning period. In conclusion, our results suggest that a later onset and longer duration of weaning followed by discontinuous feeding improved larval survival, growth and reduced deformities in pikeperch populations.

In experiment 3 (effect of population parameters), it was demonstrated that the production of juveniles of 1.8-1.9 g mean body weight can occur in 52 days as well as high levels of swim bladder inflation and tank biomass which is a marked improvement of pikeperch juvenile production in RAS conditions. Final biomass correlated with a higher initial larval density (100 larvae l^{-1}) and the use of larvae supplied by bigger females. In the comparison of the predatory behaviour of cannibals vs non-cannibals, predation tests revealed that cannibals show less predatory behaviour than non-cannibals, but they were significantly more efficient in prey capture. In the comparison of the digestive enzymatic activity of predators vs non-predators, results showed that trypsin and amylase activity values were higher in non-predator larvae than in predator larvae. Furthermore, pepsin activity values were lower in non-predators than in predator larvae. These results indicate that predatory larvae have a more developed digestive system development (higher levels of acid proteases in comparison to alkaline proteases) at the same age of non-predator larvae.

Summary of progress towards objectives (49-60 Mo):

The overall aims of WP16 were to establish an optimal combination of factors (task 16.1), which could give the best performance of pikeperch (*Sander lucioperca*) larval populations, and to develop an industrial protocol for pikeperch larval rearing (task 16.2). Based on previous results obtained in experiments 1 (effects of environmental factors), 2 (effects of feeding-related factors) and 3 (effects of population parameters), a fourth experiment was conducted in order to test an optimal combination of the most beneficial rearing factors identified (February – April 2018, see D16.4). Using a pilot scale recirculating aquaculture system (RAS), this optimal combination was repeated 7 times. It was shown that raising juveniles of 0.7-0.9 g mean body weight (mean specific growth rate of 15.1% d^{-1}) can occur in 52 days, with an average survival rate of 14.9%, an average food conversion rate of 0.66 and a high final stocking density (13.6 kg/m^3). Results were very homogeneous in all the seven tanks. Regarding the production costs it has been calculated that juveniles of 0.8 g average can be produced for 0.20 €. Concerning the behavioral study, the hypothesis was that the introduction of real prey (perch larvae) in the enclosures might lead to a decrease of the cannibalism rate. This was not the case.

Then the following objective (D16.5) was to test this optimal combination of factors in commercial conditions at a commercial situation (July – August 2018), using the facilities of P39 (F2B). However, we were not able to apply faithfully our optimal combination due to the limitations of the technical facilities and the characteristic of the pikeperch broodstock of the SME, thus the efficiency of our optimal combination of factors cannot be fully discussed. It means that we have applied an “adapted DIVERSIFY protocol” and a real validation of our optimal combination of factors in SME conditions remains to be confirmed.

Finally, based on the optimal combination of rearing factors identified in the task 16.1, the knowledge and know-how of the SME and results obtained pikeperch larvae nutrition (WP10), six recommendations have been proposed to promote an industrial protocol for pikeperch larval rearing (D16.6).

Details for each Task

Task 16.1 Optimal combinations of factors to improve larval rearing (led by UL, Pascal Fontaine).

This task was based on four successive experiments using multifactorial designs in order to integrate the effects of each simple factor tested and interactions between them, and to identify *in fine* an optimal combination of factors that significantly increase both larval survival and growth. The four experiments focused on the effects of environmental factors (deliverable D16.1), feeding-related factors (deliverable D16.2) and population parameters (deliverable D16.3), and finally the identification and evaluation of the optimal combinations of factors (deliverables D16.4 and D16.5). The first three deliverables of this task have been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 16.1 Determine effect of environmental factors on pikeperch larval rearing***, ***D16.2 Determination of the effect of nutritional factors on pikeperch larval rearing*** and ***deliverable D16.3 Determine effect of population factors on pikeperch larval rearing***. In this periodic report, the results related to the experiment 4 (identification of the optimal combinations of factors) and the test conducted on farm condition (evaluation of the optimal combination of factors) are presented.

Experiment 4: Identification of the optimal combinations of factors

The study was carried out in an experimental recirculating aquaculture system (RAS) with ten 700 l tanks. Larvae (560.000) were obtained from a local broodstock maintained at SARL Asialor (Pierrevillers, France) and transferred to the UL experimental platform (UR AFPA, Vandœuvre-lès-Nancy, France). The broodstock was the same as used for the three previous experiments. Larvae hatched from February 19th, 2018 and were distributed to 7 tanks, where water temperature was initially at 15-16°C. All larvae came from two females (3.90 and 4.47 kg body weight) where the eggs were stripped on February 10th, 2018 and then fertilized by mixing with sperm from 3 males. After hatching, the larvae from the two females were mixed and divided into 7 separate bags of 70000 larvae each for transportation to the research facility. The tanks were stocked with 70.000 larvae each (100 larvae l^{-1}). The photoperiod was fixed at 12h of light and 12h of darkness (Hamza et al. 2007) with a light intensity of 50 lx (see Deliverable D16.1) and a dimming period of 30 minutes in the morning and evening. Temperature was the same for all tanks ranging between 16 and 20°C. Temperature was initially increased incrementally by 1°C day^{-1} until 20°C. Dissolved oxygen was maintained above 6 mg l^{-1} and salinity was fixed below 1 ‰. In this experiment, environmental factors (light, water renewal rate, water current direction and cleaning period) were the same as for the second experiment. The feeding strategy and the population factors were carried out according to best protocol obtained in the second and third experiment (**Table 16.1.1**). From 0 to 25 dph a surface skimmer was placed in each tank to remove the oil on the water, which interferes with swim bladder inflation. The first feeding occurred at 4 days post hatching (dph), where larvae received *Artemia* nauplii (480 µm; Premium Artemia Cysts, Salt Lake Aquafeed, Utah, USA) until weaning. Weaning occurred between 16-24 dph with mixed feeding using an inert food (Prowean 300, BioMar, Århus, Denmark) and live preys, where the proportion of live prey was decreased over a three day interval. After this period, larvae were exclusively fed inert feed of increasing particle size. After weaning, inert feed was provided in excess over the whole experimental period. At the end of the experiment (53 dph), a feeding ratio of 4% was applied.

Table 16.1.1. Applied modality for each factor. This combination of factors was repeated in the 7 experimental tanks (n = 7).

Factor	**Modality**
Density	100 larvae L^{-1}
Sorting of fish jumper	No
Sibling or not sibling	Not sibling
Female weight	Large (> 3.3 kg)

Feeding schedule	Discontinuous
Light regime	12:12
Light intensity	50 lx
Weaning start (dph)	16
Weaning duration (days)	9
Water renewal rate (tank vol./h)	1
Tank cleaning period	Morning
Tank current direction	Bottom to top

In this experiment it was confirmed that raising juveniles of 0.7-0.9 g mean body weight can occur in 52 days. The mean specific growth rate (15.1% d^{-1}) was close to the values obtained in previous experiments with the same duration (specific growth rate: 15.6 and 15.1% d^{-1} for D16.2 and D16.3, respectively). The final mean individual body weight (816 mg) was lower than those of previous trials (average body weight: 911 and 1471 mg for D16.2 and D16.3, respectively). However, the average survival rate of 14.9%, final biomass harvested of 9.6 kg and the final stocking density (13.6 kg/m^3) were the highest of all our experiments (**Table 16.1.2**). A trade-off between individual growth performance (final individual weight) and overall tank performance (survival, final stocking density) seems evident. The increased survival rate and the resulting high final stocking density obtained in this experiment could have negatively impacted the individual final weight and length. This fourth experiment allowed us to identify an optimal and reliable combination of environmental, feeding-related and population factors that improves pikeperch larvae survival and final juvenile biomass in rearing tanks with very high swim bladder inflation and low deformities rates. Regarding the production costs it has been shown that juveniles of 0.8 g average can be produced for 0.20 €.

Table 16.1.2. Summary of performance parameter recorded in all tanks.

Tanks	Swim bladder inflation rate (%)	Initial biomass (g)	Final biomass (g)	Mean initial body weight (mg)	Mean final body weight (mg)	Survival rate (%)	SGR (%/day)	FCR
2	90.8	34.6	9526	0.49±0.02	710.0 ±161.7	19.2	14.8	0.66
3	96.9	34.6	9722	0.55±0.06	938.3 ±177.4	14.8	15.2	0.65
5	88.1	34.6	9754	0.57±0.03	945.4 ±311.9	14.0	15.1	0.65
6	94.7	34.6	9638	0.52±0.01	740.6 ±258.0	13.7	14.8	0.65
7	90.4	34.6	9658	0.47±0.03	806.8 ±259.0	14.0	15.2	0.65
8	95.5	34.6	9483	0.34±0.24	827.8 ±273.6	14.7	15.9	0.66
9	91.8	34.6	9075	0.52±0.03	740.6 ±163.4	13.7	14.8	0.69
Average	92.6	34.6	9550.9	0.48±0.13	816.0 ±248.8	14.9	15.1	0.66

In order to assess the effects of potential prey on cannibalism in pikeperch larvae, an additional experiment was performed. In 16 small enclosures (volume of 3l) belonging to two separated hatcheries, we tested the effect of potential prey on mortality rate, cannibalism, growth (total length and weight), and relationships between individuals in an enclosure. In a second experiment, we tested the influence of potential prey on the group structure through a behavioral assay. For the first experiment, 50 individuals were placed in a small enclosure under two conditions: with or without potential prey. Larval behavior was recorded on a daily basis and subsequently reviewed for analysis. For the second experiment, to study the group structure and the relationships between individuals, ten pikeperch larvae per enclosure were placed in small arena (30cm in diameter). After a 30-minute acclimatization phase their behavior was recorded for one hour. The group structure was characterized by the distances between individuals (closest distance to a conspecific, mean distance and the variance of the distances between individuals of the group): moreover, the activity (time of swimming) and relationships between individuals (contact or not) were analysed. The results of the experiment with the perch larvae as prey showed that there was no significant difference in mortality rate between the presence or absence of prey but the rate of cannibalism was higher when the prey were present (**Fig. 16.1.1**) as well as the length and biomass (**Fig. 16.1.2**). There was no significant difference in relationship parameters between individuals (attacks, pursuit, bite and ingestion) or for parameters characterizing the group structure of larvae (closest distance to conspecific, mean distance and its variance between individuals of the group) as a function of prey presence.

To conclude, this fourth experiment allowed us to identify and to validate (7 replicates) an optimal and reliable combination of environmental, nutritional and population factors in a pilot scale RAS (but in laboratory conditions) that improves pikeperch larvae survival and final juvenile biomass in rearing tanks with very high swim bladder inflation and low deformities rates.

The full description of the work and results is provided in Deliverable 16.4 “Identification of optimal combinations of factors for pikeperch larval rearing”.

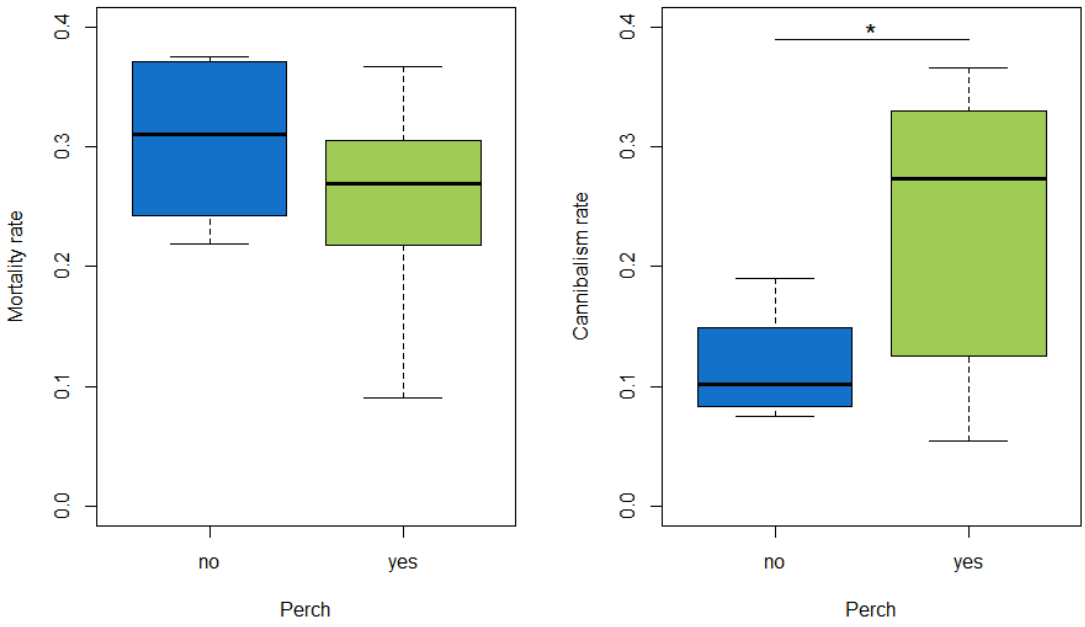


Figure 16.1.1. Rate of mortality and cannibalism in the presence or absence of prey. Box plots indicate the median, the1st and 3th quartiles. The * indicates a significant difference at the level of $p<0.05$.

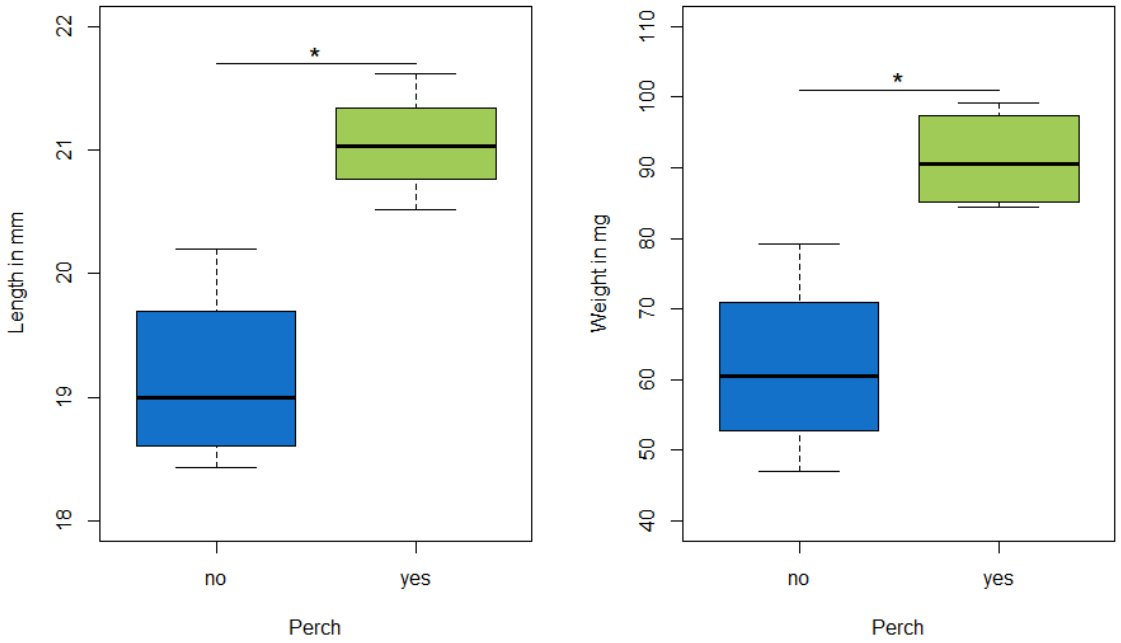


Figure 16.1.2 Length and mass of pikeperch larvae in the presence or absence of prey at the end of the experiment. Box plots gave the median and 1st and 3th quartiles. The * indicates a significant difference at the level of $p<0.05$.

Experiment 5: Evaluation of the optimal combinations of factors on farm conditions

At the facilities of P.39 F2B, the improved protocol was tested in one 500 l circular black walled tank with a white bottom and compared to the F2B commercial protocol applied in two similar tanks. These three tanks were part of a standalone filtration system. Temperature was controlled by a heat pump (Fluidra Evoline 13) while temperature and oxygen were measured using an online controller (Tecnos Oxiwifi2 with a Tecnos galvanic oxygen and temperature probe). Eggs from 4 females of 1.1 - 1.8 kg, were fertilized with sperm from 5 males and were later incubated in Weiss jars for 7 days at 14 – 16 °C. They were hatched on the 8th of July. Approximately 50 000 larvae were stocked in tank T3 ("Diversify protocol"), 100 000 in tanks T1 and T2 (F2B commercial Protocol). This resulted in concentrations of 200 larvae · l^{-1} in tanks T1 and T2 and 100 larvae · l^{-1} in tank T3. Concerning T3, only eggs from two large females (1.7-1.8 kg) were used. The trial lasted for 49 days (from the 8th of July until 26th of August 2018).

Due to the operational constrains of the partner SMEs related to its own production (spawning time of their broodstock and reproductive success) and the successive delays we met during the previous experiments done in the WP16 (see D16.1-4), it was not possible to implement this trial as foreseen initially. We were not able to apply faithfully our optimal combination due to the limitations of the technical facilities and pikeperch broodstock of the SME, thus the efficiency of our optimal combination of factors (see D16.4) cannot be fully discussed. It means that we have applied an "adapted DIVERSIFY protocol" and a real validation of our optimal combination of factors in SME conditions remains to be confirmed. Taken together, the present comparison between the Diversify and commercial protocols suggests that the continuous production of pikeperch juveniles using RAS in SME conditions continues to be limited and requires further research in order to improve survival and growth.

The full description of the work and results is provided in ***Deliverable 16.5 "Evaluation of selected rearing combinations for pikeperch on farm condition"***.

Task 16.2 Development of an industrial protocol (led by F2B, Jiri Bossuyt).

In the framework of the task 16.1, a multifactorial approach was developed to identify an optimal combination of factors to improve pikeperch larval rearing. An optimal combination of factors was proposed (**Table 16.1.1)**. However we have tested a limited number of parameters (12 parameters with two modalities per factor) and consequently some potential influential factors have not been considered and studied. Also, considering the experience of the SME in pikeperch larval rearing, including results presented in the deliverable D16.5, and the results obtained in WP10 (pikeperch larvae nutrition), the last objective of WP16 is to propose an industrial protocol and recommendations for pikeperch larval rearing integrating also new results published recently.

In order to secure the production of juveniles of pikeperch using RAS and controlled conditions from hatching until 50 days post hatching (DPH), we suggest major recommendations according to results obtained in the WP10 and WP16, or recently published during the conduct of the DIVERSIFY project:

1 - To apply the following constant environmental conditions: light intensity of 50 lux, a water renewal rate of 100% per hour, a cleaning of the tank during the afternoon and an inlet of the water at the bottom level (see D16.1).
2 – To use a feeding strategy based on a later onset (starting at 16 DPH) and longer (9 days) duration of weaning followed by discontinuous feeding of the formulated diet after the weaning period (see D16.2).
3 – To feed larvae with enriched live preys (approximatively 3 first weeks including weaning period) and a formulated diet guaranteeing an adequate supply of LC-PUFAs and phospholipids (supplied as soy lecithin) (see D10.1-2).
4 – To mix rotifers (*Brachionus plicatilis*) and *Artemia nauplii* during the initial live preys feeding period in order to increase the survival rate during the transition from an endogenous to an exogenous feeding (Policar et al., 2018).
5 – To favour larvae from big females (> 3 kg) in order to obtain initially larvae of larger size (see D16.3).
6 – To rear larvae at high initial density (100 ind.L^{-1}) (see D16.3).

The recommendations cited above are mainly issues from experiments done in a specific rearing system (RAS, sub-squared tanks of 700 L each, green tank wall color) (see D16.1-4), however the optimal combination of factors identified (D16.4) was not confirmed in the SME (P39) facilities which are very different to those used in laboratory conditions (see D16.5). As a result all these recommendations have to be validated according to the rearing system mobilized for pikeperch larvae culture. Another important point that must be considered is the production cost of a pikeperch juvenile, which is an important issue for fish farmers and a real bottleneck for their future success (see D31.31). In laboratory conditions, this cost was evaluated at 0.20 euro per juvenile (see D16.4). Some recommendations recommended here (new diet, use of rotifers) could have negative consequences on this cost, which needs further evaluation. The full description of the work and results is provided in ***Deliverable 16.6 "Proposition of an industrial protocol for pikeperch rearing***".

Deviations from Annex I and their impact:

All experiments of the task 16.1 were delayed (8 months). This delay is explained by the fact that we have repeated two experiments, because of a very high mortality caused by perch *perhabdovirus*, and observed in the first attempt in May 2016. This was the first time that this pathology was observed on pikeperch larvae.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Colchen, T., Faux, E., Teletchea, F., Pasquet,A., 2017. Is personnality of young fish consistent through different behavioural tests ? Applied Animal Behaviour Science 194, 127-134.

Colchen, T., Fontaine, P., Ledoré, Y., Teletchea, F., Pasquet,A., 2019. Intra-cohort cannibalism in early life stages of pikeperch. Aquaculture Research (Submitted).

Colchen, T., Dias, A., Gisbert, E., Teletchea, F., Pasquet,A., 2019. The onset of piscivory in fish: behavioural and physiological correlations. Physiology and Behavior (In Preparation).

Fontaine, P., Colchen, T., Ledoré, Y., Gisbert, E., Krauss, D., Hmila, S., Pasquet, A., 2019. Optimization of pikeperch Sander lucioperca L. larval rearing in RAS conditions. Fishes (In Preparation).

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:	Torstein Harboe (P7), Audun H Nerland (P7), Kristin Hamre (P7), Øivind Bergh (P7), 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 were however, a higher proportion of jaw-deformed larvae in the RAS system. Previous work with halibut yolk sac larvae in silos has strongly indicated that jaw deformities are more frequent when the larvae are exposed to water movement the first days after hatching. Most likely, there was a difference between the siloes in such water movement, which may have been responsible for the difference.

Thirty-five different *Vibrio* spp. strains were tested for virulence towards Atlantic halibut larvae in a major challenge experiment. Some of the strains are known to be associated with bacteriophages, making phage therapy possible. Other possible strains were to be used as model strains in challenge experiments with probiotics following the model by D`Alvise et al., 2012.

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

RAS first feeding: Halibut larvae, at an age of 265 day-degrees, were transferred from a yolk sac incubator to 6 first feeding tanks. Numbers of larvae were approximately 5000 in each tank. Three of the tanks were connected to a RAS system (Tropical Marine Center). The three other tanks had a standard flow through water system with water coming from 160 m depth. The tanks had a volume of 1400l and water flow of 5 $l \cdot min^{-1}$. Water temperature was 12 ± 0.3°C during the whole period. Highest growth was obtained for the larvae reared in the FT system. However, survival was not different between the FT and RAS groups. The lower growth in the RAS group was probably due to high ammonia concentrations in the RAS system.

Artemia on-growing protocol: A production protocol, based on Olsen et al., 1999, for on-grown *Artemia* was further developed, where water renewal and quality were crucial parameters. The protocol includes feeding, washing and disinfection of the *Artemia*, and has been tested both at an experimental (P7. IMR) and commercial scale (P22. SWH). The experiments that led to this protocol were followed by analyses of biochemical profiles of macro- and micronutrients of the on-grown *Artemia* that are presented in *Deliverable D11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia*

First feeding on-grown Artemia: A strategy to alleviate the slow growth in the later larval stages of Atlantic halibut and improve juvenile quality is to use on-grown *Artemia*. Ongrown *Artemia* are larger, contain more protein and phospholipids and have a different micronutrient status from *Artemia* nauplii (Hamre and Harboe, unpublished; Task 11.2). They also have a lower shell to nutrient content. Olsen et al., (1999) showed that halibut larvae fed on-grown *Artemia* develop into juveniles with better pigmentation and eye migration than Atlantic halibut fed *Artemia* nauplii. There was no difference in larval performance. Survival, measured as number of halibut fry 70 days after first feeding, was between 42 and 48% of incubated larvae. Growth data, except for the end point, has so far not been measured. However, at the end point there were no differences between the two groups. Both groups showed 100% normal pigmentation and good eye migration (score: more than 2.5/3).

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

During this period halibut fry were produced in RAS systems applied both during yolk sac incubation and first feeding stages. The RAS unit used for first feeding was started 6 weeks prior to larvae incubation and the ammonia concentration was low during the entire period, even when clay was added to create turbidity. The larvae in the RAS had better growth and survival compared to the larvae in the FT system.

Summary of progress towards objectives (49-60 Mo):

We have carried out a metagenomic analysis of the bacteriological composition of water and larvae in RAS and FT systems for both yolk sac and first feeding stages. This will provide a basis for selection of candidate probiotic bacteria for use in Atlantic halibut larviculture.

- 300-400 different bacterial genera were detected in the rearing systems
- Significant differences were detected in the microbiota composition of the RAS and FT systems: both in yolk sac silos and first feeding tanks, and in the water and the larvae.
- No obvious correlation was seen between the microbiota in the water and the microbiota of the larvae.
- Antibiotic treatment had a big influence on the composition of the microbiota.

Details for each Task

Task 17.1 Recirculation (RAS) vs Flow through (FT) systems during yolk sac and first feeding stages and the effects on larval survival, quality and growth (led by IMR, Torstein Harboe).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D17.2: Determine if RAS is a more effective protocol than FT for Atlantic halibut larvae.***

Task 17.2 The effect of probiotics on larval microbiota and survival and development of an industrial protocol (led by IMR, Audun Nerland).

Infections with opportunistic bacteria are a severe problem in aquaculture, especially in marine larviculture used for the production of juvenile fish for commercial fish farming. While at later life stages the frequency of bacterial infections can be reduced by preventive measures such as vaccination and good management practices, the very young larvae and small fish have an immature immune system and cannot be protected by vaccination. Very often infections in larviculture are treated by antibiotics. However, this is not a sustainable practice since bacterial antibiotic resistance will develop and antibiotic-contaminated effluents

are deleterious to marine ecosystems. Therefore, alternative strategies for preventing bacterial infections in fish larvae, such as pathogen-reducing probiotic bacteria or bacteriophages are highly needed. The commercial production of Atlantic halibut (*Hippoglossus hippoglossus*) fry is currently carried out in flow through systems (FT), while there is a growing consensus that Recirculating Aquaculture Systems (RAS) would offer more stable environmental and chemical water parameters that would lead to improved larval performance. In this task, we have carried out a metagenomic analysis of the bacteriological composition of water and larvae in RAS and FT systems for both yolk sac and first feeding stages. This will provide a basis for selection of candidate probiotic bacteria for use in Atlantic halibut larviculture.

Metagenomic analysis of bacterial composition:
Sampling of bacteria from water: Water samples of 45 ml were taken from the silos or the tanks (n=3 from each unit per sampling) and centrifuged at 3200 g for 30 minutes at 4°C. The pellets were resuspended in 1 ml SLB (sucrose lysis buffer: 20 mM EDTA, pH 8.0; 400 mM NaCl; 0.75 M sucrose; 50 mM Tris-HCl, pH 9.0) and kept at – 20°C until further processing for DNA isolation.

Sampling of larvae: Individual larvae (n=4 from each unit per sampling) were transferred to 2 ml Eppendorf tubes (omitting carrying over seawater) and frozen at – 20 °C until further processing for DNA isolation. For larger larvae at the end of start-feeding, individual larvae were homogenized using a Kontes pestle, and 200 µl of the homogenates were kept at – 20 °C.

DNA was isolated from the samples by using the CTAB (hexadecyltrimethylammonium) method as described by Zhou et al 1996. Briefly, starting with 200 µl samples, 2 volumes of 1% CTAB buffer (1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 mg per ml) were added to the SLB preserved samples and incubated for one hour at 60 °C. Then SDS (final concentration 2%) was added and incubated further for one hour at 60 °C, before extraction once with phenol/chloroform, then twice with chloroform and finally precipitation of the DNA with ethanol and resuspension in 30 µl pure water.

Taxonomic level: The method gives information about the ratio between different bacteria, down to species level. However, as one moves down the taxonomic levels (phylum, class, order, family, genus and species) the uncertainty of the classification will increase. For this reason, we chose to discuss our findings mostly at the genus level.

Summary and conclusions:

- 300-400 different bacterial genera were detected in the rearing systems
- Significant differences were detected in the microbiota composition of the RAS and FT systems: both in silos and tanks, and in the water and the larvae.
- No obvious correlation was seen between the microbiota in the water and the microbiota of the larvae.
- Antibiotic treatment had a big influence on the composition of the microbiota.

Task 17.3 Production of on-grown *Artemia* (led by IMR, Torstein Harboe).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D17.1 Production of on-grown Artemia and D11.1 Report on the nutrient profile of Artemia nauplii and on-grown Artemia***

Task 17.4 Comparison of feeding on-grown *Artemia* versus *Artemia* nauplii on larval performance (led by IMR, Torstein Harboe).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D17.4 Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance.***

Deviations from Annex I and their impact:
The original plan for Task 17.2 was to perform *in vitro* challenge trials with probiotic candidates for use in larval rearing systems. However, addition of probiotics has proven to be problematic in cold-water systems and an alternative strategy for finding candidates was chosen, based on new and more specific molecular methods (metagenomics) that have recently become available for characteriszation of bacteriological environments both within ecosystems, water and individual larvae. Further, as interest for using RAS in mariculture is increasing, we tested how the microbiome in these systems develops in yolk sac and first feeding larvae, as an alternative way of establishing a probiotic effect.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Harboe, T., Mangor-Jensen R., and Norberg B. Comparison of Flow through vs Recirculation in halibut juvenile production. Effects on survivals, growth and juvenile quality. (*in preparation*)

Nerland, A.H. Norberg, B., Olausson, S. K., and Harboe, T. Comparison of Flow through vs Recirculation in halibut juvenile production. A metagenomic analysis on bacterial composition of rearing water and larvae. (*in preparation*)

WP 18 Larval husbandry – wreckfish

WP No:	18	**WP Lead beneficiary:**		P8. IEO
WP Title (from DOW):	Larval husbandry – wreckfish			
Other beneficiaries (from DOW):	P1. HCMR	P19. CMRM	P32. MC2	
Lead Scientist preparing the Report (WP leader):	Blanca Álvarez-Blázquez			
Other Scientists participating:	Nikos Papandroulakis, Ioannis Papadakis (P1), Fatima Linares, Jose Luis Rodriguez Villanueva (P19), Antonio Vilar Peron (P32), Evaristo Perez Rial, Montse Perez (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 the broodstock of MC2 spawning was obtained, possibly due to the young age of the fish in the different stocks, and also unexpected oceanographic variations in seawater temperature.

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

Task 18.1 Development of feeding methodology. The objective of this Task is to test different feeding regimes (prey densities and succession of prey type) in order to develop a feeding protocol and avoid periods of food deprivation. Testing includes rearing in semi-intensive culture system (Mesocosm with 40000 l) tanks, from the end of endogenous feeding to the change to inert feeding (weaning phase). The culture system will be evaluated in terms of ontogeny of larval digestive and visual system (influenced by feeding) through histological and image analysis procedures.

During the reported period some preliminary trials were performed due to the low availability of eggs that did not allow the implementation of a full-scale trial.

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

Important advances have been made in the understanding of ontogeny and larval development (**D18.**1) as well as the initial stages to develop an adequate larval feeding protocol. Knowledge of the optimal incubation temperature and larval culture (**Subtask 18.2.1**) has also increased. Improving the technical conditions of culture that include aeration, water flow rate and tank circulation as well as continuing to investigate the high percentage of larval malformation (**Subtask 18.2.2**), will be objectives during the last

period of the project and comply with the delivery of the following deliverables that have been delayed: **D18.2**, **D18.3** and **D18.4**.

Summary of progress towards objectives (49-60 Mo):

A feeding protocol was developed based on studies of the different stages of the larval development after hatching; the yolk sac consumption, acceptance of exogenous food and duration of larval development and growth until the acceptance of inert food (weaning). The sequence of live food consumption will be determined as a function of age and the time of weaning. Results on survival and viability will also be provided, as well as morphometric determinations during larval culture

Although no industrial protocol was produced the achieved results are of great significance as for the first time individuals survived the larval rearing phase and were weaned into artificial diets. It is worth mentioning that, in the IGAFA facilities, there are 25 juveniles that reached mor than 150 dph that were cultured at approximately 18ºC and represents a significant step forward in wreckfish larval culture, providing a basis for further studies

The growth in length of the wreckfish larvae until weaning was knew, increasing from 5.6 mm to 12.8 mm at 5 and 65 dph, respectively. Some data about behavior were known during the project, peak of mortality, larvae deformities and age (days post hatching) in that occurs, age of start eating (open mouth), etc.

The study of the technical conditions and the adequate parameters regarding the aeration, the flow and form of creating an adequate water circulation, as well as continue investigating the larval malformations that occur in a high percentage are needs for the immediate future.

Very important results were achieved in larvae feeding sequence in RAS system culture. These data could be the starting point for future experiments and a reality to propose the cultivation of wreckfish as a possibility as diversification of aquaculture

Details for each Task

Task 18.1 Development of feeding methodology (lead by HCMR, Nikos Papandroulakis)

Part of this task has been completed during the previous reporting periods and the full description of the work and results have been provided in deliverable ***D18.1 Development of the digestive system of wreckfish*.** No further work was done towards the development of a feeding methodology, due to lack of good quality eggs from the broodstock of **P1.HCMR**, and failure to ship eggs from the other facilities in Spain.

However, work related to this task was carried out at **P8.IEO**, **P32.MC2** and **P19.CMRM** where trials tested different food types, doses and feeding sequences that are described in the following **subtask 18.2.2**.

Task 18.2 Defining optimum conditions for larval rearing (lead by IEO, Blanca Álvarez-Blázquez)

Sub-task 18.2.1 Testing (IEO, MC2) the effect of two temperatures ranges (14-17 abd 19-22ºC) in triplicate trials. Data obtained during the previous period (31-48 Mo) suggested that the optimal water temperature for artificial incubation of wreckfish eggs is in a range of 16.5-19.5ºC. Lower temperatures (14±0.5ºC) promote more deformed larvae and lower hatching rates, with more egg mortalities during the first three days of incubation (**Fig. 18.2.1**)

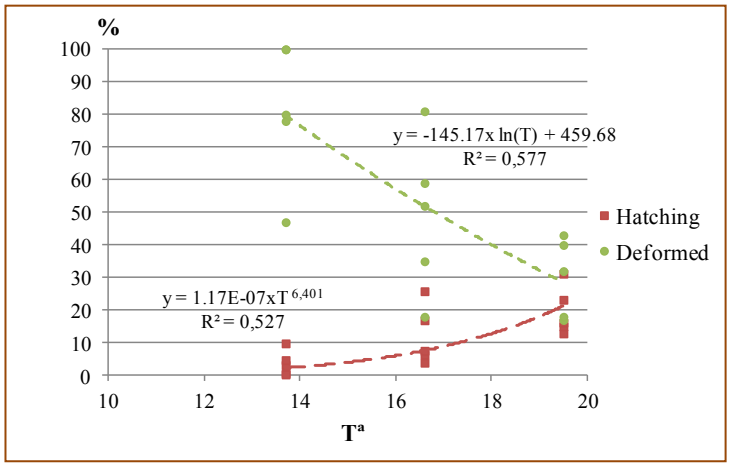


Figure 18.2.1. Regression adjustment for percent of hatched and deformed larvae as a function of incubation temperature.

Regarding higher water incubation temperatures, it is possible to incubate at 19±0.5ºC with acceptable results in terms of deformities and hatching rates. However, due to the large size of wreckfish larvae and the density of culture tested, using very high temperatures increases the levels of ammonium, pH and opportunistic species (ciliates) that significantly alter the optimal conditions of larval husbandry. Larval rearing between 15.5±0.5ºC and 19.1±0.6ºC showed good performance of larvae up to 26 dph, mainly at the highest temperature where larvae of 0.47 mg dry weight were obtained (**Fig. 18.2.2**)

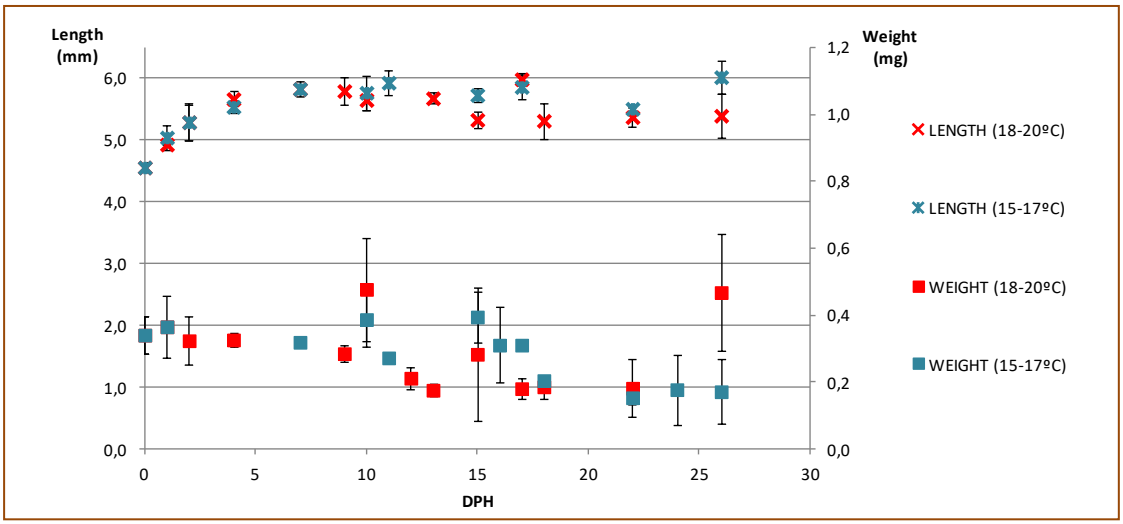


Figure 18.2.2. Length (mm) and dry weight (mg) of larvae cultured at different temperatures.

The joint action of incubation between 16.5-19.5ºC and larval culture between 15.5 - 19.1ºC suggest that a temperature range between 16-18 ºC may be adequate to improve survival and growth in wreckfish. The full description of the work and results is provided in deliverable ***D18.2 "Determine optimum temperature condictions for rearing wreckfish larvae"***.

Sub-task 18.2.2. Test of two culture systems RAS (P19.CMRM) and flow-through (P8.IEO) in terms of larval culture conditions and feeding protocols.

During 2018, more advances in achieving natural spawns and larval husbandry have been achieved in the three Galician wreckfish stocks resulting in very good larval hatching (42-82%) and survival until 34-37 dph. At Instituto Galego de Formación en Acuicultura, IGAFA (CMRM) two batches of larvae, one from natural spawns at the Instituto Español de Oceanografía (IEO) and the other from natural spawns at the MC2 produced larvae and juveniles that survived over 6 months.

More than 50 experiments of larval culture have been carried out in the IEO facilities. Several rearing conditions such as photoperiod, water circulation, use of aeration or sequence of feeding used were modified as the season progressed (**Table 18.2.1**).

Table 18.2.1 Larvae cultures at IEO facilities during the spawning season of 2018.

STOCK	SPAWN	DATE	LARVAE (nº)	LARVAL DENSITY (nº Larv/l)	MEAN Tª	FOOD	SURVIVAL (dph)	WATER SYSTEM	TANK VOLUME (L)
IEO	1	09-03-18	171921	21	15,2	ROT 10-26 dph. A0 11-28 dph	29	FT. NATURAL Tª	8000
	2	02-04-18	110740	7,1	17,2	ROT ORIGREEN 7-14 dph. A0 11-13. A-1 ORIGREEN 14-17 dp	17	CC. HOT WATER	8500
	3	07-04-18	134802	5,5	17,5	ROT 7-10 DPH. A0 8-11 dph.	23	CC. HOT WATER	8000

STOCK	SPAWN	DATE	LARVAE (nº)	LARVAE DENSITY (nº /l)	MEAN Tª	FOOD	SURVIVAL (dph)	WATER SYSTEM	TANK VOL (L)	COLOUR BOTTON TANK
IEO	1	16/02/2018	3429	7	18,3	ROT ARAq 12-18 dph	18 DPH	CC until 14 dph	500	WHITE
	2	21/02/2018	7500	13	17,9	ROT ARAq 11- 24dph. A0 15-24 dph	24 DPH	CC until 11 DPH	1000	WHITE
	3	27/02/2018	73260	36	16,8	ROT ARAq 10-19 dph. A0 13-23 dph	23 dph	CC until 12 dph	2000	WHITE
	4	04/03/2018	7282	1,8	15,4	ROT ARAq 12-27 dph. A0 20-27 dph	27 DPH	CC until 11 dph. FT 120 cc/15 seg	500	BLACK
	5	10/03/2018	37844	18,9	15,6	ROT enrich. T-Iso 11-25 dph. A0 19-27 dph	27 DPH	FT 500 cc/15 seg	2000	WHITE
	6	19/03/2018	102570	39	16	ROT ARAq 10-20 dph. A0-A1: 12-24 dph	24 DPH	FT 500 cc/15 seg	800	WHITE
				26	15,9	ROT enrich. T-Iso 10-18 dph. A0 11-18 dph	19 DPH	CC until 9 dph	800	WHITE
	7	24/03/2018	23320	10	16,5	ROT CONTROL 9-17 dph. A0 12-20 dph	21 DPH	FT	400	
	8	24/03/2018	21888	33	18,9	ROT enrich T-Iso10-15 dph. A 0 12-17 dph	19 DPH	FT	1750	WHITE
				4,5	17,6	ROT CONTROL 9-14 dph. A0 11-13. A-1 T-ISO 13-22 dph	22 DPH		400	WHITE
	9	28/03/2018	11170							
	10	29/03/2018	71524	46	17,1	ROT enrich. T-Iso 8-15 dph. A0 11-14. A1-15-22 dph	22 DPH	FT	2000	BLACK
			45182							
	11	29/03/2018	47456	24	16,8	ROT ORIGREEN 7-15 dph. A0 10-14. A1 ORIGREEN 14-19 dph	19	CA	2000	WHITE
	12	07/04/2018	134802	5,5	15,1	ROT ORI-GREEN 7-10 dph. A0 8-11 dph.	11 DPH	FT	8000	WHITE
	13			20,4	18,4	ROT ORI-GREEN 7-10 dph. A0 8-10. A1 10-18 dph	18 DPH	C C + AIR	2000	WHITE
	14			14,9	18,2	ROT ORI-GREEN8-10 dph. A0 9-10. A1 11-16 dph	18 DPH	C C + AIR	900	WHITE
	15	12/04/2018	54116	12,8	17,6	ROT ORI-GREEN 7-14 dph. A0 7-9, A1 10-20 dph.	20 DPH	FT	600	WHITE
	16	16/04/2018	67428	14	18,7	ROT ORI-GREEN 6-11 dph. A0 7-9. A1 10-20 dph	23 DPH	FT	2000	WHITE
				18,6	19,1	ROT ORI-GREEN 6-11 dph. A0 6-9. A1 10-20 dph	21 DPH		400	WHITE
				16,4	18,8	ROT ORI-GREEN 6-13 dph. A0 6-9. A1 10-17 dph	17 DPH		400	WHITE
				20,4	18,4	ROT ORI-GREEN 6-13 dph. A0 6-9. A1 10-17 dph	17 DPH		800	WHITE
	18	25/04/2018	39798	5,7	19,2	ROT ARA 6-7 dph	8 DPH	FT	3X100 L	WHITE
				5,7	19,2	ROT CONTROL 6-7 dph	8 DPH		3X100 L	WHITE
				19,2	19,1	ROT T-ISO 5-8dph. A0 9-10. A1 11-22dph	22 DPH		400 L	WHITE
				18,3	18,9	ROT T-ISO 5-8 dph. A0 9-10. A1 18 dph	18 DPH		400 L	WHITE

The first experiments were carried out in non-flow through up to 6-12 dph, photoperiod of 12h L:12h D, with moderate aeration in the center of the tank, green water from day zero and a temperature between 15-18ºC. Larvae density ranged from 2 to 30 larvae l^{-1} and survival varied between 4 and 30 dph. Rotifers (10-25 dph) and *Artemia nauplii* (11-30 dph) were supplied. Dissolved oxygen was between 7.2-8.4 $mg.l^{-1}$.

In a second stage, new tests were carried out and some of the cultivation parameters were readjusted. Larvae of 1 dph were introduced at densities between 6-20 larvae l^{-1} in the larval culture tanks and kept in darkness until 7 dph. Temperature between 17-20ºC, a flow through system (2.5 renewals d^{-1}) with no aeration was maintained. A photoperiod of 10L: 12D and green water culture conditions were applied from 7dph. Feeding of rotifers (7-13 dph), *Artemia* nauplii (8-11 dph) and enriched metanauplii (10-29 dph) were used. Survival between 8 and 30 dph were reported.

In the last tests, some variables were modified. The culture density was maintained between 5-15 larvae l^{-1}, temperature was controlled at 17.9 ± 1ºC, the dissolved oxygen was between 7.0 and 8.4 $mg.l^{-1}$, the intensity during the light period was adjusted to 400 lux on the surface of the tank and a new feeding protocol was supplied: rotifer (7-18 dph), *Artemia* nauplii (12-19 dph) and enriched metanauplii (17-30

dph). The rest of the culture conditions were the same as those described in the previous paragraph. Survivals between 18 and 34 dph were observed.

For all the experiments carried out during 2018, live prey concentrations were adjusted between 3-10 ml^{-1}, 0.5-1 ml^{-1} and 0.5-2 ml^{-1} for rotifer, *Artemia* nauplii and metanauplii, respectively.

In **P32.MC2** facilities larvae culture was focused in conditions of FT (**Table 18.2.2**), with water temperature between 17.5 and 18.8ºC and live food based on rotifer and copepods (*Acartia*) since 6 to 22 dph. Larval density was between 0.2 and 11.1 larvae l^{-1} and survival until 22 dph.

Table 18.2.2 Larval culture conditions in MC2 facilities during trials in 2018.

STOCK	TRIAL	SPAWN	LARVAE nº	LARVAE DENSITY	MEAN Tª	FOOD	SURVIVAL dph	Water System	Tank Vol	TANK COLOUR	TANK SHAPE	AIR	Light
MC 2	1	28/03/2018	30000	3	17,5	Enrich rot and Acartia 6-20 dph	20	FT 30 Lh^{-1}	1000	Black	CYLINDER gently cone-shaped botom	Botom buble	Natural light
	2	02/04/2018	0		18,5	Enrich rot and Acartia 6-20 dph	12	FT 30 Lh^{-1}	180	White	Cone-bottom, cylindrical tank	Botom buble	Natural light
	3	07/04/2018	200	0,2	18	Enrich rot and Acartia 6-20 dph	17	FT 30 Lh^{-1}	1000	Black	CYLINDER gently cone-shaped botom	Botom buble	Natural light
	4	24/04/2018	10000	10	18,5	Rot <6 dph	7	FT 30 Lh^{-1}	1000	Black	CYLINDER gently cone-shaped botom	Botom buble	Natural light
	5	30/04/2018	2000	11,1	18,8	Enrich rot and Acartia 6-20 dph	19	RAS	6 x 3 1x 2	White	Jars	Botom buble	Natural light

Different culture systems were used in IGAFA (**P19.CMRM**) facilities (**Table 18.2.3**). The best results were achieved with RAS conditions with two batches, one from IEO and another one from MC2 facilities. Water temperature was 17.6 and 17.9ºC, in tanks volume of 400 L, without air and natural photoperiod since 7 and 9 dph. Life food was based on rotifer, *Artemia* nauplii and enriched metanauplii with dry microalgae supplemented with arachidonic acid (see D12.1). Both larval batches achieved the weaning period and dry food was provided since 40 dph at the same time that A1. At 48 dph larvae were weaning completely. In IGAFA facilities juveniles alive until now were reared at ±18ºC of water temperature, achieving the first results in larval and juvenile growth until 6 months of age.

Table 18.2.3. Larval cultures at IGAFA (**P19.CMRM**) facilities during 2018.

STOCK	TRIAL	SPAWN	LARVAL DENSITY (nº Larv/l)	MEAN Tª	FOOD	SURVIVAL (dph)	WATER SYSTEM	TANK VOL.	TANK COLOUR	TANK SHAPE	AIR	LIGHT
IGAFA (CMRM)	1	27-02-18	10,0	16,3	Enrich. Copepod Isoch 11-23 dph	24 DPH	CLOSED	200	WHITE	ROTATION (SMILAR KREISLER)	NO	since 11 dph
	2	02-04-18	13,0	16,9	Enrich. Rot. Araq 13-28 dph. A0 15-28 dph	28	CLOSED	200	WHITE		NO	since 9 dph
	3	22-03-18	10,0	15,9	Enrich Rot control and araq. 9-15 dph. A0 12-17 dph. Enrich A1 control and araq. 17-21	21 DPH	CLOSED	200	WHITE		NO	NO
	4	27-03-18	11,0	15,1	NON	3 DPH	RAS	400	BROWN	FLAT	YES	NO
	5	02-04-18	12,5	17,6	Enrich rot araq 9-17 dph. A0 15-18 dph. Enrich A1 araq. 18-48 dph. Dry food 40DPH until now	ALIVE	RAS	400	BROWN	FLAT	NO	since 9 dph
	6	08-04-18	10,0	15,6	Enrich Rot araq 10-25 dph	25 DPH	RAS	400	BROWN	FLAT	NO	since 10 dph
	7	08-05-18	9,0	19,0	Enrich Rot araq 10-25 dph	4 DPH	CLOSED	200	WHITE	ROTATION	NO	NO
	8	10-05-18	12,5	17,9	Enrich Rot araq. 8-19 dph. A0 18-23 dph. Enrich araq. A1 23-48 dph. Dry food 40 dph until now.	ALIVE	RAS	400	BROWN	FLAT	NO	since 7 dph

Conclusions and perspectives:

The mean weight in growth and length during trials at **P8.IEO** were reflected in the **Fig.18.2.3**. Growth in weight and length were adjusted to potential regression curves. More data of weight will be needed to achieve a clear conclusion about growth in weight of wreckfish larvae.

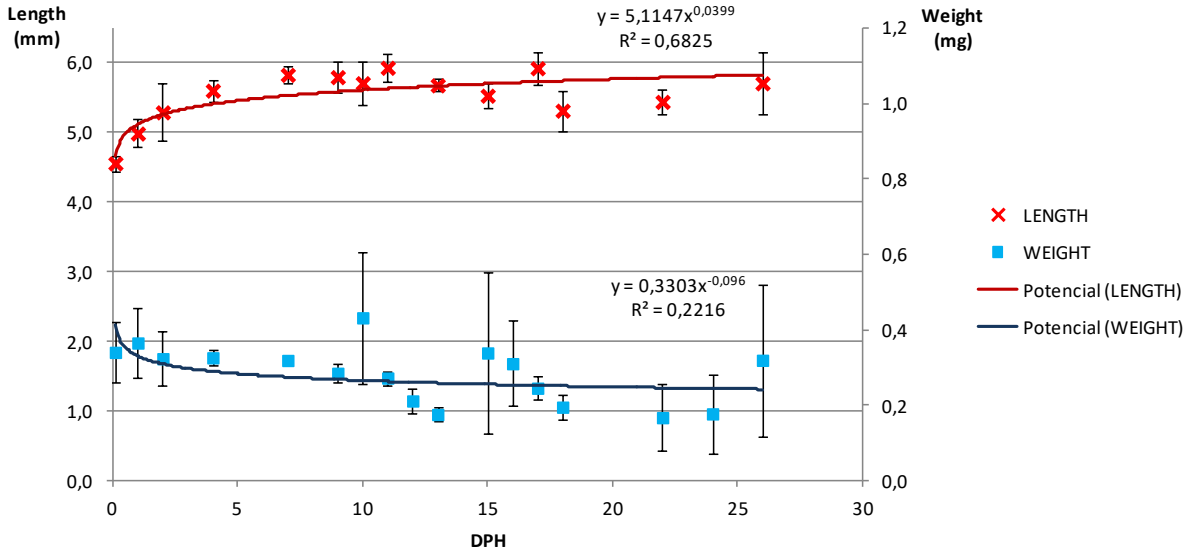


Figure 18.2.3. Growth in weight and length of wreckfish larvae from 0 to 26 dph

With data of length obtained in IGAFA facilities until 65 dph, the growth in length of the wreckfish larvae until weaning was from 5.6 mm to 12.8 mm at 5 and 65 dph, respectively (**Fig.18.2.4).**

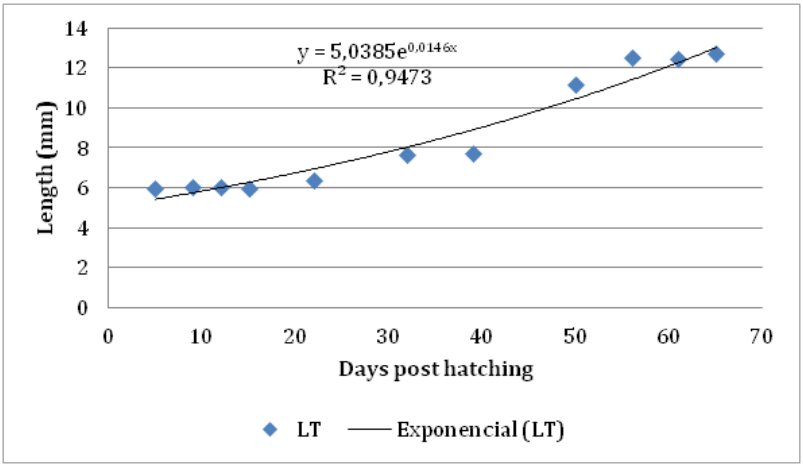


Figure 18.2.4. Wreckfish larvae length increasing (mm) from 5 to 65 dph.

Most important advances were made in the feeding sequence with trials in IGAFA (CMRM) in RAS, where the best survival results were obtained **(Fig. 18.2.5)**.

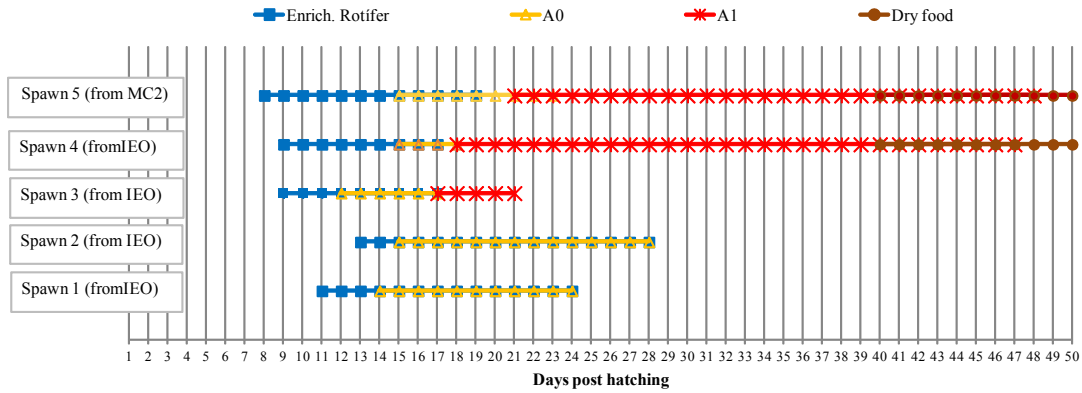


Figure 18.2.5. Larval rearing protocol for wreckfish used in the CMRM facilities.

As a result, we can conclude that the optimal feeding sequence for the larval culture of wreckfish in RAS at 12.5 larvae l^{-1}, 17.5-18ºC of water temperature, 36.4±1.7.5 ‰ of and dissolved oxygen bewteen 7.2-8.4 $mg.l^{-1}$, without air and natural photoperiod from 7-9 dph, was the following:

- Enriched rotifer (arachidonic supplemented): 8-19 dph (4-6 $rot.ml^{-2}$)
- *Artemia* nauplii: 15-23 dph (0.2-0.7 $A0.ml^{-1}$)
- *Artemia* metanauplii (arachidonic supplemented): 18-48 dph (0.2-0.7 ml^{-1})
- Dry food: from 40 dph.

Some data about behavior were known during the project:

- Peak of mortality: between 7-11 dph.
- Air vesicles and deformities: from 10 dph (with bubble pump).
- They eat voraciously from 6 dph at 18-19ºC (open mouth)
- Remain at the bottom of the tank and on walls up to 10 dph
- They remain near the surface between 7-15 dph where they remain the rest of the time.
- Wreckfish larvae have a large yolk sac and the total absorption of endogenous reserves occurs ca. 20 dph at 17 ± 0.5°C. The presence of the large yolk sac and the large oil droplet, indicates the presence of a long endogenous feeding stage.

This was the first time in the project that we succeeded in producing juveniles weaned to inert food, and this signifies a milestone in the efforts to produce wreckfish under aquaculture conditions as well as knowledge about the feeding protocol and the specific behavior and metamorphosis of wreckfish larvae. The study of the technical conditions and the adequate parameters regarding the aeration, the flow rate and water circulation, as well as the considerable numbers of larval malformations must be investigated. In conclusion, advances in the wreckfish larval rearing during this project were very encouraging, but much more work is needed in the development of practical protocols for larval and juvenile production.

The full description of the work and results is provided in deliverables ***D.18.3 Develop a feeding protocol for wreckfish larvae***, and ***D18.4 "Determine the most effective culture system (RAS vs flow-through) for wreckfish larvae.***

Deviations from Annex I and their impact:

No further work was done towards the development of a feeding methodology in the 4th RP at P1. HCMR, due to lack of good quality eggs from the broodstock of this partner, and failure to ship eggs from the other facilities in Spain. However, the work related to this task was carried out in the different facilities where the stocks of broodstock of the Atlantic area are found: IEO, MC2 and MCMR, carrying out trials with different food, doses and sequences that were described in the **Subtask 18.2.2**.Test of two culture systems RAs (CMRM) and flow-through (IEO).

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Pérez Rial, E., Linares, F., Rodríguez Villanueva, J.L., Vilar, A., Mylonas, C.C., Fakriadis, Y., Papadaky, M., Papandroulakis, N., Duncan, N., Robles, R., Lluch, N., Pazos, G., Méndez, B., Sigelaki, I., Gómez, C., Pérez, M. and Álvarez-Blázquez, B. 2019. Wreckfish (*Polyprion americanus*). New knowledge about reproduction, larval culture and nutrition. Promise as a new species for aquaculture. Fishes IP. (Submitted)

WP 19 Larval husbandry – grey mullet

WP No:	19	**WP Lead beneficiary:**	P4. IOLR
WP Title (from DOW):	Larval husbandry – grey mullet		
Other beneficiaries (from DOW):	P2. IRTA	P25. DOR	
Lead Scientist preparing the Report (WP leader):	Bill Koven		
Other Scientists participating:	Alicia Estevez, Enric Gisbert (P3), Hagay Sarusi (P25)		

Objectives

1. Investigating environmental and nutritional factors that affect larval rearing.
2. Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production.
3. Determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding.

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

The experiment addressing Task 19.1 Effect of algal type and concentration on larval performance and the Sub-task 19.1.1 (IOLR) Determine the effect of algal type and concentration in rearing tanks on larval performance had begun on Oct 31st 2014 and was being carried out at the writing of the previous report. Consequently, only the experimental design was reported then but will now be presented in the present report.

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

In sub-tasks 19.1.1 and 19.1.2, two tank turbidity levels (0.76 and 1.20 NTU) from two algal species (*Nannochloropsis oculata* and *Isochrysis galbana*) and the no-algae control (0.26 NTU) were tested on 2-25 dph grey mullet larvae. This study concluded that rotifer consumption and survival of grey mullet larvae and juveniles were dependent ($P<0.05$) on algal turbidity but independent of algal type. (2) Rotifer consumption in early development markedly influences later juvenile survival. (3) Higher survival resulted in large numbers of smaller fish which contributed to a skewed size distribution in the population.

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

In **Sub-task 19.1.1**, which determined the effect of algal type and concentration in rearing tanks on larval performance demonstrated that the higher turbidity (1.2 NTU) increased rotifer consumption independently of algal type although common biochemical factors between Nannochloropsis oculata and Isochrysis galbana may still influence larval performance. The significant ($P<0.05$) effect of turbidity level on rotifer consumption in 5 dph larvae was markedly similar to the treatment effect on survival in 51 dph fish despite that more than three weeks had elapsed since the fish were exposed to the algal treatments suggesting the long term effect and importance of rotifer feeding. The results of this study were described in the submitted

deliverable **D19.1** *Determine most effective type and concentration of algae used in grey mullet larval rearing.*

Sub-task 19.1.2 determined if the benefit of algal addition to rearing tanks was due to background lighting or other factors that contribute to larval performance. The results verified that high *N. oculata* turbidity (1.2 NTU) improved larval performance over the low *N. oculata* turbidity treatment (0.8 NTU). Moreover, larvae in the high *N. oculata* turbidity treatment significantly consumed more rotifers, as well as displaying better growth and survival than larvae exposed to the same turbidity derived from clay. This suggests a further advantage that live algae provides over its ability to produce turbidity in the larval rearing of grey mullet.

Task 19.2 compared the selected microalgae type and protocol (**Task 19.1**) with a lyophilized substitute demonstrated that larvae exposed to lyophilized and live *Nannochloropsis oculata* , which gave a turbidity of ca 1.2 NTU, in the rearing tanks resulted in very similar larval performance in terms of rotifer ingestion rate, swim bladder inflation, growth and survival. These results suggest that lyophilized algae, which are an available commercial product, can be used to replace live algae. This would lead to a significant saving in time, labour and infrastructure. Worthy of note is that the advantage of algae over clay is not lost during the lyophilisation process. Consequently, the results of the present study recommends the use of lyophilized *Nannochloropsis oculata* in the larval rearing of grey mullet.

Task 19.4 Determined when to wean larvae and which weaning diet to use according to DT maturation and the shift from carnivorous to omnivorous feeding. The results showed that mullet juveniles grew significantly less, in terms of length and final weight, when fed only an ulva based herbivorous diet compared to a commercial carnivorous feed, while fish fed the 1:1 omnivorous mix of ulva and the commercial feed exhibited markedly ($P<0.05$) superior growth than all the treatments. Fish fed the herbivorous diet demonstrated significantly higher numbers of smaller fish (<100 mg), than the carnivorous and omnivorous diet fish and, in general, exhibited a population skewed to slower growing individuals. Conversely, 200-300 mg carnivorous and omnivorous treatment fish represented a significantly ($P<0.05$) higher percentage of the population than the herbivorous diet fed fish. Mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch rich microalgae and macroalgae, as well as benthic protein rich organisms characterizing the lower salinity estuarine waters they move into at this developmental stage. Furthermore, the high amylase and maltase activity in the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing resulting in improved growth. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds

Summary of progress towards objectives (49-60 Mo):

Task 19.5 and **Deliverable *D19.5 Testing the improved grey mullet larval rearing protocol in a commercial hatchery (originally led by DOR, changed to led by IOLR).*** The summary of spawning, egg stocking, juvenile and survival and production details in 2017 were presented. The production of 78,704 juveniles carried out in 6 m^3 semi-commercial tanks does not include the juveniles harvested from experimental tasks and research studies that were carried out within the framework of Diversify. This meant that the entire juvenile production at **P4.IOLR** for 2017 was *ca.* 200,000 fish. Survival was *ca.* 20% from egg to 60 dph during the planned studies.

In ***Task 19.2 Comparing the selected microalgae type and protocol with lyophilized substitute (led by IRTA)*** and ***Deliverable D19.4 Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing,*** the use of live *N. oculata* and the best turbidity performing protocol was compared with lyophilized *N. oculata* to determine if the benefit of this protocol would be conserved if the algae were freeze dried. The lyophilized and live algae treatments provided almost identical daily turbidities during the 3-30 dph experiment. This resulted in very similar larval performances in the two treatments in terms of rotifer ingestion rate, swim bladder inflation, growth and survival. In this study, larval digestive tract enzyme activity was also very similar in the lyophilized and live *Nannochloropsis oculata* algae treatments.

Task 19.3 **and deliverable** ***D19.4 The effect of co-feeding copepods and rotifers on digestive tract maturation and enzyme production.*** The protocol for the production of *Tisbe* spp. copepods was developed at **P4.IOLR.** However, before feeding them to larval mullet, these zooplankters must be disinfected as they can be a vector for the red-pigmented pathogenic bacterium *Pseudoaltermonas* spp. The best disinfectant treatment, from three that were tested, was ozone with a CT value of 0.3 to 1.41. Unfortunately, complete mortality followed three attempts to transfer eggs, pre-larvae and end of rotifer feeding larvae, which were of high quality and robust. The results suggest that mullet larvae are extremely sensitive and all handling should be avoided before 25 dph.

Details for each Task

Task 19.3 Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production. was changed to the effect of co-feeding copepods and rotifers on digestive tract maturation and enzyme production (lead by IOLR) was completed and the full description of the work and results have been provided in ***Deliverable D19.2 Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production*** and is presented here in brief.

Zo-Opt Ltd of Geva Ada, Israel had established know-how to sustainably mass produce, suitably sized (ca 60 µm), highly concentrated (50,000 individuals ml^{-1}) and clean cultures of ciliates (*Euplotes* spp.) as a first feed for the rearing of marine fish larvae. Unfortunately, Zoopt closed a few years after the project "Diversify" began and it was impossible to carry out this task as originally planned. Consequently, **P4.IOLR** received permission from the EU to conduct a similar study but using copepods (*Tisbe* spp.) instead. At the time, **P4.IOLR** was developing protocols to produce sufficient quantities of these copepods in order to carry out small scale experiments that would co-feed these zooplankters with rotifers to early developing grey mullet larvae. The aim was to improve grey mullet larval survival, growth and advance the ontogeny of pancreatic and brush border enzymes.

Materials and methods

Copepod culture

The protocol for the production of *Tisbe* spp. copepods was developed at **P4.IOLR**, where the culture system consisted of 40 plastic carboys where the top had been cut off to give a volume of *ca*. 17 l. The copepods were given a mixture of *Isochrysis* and *Tetraselmis* spp. microalgae every 3 days while every 5 days the water was changed through filtering the copepods on a 40 µm mesh. However, before filtering, washing the copepods in filtered, UV treated seawater and feeding them to larval mullet, these zooplankters must be disinfected as they can be a vector for the red pigmented pathogenic bacterium *Pseudoaltermonas* spp., which can decimate or eradicate larval populations of grey mullet and other species.

Table 19.2.1 the weight, length and width of reared Tisbe copepods reared at P4.IOLR

	<100 µm	>300 µm	replicates
DW (µg)	3.21±0.05	10.87±0.06	50
Length (µm)	98.9±10.2	782.7±264	13
Width (µm)	30.7±2.7	244.3±141	13

Copepod disinfection studies

Four experimental protocols to disinfect the copepods were tested. In experiment 1 the copepods in water medium were treated with the commercial product "Micro-control (Rich S.A., Faliro, Greece), which is a multipurpose aquaculture disinfectant for algae, rotifers and *Artemia*. A range of concentrations (ppm or µg l^{-1}) from 25 to 300 ppm were tested on copepod cultures with exposure periods of 5, 10, 15 and 20 minutes.

Experiment 2 tested 5 minute commercial polydine (10%) treatments using different concentrations of this product (0.5, 1.0, 3.0 and 5.0 ml l^{-1}) which resulted in total copepod mortality and therefore unsuitable as a disinfectant for this zooplankter. Experiment 3 tested the effect of the antibiotic nitrofurazone on the copepod culture. Various concentrations (1, 3, 5, 8 ppm) were added to the copepod medium where the zooplankters were exposed for 22 h.

Experiment 4 used ozone as a disinfectant and was the most successful of all these trials. Two concentrations (0.1 ppm and 0.47 ppm) were tested with a 3 min exposure period (CT values were 0.3 and 1.41, respectively). The ozone in the water medium was neutralized by using sodium thiosulphate (1 ppm O_3/2 ppm $Na_2S_2O_3 \cdot 5H_2O$). Copepod samples were taken and plated on agar plates, which were incubated at 25 ^oC for 22 h in order to monitor any bacterial growth.

Experimental system

The experimental system was comprised of fifteen 17 l aquaria, from a 60 aquaria assay system (**Fig. 4**), fed by filtered (10 µm), UV treated diluted seawater (25 ‰) at 25 ± 0.5 ^oC with an exchange rate of 3 aquaria volumes per day. This allowed the testing of the three rotifer: copepod ratio treatments; (1) control-10 rotifers ml^{-1}: 0 copepods ml^{-1}, (2) 5 rotifers ml^{-1} : 30 copepods ml^{-1}, (3) 0 rotifers ml^{-1} : 60 copepods ml^{-1} in triplicate aquaria per treatment.

Larval stocking

On 4 separate occasions the 15 aquaria were stocked with hand-counted eggs or larvae; 1. eggs (100 eggs l^{-1}), 2. newly hatched larvae (1000 larvae $aquarium^{-1}$), 3. 14 dph larvae (1000 larvae $aquarium^{-1}$), 4. 14 dph larvae (500 larvae $aquarium^{-1}$). A fifth attempt was made with 25 dph larvae (50 larvae $aquarium^{-1}$), which did survive in the aquarium system, to test the effect of replacing *Artemia* nauplii with larger copepods (>300 µn). However, the copepod production could not produce sufficient numbers of these larger zooplankters to feed the fish in the aquaria according to the experimental design. On the other hand, grey mullet have already started weaning at 25 dph onto a dry food which is completed by 35 dph. As a result this study, had it succeeded, would be of limited value. Unfortunately, all eggs and larvae died within 24 h of the start of these aquarium trials and the objective of this study could not be completed.

Results and Discussion

The results of testing the "Micro-Control" product with exposure periods of 5, 10, 15 and 20 minutes with concentrations of 100, 190 and 300 ppm are shown in **Table 2**. All concentrations and exposure periods tested resulted in significant mortality and/or moribund copepods even at concentrations well below that recommended for disinfecting rotifers (50 ppm). In addition, the use of 10% polydine proved lethal to use and deemed unsuitable for disinfecting copepod cultures. In contrast, there was very little mortality in the nitrofurazone treatments but it was problematic to remove this antibiotic from the water, which is necessary as it is lethal for the larvae. The copepods could be removed from the medium through a filter but this was also a long process and the extended exposure to this antibiotic presented a potential health hazard to staff.

However, the ozone treatments were very promising at both CT values. The results are shown in **Fig. 5**, where **Fig. 5a** demonstrated that the filtered UV treated hatchery seawater, used for washing the copepods, had no bacterial growth, while the copepod medium that was not treated with disinfectant had a considerable number of colonies (**Fig. 2b**). On the other hand, the plates from the treatments having CT values of 0.3 and 1.41 demonstrated very few and no bacterial colonies, respectively (**Fig. 5c, d**). **Fig. 6** shows the copepod production after ozone treatment and washing with fresh, filtered, UV treated seawater.

The complete mortality following three attempts to transfer eggs, pre-larvae and end of rotifer feeding larvae to the aquaria was extremely frustrating as a protocol for the culturing of copepods of high quality with a low bacterial load was established and ready to be implemented. Although eggs and larvae transferred to the aquaria appeared of high quality and robust, we can only conclude that grey mullet larvae are extremely sensitive and all handling should be avoided before 25 dph. Future studies should endeavor, when copepod production has been scaled up, to use the same tanks for stocking eggs and co-feeding the larvae with rotifers and copepods.

Table 19.2.2 The effect of different concentrations and exposure periods of copepods to the disinfectant product Micro-control (Rich S.A., Faliro, Greece)

Time	100 ppm	190 ppm	300 ppm
5	no mortality	no mortality	no mortality
10	no mortality	partial mortality	partial mortality
15	no mortality	partial mortality	complete mortality
20	complete mortality	complete mortality	complete mortality

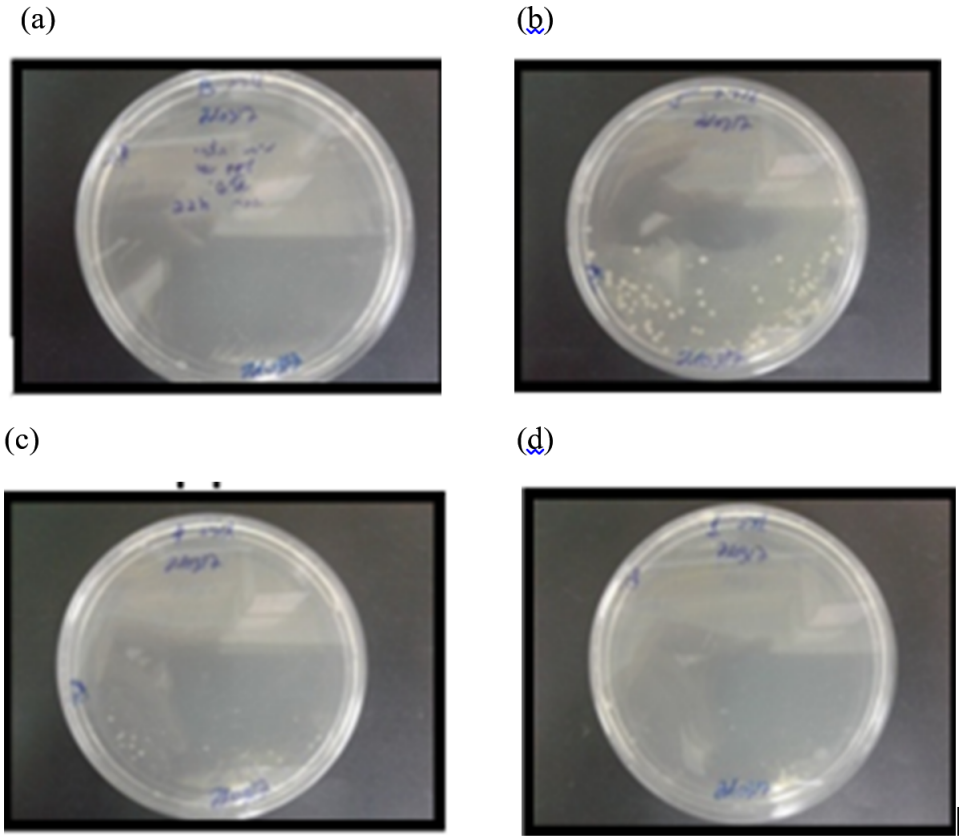


Fig. 5 Bacterial colonies on plates streaked with (a) filtered, UV treated water used for washing copepods, (b) copepods and medium without disinfectant treatment, (c) copepods and medium with CT 0.3 and (d) CT 1.41 ozone treatments

Task 19.5 *Testing the improved grey mullet larval rearing protocol in a commercial hatcher* has been completed and the full description of the work has been provided in the **Deliverable D19.5** ***Evaluate an improved grey mullet larval rearing protocol in a commercial hatchery*** which has been submitted in the 4th Reporting period and is presented here in brief.

A protocol for the larval rearing of grey mullet has been developed at **P4. IOLR** and is based on the experience garnered by the IOLR group over the last 4 years of project "Diversify". The protocol has also been tested and refined in both 400 and 1500 l conical V-tanks and has frequently delivered survival at the juvenile stage of *ca.* 20%. The results presented here are the performance of five 6000 l V-tanks which were used to carry out commercial rearing from egg to *ca.* 60-70 dph during 2017. It should be emphasized that this protocol requires control over salinity and temperature in the rearing tanks.

Materials and methods

Five 6000 l V-tanks in a flow through system where filtered (10 µm), UV-treated, 40 ‰ seawater (25 ºC) entered from the tank bottom and exited through filters near the top of the tank were used in these commercial trials. Rotifer (*Brachionus rotundiformis*) and *Artemia* nauplii were enriched with the commercial product "Red Pepper" (Bernaqua, Belgium) and the weaning diet used was a 1:1 combination of dried *Ulva lactuca* produced by **P4.IOLR** and the commercial product "Caviar" (Bernaqua, Belgium).

Results and Discussion

The summary of spawning, egg stocking, juvenile and survival and production details in 2017 are presented. The production of 78704 juveniles does not include the juveniles harvested from experimental tasks and research studies that were carried out within the framework of Diversify. This meant that the entire juvenile production at **P4.IOLR** for 2017 was *ca.* 200,000 fish. Survival was *ca.* 20% from egg to 60 dph during the planned studies. This was somewhat higher than the 10-16% in the commercial trials and may have been a result of variable egg quality. The desired egg stocking level is 50-70 eggs/l at the ambient salinity of 40 ‰ and temperature of 25-26 ºC. This was not strictly adhered to among the tanks of the commercial trials as an attempt was made to use all available eggs. It is advisable to stock eggs in darkness until hatching, where there is a 400 % tank exchange/day to wash out organic material from the hatching process. After hatching and during the pre-larval stage, the salinity is then gradually reduced to 25 ‰ and the exchange rate lowered to a 100 % tank exchange/day. Swim bladder inflation begins at 3 dph and there is ca. 100% inflation by 5 dph. Siphoning the tank bottom should be carried out each morning. Rotifer (*Brachionus rotundiformis*) enrichment (1000 rots/ml) takes place in 28 ºC seawater with a salinity of 20-25 ‰ and Red Pepper is added in two rations over 8 h according to manufacturer's instructions (0.36 ml/1 x 106 rotifers) together with 400 mg taurine/l. *Artemia* enrichment takes place in 28 ºC seawater (40 ‰) where 0.6 nauplii/ml are fed Red Pepper in two equal rations over 16 h according to manufacturer's instructions (1.5 ml/1 x 106 nauplii). The greening of the tanks using *Nannochloropsis galbana* and rotifer feeding, unenriched and enriched *Artemia* feeding as well as weaning from *Artemia* onto a dry food is described.

Task 19.2 Comparing the selected microalgae type and protocol with lyophilized substitute has been completed during the previous reporting periods and the full description of the work and results have been provided in deliverable ***D19.4 Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing*** which was submitted during the 4th periodic report **and is presented here in brief.**

Sub-tasks 19.1.1 and 19.1.2 concluded that a turbidity of 1.2 NTU in the rearing tanks of 2-25 dph mullet larvae significantly improved rotifer consumption and survival of juveniles independent of whether *Isochrysis galbana* or *Nannochloropsis oculata* was used. In **Task 19.2** the use of live *N. oculata* and the best turbidity performing protocol was compared with lyophilized *N. oculata* to determine if the benefit of this protocol would be conserved if the algae were freeze dried.

Materials and methods

The testing of live or lyophilized *Nannochloropsis oculata* derived turbidity (*ca* 1.22 NTU or concentration of 0.5 x 10^6 cells/ml) on 2-30 dph larval performance in replicates of 4 tanks per treatment was carried out under a photoperiod of 14D/10L with a light intensity of 500 lux. Turbidity was measured (Turbicheck, Lovibond, Amesbury, England) after filtering (40 µm) during the morning and afternoon addition of algae. The larvae were fed rotifers enriched on taurine and the commercial enrichment "Red Pepper" (Bernaqua Ltd., Belgium) and then *Artemia* from 15 to 21 dph, which were enriched with the same protocol. From 22 to 30 dph, the fish were fed a 1:1 (DW) combination of dried ulva and the commercial weaning diet

Caviar™ (Bernaqua, Belgium). On 30 dph, larval samples were also collected for pancreatic, brush border and cytosolic enzyme analyses.

Results and Discussion

The lyophilized (Nanno-D) and live (Nanno-L) algae treatments provided almost identical daily turbidities during the 3-30 dph experiment. This resulted in very similar larval performances in the two treatments in terms of rotifer ingestion rate, swim bladder inflation, growth (TL and DW) and survival. The effect of using live or dry algae (*Nannochloropsis oculata*) on the brush border and cytosol maturation markers; alkaline phosphatase and leucine alanine peptidase, respectively in the larvae of grey mullet. L and D values of an enzyme having different letters were significantly ($P<0.05$) different. Levels of leucine alanine peptidase having an asterisk were significantly ($P<0.05$) higher than levels of alkaline phosphatase within the live or dry *N. oculata* treatments. In this study, larval digestive tract enzyme activity was very similar in the lyophilized or live *Nannochloropsis oculata* algae treatments. On the other hand, the dry or lyophilized algae markedly ($P<0.05$) increased the activities of alkaline phosphatase, the brush border membrane (BBM) marker enzyme for gut maturation as well as the cytosol intracellular digestion marker leucine alanine peptidase over these enzymes in live algae. This would also mean that both BBM and cytosol digestion are active in this age larvae, which was consistent with the findings in older fish of the turbidity deliverable (**D19.1**, ***Determine most effective type and concentration of algae used in grey mullet larval rearing***). In the present study, freezing the *N. oculata* cells before lyophilization, particularly if it was not a rapid process can cause large ice crystals and rupturing of the cell walls. This may have allowed more microalgae cell content to be available to the larvae once ingested through drinking. On the other hand, the live, intact *N. oculata* cells may have passed through larvae with only partial or no release of their contents. Taken together, the results of this study clearly show that lyophilized *N. oculata algae* can be used to replace live algae which would be a significant saving in time, labour and infrastructure and may have expressed a growth advantage in older fish. Consequently, the results of the present study recommend the use of lyophilized *Nannochloropsis oculata* in the larva rearing of grey mullet. Nevertheless, more work is needed to determine the algal compounds that contribute to gut maturation and provide an improvement to grey mullet larvae and juvenile performance.

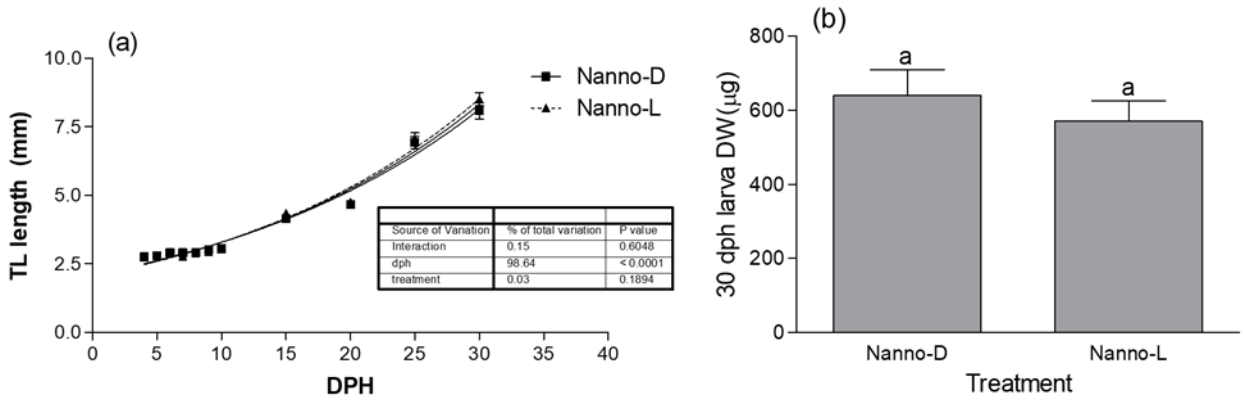


Figure 3 The effect of lyophilized and live algae (Nanno-D, Nanno-L, respectively) turbidity on (a) 3-30 dph larval length; n=4.

Deviations from Annex I and their impact:

D19.2 Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production was the original deliverable in the DOW. The ciliates were supposed to be supplied by Zoopt Ltd., who was being sub-contracted. Unfortunately, this company closed during the project. EU permission

was given to change the task and deliverable to ***Task 19.3*** and deliverable ***D19.2 Determining the effect of co-feeding copepods and rotifers on digestive tract maturation and enzyme production (lead by IOLR).*** However, after complete larval mortality following 4 attempts to stock the experimental aquarium system, this study was not completed although developing methodology to culture *Tisbe* spp. copepods and a disinfectant protocol for mullet eggs, which were part of this deliverable, were completed. ***D19.5 Evaluate an improved grey mullet larval rearing protocol in a commercial hatchery*** was meant to be carried out by **P25.DOR** but this commercial hatchery closed during the project and the task and deliverable was carried out at **P4.IOLR**. ***D19.4 Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing*** was completed on month 55 and not on month 48 due to technical problems in the performing of the experiment and analyses.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Koven, W., Gisbert, E., Nixon, O., Solovyev, M.M., Gaon, A., Allon, G., Meiri-Ashkenazi, I., Tandler, A., Rosenfeld, H. 2019. The effect of algal turbidity on larval performance and the ontogeny of digestive enzymes in the grey mullet (*Mugil cephalus*). *Comparative Biochemistry and Physiology - Part A* 228,71-80.

Koven, W., Gisbert, E., Estevez, A., Nixon, O., Meiri-Ashkenazi, I., Tandler, A., Rosenfeld, H. Comparing the efficacy of adding live or lyophilized *Nannochloropsis oculata* on grey mullet (*Mugil cephalus*) larval performance. (in preparation)

Koven, W., Gisbert, E., Meiri-Askenazi, I., Nixon, O., Tandler, A., Rosenfeld, H. Determine the efficacy of weaning diet type during the carnivorous-omnivorous transition of juvenile grey mullet (*Mugil cephalus*). (in preparation).

Group Work Packages

Grow out husbandry

For meagre, during the last period the analysis of data generated was completed and the relevant deliverables were submitted for determining optimum conditions for the cage rearing. From the shading experiment, no differences were found in the parameters monitored. The optimal feeding methodology that adjusts to the biological characteristics of meagre during grow out should aim to feed when light intensity is low (dusk, dawn and night) using stimuli to ensure a good feeding response while the fish should be left to digest during periods of high light intensity (daytime – particularly mid-day). Recommended feeding stimuli are both air bubbles and light or a combination of the two, that can be used in an industrial setting. Their application can be implemented and managed easily with existing technologies in sea cages.

For greater amberjack all tasks were completed and relevant deliverables submitted. During the period four experiments were performed. At the cage trial implemented in Greece mortalities due to parasite infection (*Zeuxapta seriolae*) were observed and a treatment was developed with oxygen peroxide. Groups presented high heterogeneity and size sorting was required for effective husbandry. Regarding growth performance, it was high (apx 3.5g d^{-1}) during the first 4 months and decreased later with a rate of 1.25 g d^{-1} to 1.83 g d^{-1}. For the trial in CANEXMAR fish growth along the trial showed a slow increase during the first period (December to April) with a higher slope response after April, fish needing an acclimation period after stocking in the sea. Temperature effects on the performance of g. amberjack was not significant for the ranges studied (20 & 23 ^{o}C) on fish feed intake and growth. Regarding the stocking density, no negative effects on growth were observed in fish reaching a final stocking density of 6.8 kg m^{3}.

The objective for pikeperch was to validate in commercial farm the conditions identified as optimal by assessing growth related parameters and physio-immunological status. Light spectrum (red or white) did not significantly affect the growth parameters or stress level. However, red or white light acted differently to immune functions, but it is not clear if they could induce a different level of immunocompetence. The test on stress sensitivity of fish under red or white light conditions showed no statistical differences. These results better defined that pikeperch juveniles are highly sensitive and frequent manipulations may be the major factor affecting their immunocompetence. Furthermore, the study on the effects of the domestication level and geographical origin on physiological stress response and immune status of pikeperch was concluded. While basal stress level did not differ between Czech F0 and F4 populations, the difference in response to acute stress, indicated that domestication level increased stress response in pikeperch. Moreover, the highest stress sensitivity of F4 populations observed in the current study was associated to the best immune status.

WP 20 Grow out husbandry – meagre

WP No:	20	**WP Lead beneficiary:**		P3. IRTA
WP Title (from DOW):	Grow out husbandry – meagre			
Other beneficiaries (from DOW):	P1. HCMR	P23. ARGO		
Lead Scientist preparing the Report (WP leader):	Neil Duncan			
Other Scientists participating:	Alicia Estévez (P3), Ignasi Gairín (P3), Nikos Papandroulakis (P1), Yannis Papadakis (P1), Tasos Raftopoulos (P23)			

Objectives

1. Adaptations in the existing methodology for grow out in cages related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed,
2. Development of an appropriate feeding method that respects the species' specificities.

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

The task related to meagre was targeted to adaptations of the existing methodology for grow out in cages. In particular the conditions related to the rearing environment (depth and light conditions) and improvements related to the size dispersion that is frequently observed will be studied. Also the development of an appropriate feeding method that respects the species specificities will be addressed.

Size variability of meagre juveniles: the first trial showed that (a) high cannibalism at the early stages may result in significant size variability of juveniles and (b) different size classes performed similarly following grading. For the definition of the optimum rearing environment during on growing, there is an on-going trial and another one in preparation. The results obtained until now show that depth of the cage net during on-growing affect the behaviour of the fish, but it is not yet clear whether it has any effect on growth. There is also a significant difference of fish behaviour during day and night.

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

As before, the tasks related to meagre were targeted to the development of appropriate feeding to provide improvements related to the size dispersion that is observed frequently. Regarding size variability of meagre juveniles (Task 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 was followed in

the deep and shallow cages throughout the year and glucose, lactate and lysozyme were significantly elevated in fish in shallow cages. No differences in mortality were observed between shaded and unshaded cages. Behaviour profiles of the fish described a significant difference of fish behaviour during day and night with fish being close to the bottom during the day and dispersed throughout the water column at night. There was also evidence of feeding during the night and this will be explored in the feeding methodology (**Task 20.3**) experiments programmed for the third reporting period.

Finally, a feeding behavior study demonstrated that (a) meagre is able to learn, to be trained and to remember specific stimuli that are associated with feeding time, (b) light is an acute stimulus to which the fish respond very quickly (from the second day of its application) and (c) environmental conditions, particularly light intensity, affect meagre feeding behavior.

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

The work continued to define optimal grow out conditions and develop appropriate feeding method for meagre. **Task 20.1** was completed and ***Deliverable 20.1. Methodology to avoid size variability in meagre juveniles.*** was presented. **Task 20.2 Effect of rearing environment** continues. **Sub-task 20.2.1** was completed and reported during reporting period 2. **Sub-task 20.2.2** examined the effect of shading and light intensity on grow out in cages. The work was completed on schedule and the data is being processed. A substantial amount of work was completed on **Task 20.3 feeding methodology** during the reporting period. **Sub-task 20.3.1** on feeding stimuli has been completed. Both visual (light) and mechanical (aeration) ques stimulated feeding in small (50-100 gr) and large fish (700-900 gr). Natural sunlight was also observed to affect feeding behaviour. The structure of the visual system was closely connected to the development of different behavioural patterns of meagre. A high proportion of rod cells indicated that meagre are a nocturnal species that prefers low light intensity environments. Sub-task 20.3.2 on different feeding methods was completed. All three feeding systems, self-feeding, hand feeding and automatic feeding gave similar and satisfactory growth in small (50-100 gr) and large fish (700-900 gr). The self-feeding indicated an increased feeding period at lower light intensities. A total of 50% of the stomach content had been transferred to the rest of the digestive channel after 8 hours. **Sub-task 20.3.3** on two feed distribution methods in cages was initiated. Similar growth was obtained in cages fed during the day and night. Feeding methods that delivered feed at the surface and at the bottom of the cage were compared and the data is being analyzed. The experimental work for **sub-task 20.3.4** to compare automatic and demand feeding in tanks has been completed. During an entire year the growth was similar between fish feed with automatic and demand feeders. Demand feeding fish feed during the entire 24 hour period throughout the year. Together these results indicate that cage feeding can be automated with feed delivery early and late in the day when light intensity if lowest.

Summary of progress towards objectives (49-60 Mo):

All the experimental work for all the tasks was completed during the previous reporting periods. However, during the 4th Reporting Period (49-60 Mo) the analysis of data generated during the previous reporting periods was completed and the deliverables, ***Deliverable 20.2 Definition of the optimum conditions for cage culture of meagre.*** and ***Deliverable D20.3 Methodology for meagre feeding*** were submitted. The analysis of the data generated in **Sub-task 20.2.2 Effect of light intensity in the cage** determined the best conditions for the cage rearing of meagre. The experiment focused on the effect of light during the rearing by applying or not shading on the cages. This second trial, implemented in a commercial farm, was repeated in two successive production cycles. No differences were found in the parameters monitored during the shading experiment. In conclusion recommendations were made for a feeding system for industrial application. The optimal feeding methodology that adjusts to the biological characteristics of meagre during grow out should aim to feed when light intensity is low (dusk, dawn and night) using stimuli to ensure a good feeding response from fish that can often not be observed and the fish should be left to digest during periods of high light intensity (daytime – particularly mid-day). In addition, feeding stimuli were recommended for feeding. Both air bubbles and light or a combination of the two can be used in an

industrial setting to stimulate and coordinate meagre feeding. The systems to apply these stimuli can be manufactured, implemented and managed easily with existing technologies in sea cages.

Details for each Task

Task 20.1 Methodology to avoid size variability in meagre juveniles (led by IRTA, Alicia Estévez and Neil Duncan**)**

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 20.1. Methodology to avoid size variability in meagre juveniles.***

Task 20.2 Effect of rearing environment (led by HCMR, Nikos Papandroulakis**)**

Sub-task 20.2.1 Effect of cage depth (HCMR, Nikos Papandroulakis).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 20.2 Definition of the optimum conditions for cage culture of meagre.*** During the reported period the analysis of the collected data was performed.

Sub-task 20.2.2 Effect of light intensity in the cage (ARGO, Tasos Raftopoulos; HCMR, Nikos Papandroulakis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 20.2 Definition of the optimum conditions for cage culture of meagre.*** Although the work was completed during the 3rd reporting period the results were being analysed and were not reported during the report of the third reporting period. Therefore, the results are briefly reported here.

MATERIALS AND METHODS

The objective of the trial was to test the rearing of meagre in cages with and without shading, at the installations of the fish farm ARGO, applying standard commercial procedures for two rearing periods. Homogenized groups were created and transferred to the cages at the beginning of the trial. The first trial started on November 20, 2014 and lasted until April 29, 2016. The second trial started on October 20, 2016 and lasted until August 20, 2017. Two rectangular cages of 10x10x8 m (V= 800 m^3) were used for each trial. One of them was covered with a net of 90-95% shading (**Figure 20.2.1**) while the second was covered only with a bird protecting net.

(a)  (b) 

Figure 20.2.1 Experimental cages at ARGOSARONIKOS SA. Shaded (a) and not shaded (b).

The temperature during the trials ranged from summer highs of 26ºC to winter lows of 14ºC. For the second trial the initial fish groups were 10,940 and 10,200 in each cage. The initial weight was 270 and 240 g respectively. Groups were fed manually, 3 times per day, with standard commercial diets.

RESULTS

During the second trial, both groups performed significantly better than the first trial (**Figure 20.2.2**), but again no difference was observed between the experimental conditions. Growth rates were 1.64 g d^{-1} and 1.68 g d^{-1} for the fish in the shaded cage and the non-shaded cage respectively.

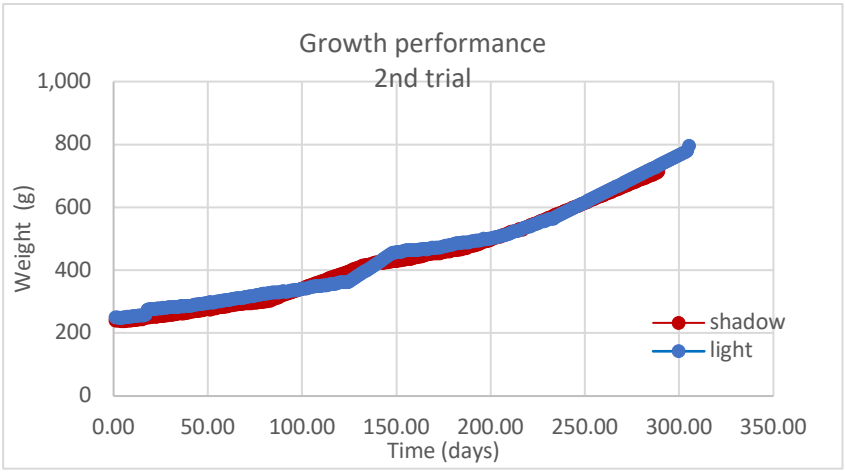


Figure 20.2.2. Growth performance of the experimental groups.

At the end of the trial a large sample of 100 individuals was taken from each group in order to estimate both mean weight but also the growth variance of the weight distribution. The results are presented in **Table 20.2.1** and in **Figure 20.2.3**.

Table 20.2.1 Final body weights of the experimental groups

Final Body Weight	Trial 2	
	Shadow	Light
Average (g)	714.3	796.5
Standard Deviation (g)	169.8	181.9
Coefficient of variation	23.8%	22.8%

Survival rate was 98.3% and 93.3% for the shaded and the non-shaded cage respectively. Finally, the results concerning feed consumption and feeding efficiency showed no significant difference in FCR being 2.0 and 1.8 for the shaded and not shaded group respectively.
Although there was a significant difference between the first and the second trial, no difference between the tested conditions was observed.

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).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D20.3 Methodology for meagre feeding.***

Sub-task 20.3.2 Test of different feeding methods (HCMR, Yiannis Papadakis).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D20.3 Methodology for meagre feeding.***

Sub-task 20.3.3 Test in cages of 2 feed distribution methods (HCMR, Nikos Papandroulakis).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D20.3 Methodology for meagre feeding.***

Sub-task 20.3.4 Comparison of automatic and demand type feeding in tanks. (IRTA, Neil Duncan and Alicia Estevez)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D20.3 Methodology for meagre feeding.***

Sub-task 20.3.5 Development of feeding system for industrial application (HCMR, Nikos Papandroulakis).

This task has been completed and the full description of the work and results has been provided in ***Deliverable D20.3 Methodology for meagre feeding.***

All the results from Tasks 20.1, 20.2 and 20.3 were combined and analysed to provide a recommended feeding system for industrial application. Briefly the following conclusions were drawn that can be recommended to the industry.

The meagre were non-aggressive towards conspecifics and stayed low in the cage or tank during the day, compared to the night when the entire cage or tank volume was used from the surface to the bottom. Meagre were feeding during the whole day (all 24 h) as shown by the results from the self-feeder experiments. Meagre, however, presented no differences in growth performance or feed utilization between the tested conditions (timing of feeding – continual, meals during the day or night, position of feed delivery – surface or low in the cage) and, therefore, it seems that the species easily adapted to different conditions without problem. However, despite of this adaptation to different conditions, meagre exhibited higher activity and feeding during the night. Eye morphology indicated an organism adapted for nocturnal vision. Feeding and activity was suppressed by high light intensity. Meagre that were fed at depth had improved immune parameters compared to meagre fed during the day that rose to the surface during periods of higher light intensities. The digestive system evacuated in 8-12 hours for 200g fish at 19ºC. Optimal growth and feed conversion was observed at stocking densities below 20 kg/m3 and temperatures from 16 – 23 ºC. Stocking densities above 25 kg/m3, temperatures <15ºC and possibly >25ºC appeared to have negative effects on growth and FCR. Together these biological characteristics recommend that meagre are fed when light intensity is low (dusk, dawn and night) and are left to digest during the hours of high light intensity (daytime– particularly mid-day).

Air bubbles and light were quickly learnt and responded to as feeding stimuli. Both air bubbles and light or a combination of the two can be used in an industrial setting, as systems to apply these stimuli can be manufactured, implemented and managed easily with existing technologies in sea cages. However, the conditions under which the rearing is carried out should be taken into account in order to select the appropriate stimulus. Thus, the use of bubbles could easily be incorporated into the rearing of meagre, whereas the light stimulus could be used in conditions where feeding is performed under relatively low light intensities (early morning, afternoon, evening), or in cases where in general the rearing takes place under low lighting, e.g. in covered tanks or cages. Additional studies using different stimuli would be useful as the general information about the behavior of meagre is considered limited. For example, it might

be advisable to study the response of meagre in other types of stimuli, such as acoustic stimuli and test their effectiveness when used as stimuli for feeding.

Therefore, the optimal feeding methodology that adjusts to the biological characteristics of meagre during grow out should aim to feed when light intensity is low (dusk, dawn and night) using stimuli to ensure a good feeding response from fish that can often not be observed and the fish should be left to digest during periods of high light intensity (daytime – particularly mid-day).

Deviations from Annex I and their impact:

There were no deviations from the updated DOW.

Manuscripts that resulted from these Tasks (if not published, indicate Submitted, Accepted or In Preparation**)**

Duncan, Neil, Alicia Estevez, Ana Roque, Elvira Fatsini, Jordi Comas. Auto-demand feeding and growth of meagre (*Argyrosomus regius*) held in tanks during the early grow out phase. (In Preparation)

Papadakis, Ioannis, Alkioni Sfendouraki, Nikos Papandroulakis, Manolis Vasilakis, Veronica Camporesi, Karamanlidis Dimitris, Theodor Stevens, Constantinos C. Mylonas. The effect of different stimuli in the feeding behavior of meagre (*Argyrosomus regius*). (In Preparation)

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	P23. ARGO
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), Tasos Raftopoulos (P23)			

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 work reported in the previous Reporting Period (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 juvenile 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.

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

Four experiments were performed during the third period for (1) the definition of cage aquaculture in Greece, (2) the determination of minimum-maximum temperature ranges for individuals of 350 g, (3) a trial for the description of the effects of temperature on the digestive characteristics of the species during on grow**ing** and, (4) a trial for the definition of optimal stocking density for juveniles of 5g.

The main results achieved so far can be summarized as follows.
Cage rearing is important for the industrial application of the rearing, but appears to be challenging. The first trial implemented in Greece resulted in significant mortalities due to parasite infection (*Zeuxapta seriolae*) that forced a change in the objectives of the trial. Hence instead of testing the different rearing volumes (and the cage depth) information on the husbandry practices in cage aquaculture of g. amberjack was gathered. Methods to treat parasites were developed with oxygen peroxide that resulted in survival of more than 65% of the originally introduced individuals. Regarding the growth performance, during the first period of the rearing growth was high (apx 5g d^{-1}). Significant differences in growth were presented between the individuals resulting in size variability of almost 100% a problem that requires further investigation.

Environmental temperature significantly affects the performance of g. amberjack and the study implemented during the reported period was with individuals with 350g mean body weight. Fish held at 21º C showed significantly higher body weight compared with fish held at 26ºC while fish held at 16ºC showed the lowest final body weight. The survival rate was higher at 16ºC but there was no significant difference in the FCR for the whole experimental period (3 months). Plasma Cortisol levels were analogous to temperature and showed a high inter-individual variability, illustrated by high standard deviation values and consequently high coefficients of variation, which ranged from 97.2 ± 41.3% for 21 ºC to 157.3 ± 41.3% for 16 ºC and 119.7 ± 46.1 for 26 ºC. Nutrient digestibility values of amberjack were in line with the observations made in earlier studies. Overall, the digestibility coefficients were high indicating the good quality of the diets. Although temperature is one of many parameters affecting gut transit time it did not affect energy fat, protein and dry matter digestibility of amberjack.

For the digestive characteristics of the species during on growing, results showed, as a conclusion from these preliminary assays, that the optimal range for the digestion of the amberjack is between 22ºC and 26ºC and the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the

enzymatic activity in that range is maximum, while in the intestine the maximum activity range is between 12 and 18h.

Regarding the stocking density, for greater amberjack juveniles, the conditions tested was for values 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium-Low (MLD), Medium-High (MHD) and High (HD) densities, respectively. The results showed that stocking density affects growth rates and feed intake. Fish maintained at High density presented better specific growth rate although not significantly different, while the condition index presented no difference between the groups. Further to this, results showed that stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four month during which the feed intake decreased with the increase of density, although no significantly.

These results are contributing to the overall objectives of the work package in order to define the optimum conditions for the rearing of greater amberjack in cages.

Summary of progress towards objectives (49-60 Mo):

Four experiments were performed during the 4th period for (1) the definition of cage aquaculture in Greece, (2) a cage culture in the Canary Islands, (3) the determination of minimum-maximum temperature ranges for individuals of >550 g and (4) the definition of optimum stocking density for individuals of 150g.

The main results achieved so far can be summarized as follows.
The second trial implemented in Greece resulted again in significant mortalities due to parasite infection (*Zeuxapta seriolae*). Methods to treat parasites were developed with oxygen peroxide. As groups presented high heterogeneity they were reorganized in small and big and the trial was performed with the objective of monitoring the biological performance of the groups. Regarding the growth performance, during the first period of the rearing growth was high (apx 3.5g d^{-1}). During the trial the growth rate was 1.25 g d^{-1} for the small group and 1.83 g d^{-1} for the big one. For the trial in CANEXMAR fish growth along the trial showed a slow increase during the first period (December to April) with a higher slope response after April, fish needing an acclimation period after stocking in the sea.

Environmental temperature significantly affects the performance of g. amberjack and the study implemented during the reported period was with individuals with 500g that were held at Semi-closed recirculation system tanks (6 tanks, 10m3 each). Fish where manually fed to apparent satiation 2 times per day (08:30 and 14:30), from Monday to Saturday, with a commercial high protein diet (51% protein, 20% lipid). The effect of the diet quality on fish growth response at higher temperatures was moreover monitored during last part of the first trial, being fish changed to fed a commercial lower protein diet (40% protein, 20% lipid). As a conclusion, and with no significant effects observed in the 2 trials for the temperature studied (20 & 23) on the fish feed intake and growth.

Regarding the stocking density, no negative effects on growth were observed in fish of 150 g initially stocked at 3.2 kg m^3 reaching a final stocking density of 6.8 kg m^3. The absence of relevant changes among the biochemical and immune parameters assessed mainly for 150 g initial size fish, suggest that *Seriola dumerili,* reared at the higher stocking density and under the current culture condition employed are not under a stressful condition.

These results are contributing to the overall objectives of the work package in order to define the optimum conditions for the rearing of greater amberjack in cages.

Details for each Task

Task 21.1 Development of rearing method in cages (led by FCPCT, Lidia Robaina)

Experiment in cages. A first trial was implemented in the commercial cages of P27. FORKYS for a period of 12 months starting from September 2016 until the July 2017. Although for the implementation of the

Task 2 it was planned a second trial at the same facilities, the company decided that it is not possible to proceed due to administrative issues and left the project. As a contingency plan FORKYS's activities were transferred to another partner (P23. ARGO), member of the consortium and with the required facilities. The second trial, in P23. ARGO fish farm was organized and started in September 2017. The work performed in the task is fully presented in ***Deliverable 21.2***. Below a summary of the work performed during the period is presented.

21.1.1 ARGO Cage rearing (Greece)

The objective of the trial was to test the rearing of greater amberjack in cages, applying standard commercial procedures. Juveniles from HCMR and from a private hatchery (NIREUS SA) were used and approximately 26.500 individuals were delivered to the farm. Homogenized groups were created and transferred to the cages at the beginning on September 2017. Two rectangular cages of 10x10x8 m (V= 800 m^3) were used for each trial. The temperature of the area during the experimental period is presented in **Figure 21.1.1.1**

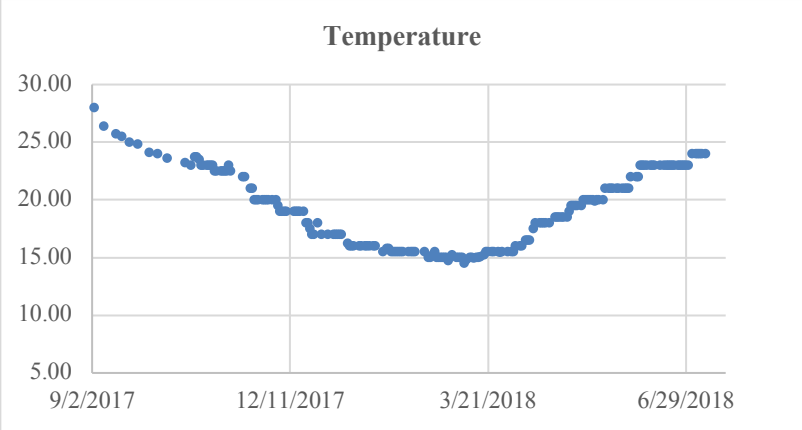


Figure 21.1.1.1. Temperature at 3 m depth at ARGOSARONICOS SA during the experimental period.

The trial started with groups of 12.000 individuals of mean weight 23 g and 14.500 of 15.5 g respectively. Groups were fed manually, 3 times per day, with commercial diets. Samples to estimate growth rate were regularly taken. During the trials, every fourth month blood samples were taken for haematological, biochemical, immunological and hormonal evaluation.

Results

During the second trial a significant incidence of parasitism occurred with *Z. seriolae* during November 2017 resulting in the loss of more than 50% of one group. The second was successfully treated with hydrogen peroxide following the procedures gained during the first trial. Furthermore, both groups have developed high heterogeneity in size and sorting was required. In the following **Fig. 21.1.1.2** the size distribution before the sorting is presented.

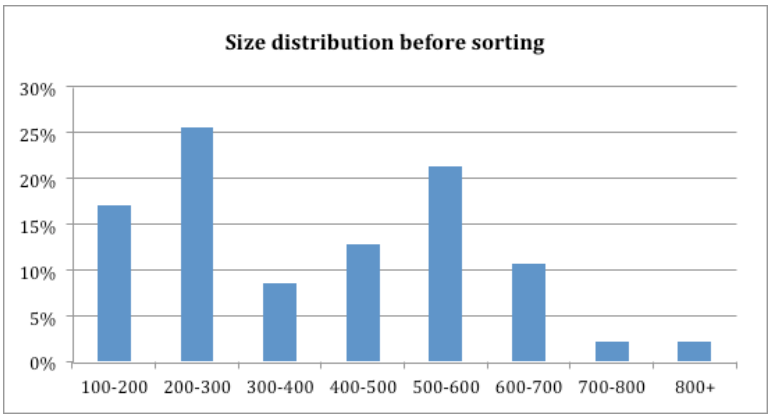


Figure 21.1.1.2 Size distribution in the ARGO trial.

In January 2018, two groups were formed of 4,700 each with a mean weight of 406±40g and 607±23g mean weight respectively. They were transferred in two cages with similar density (2.2 Kg m^{-3}) and their performance was monitored until the end of June 2018. During the first course of the trial (until the sorting) individuals presented a liner growth of 3.45g d^{-1}. The mean FCR for the period was 1.47. The rearing continued until Jul 18 when 4,870 individuals with a mean weight of 597±191g and 4,500 with mean weight of 955±189g were remaining in the cages. In **Fig. 21.1.1.3** the growth performance of the two groups is shown.

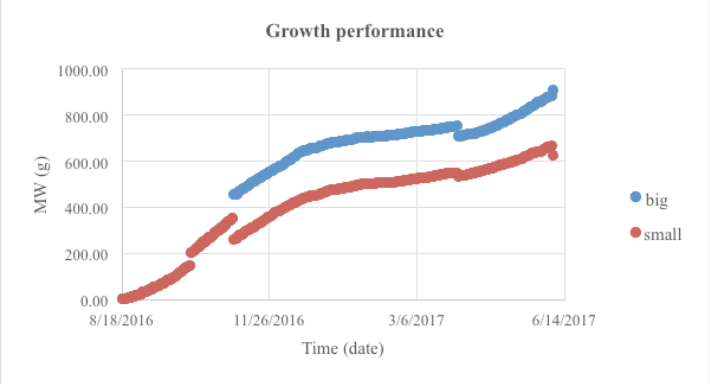


Figure 21.1.1.3. shows the growth performance observed in the experimental period.

During the trial the growth rate was 1.25 g d^{-1} for the small group and 1.83 g d^{-1} for the big one. Regarding other performance indicators, **Table 21.1.1.1** shows the mortality (%) and the food conversion ratio (FCR). No significant differences were observed although the big group performed slightly better.

Table 21.1.1.1. Performance indicators during the experimental phase

	Small	Big
Mortality (%)	2	3
FCR	2.46	2.35

The purpose of the experiments was to determine the best conditions for the rearing of greater amberjack in cages. The rearing of greater amberjack in commercial cages although is thought to be easy remains still a challenge to be resolved. The main difficulty relies on the species-specific parasites that this fish is facing. Even though the treatment of the parasite with peroxide is well established and confirmed, still the application is not easy and appropriate methodologies especially for big cages should be developed.

21.1.2 CANEXMAR cage rearing (Canary Islands, Spain)

A rearing trial in sea cages was performed in P28. CANEXMAR, located in the Canary Islands (Spain). The experiment was done during 2017, from December 2016 to December 2017. Greater amberjack juveniles of 52.92 ± 23.86 g (body weigh) (10.000) were transported from FCPCT facilities to Taliarte harbor (Gran Canaria, Canary Islands, Spain) for their transference to the farm to be stocked in their experimental cages, according to a previous agreed protocol. During the growth in the cages, a sampling schedule for proximately every 90 days was agreed with the company, although it was determined by sea overview and water current. Temperature during the growing period is showed in **Fig. 21.1.2.1**.

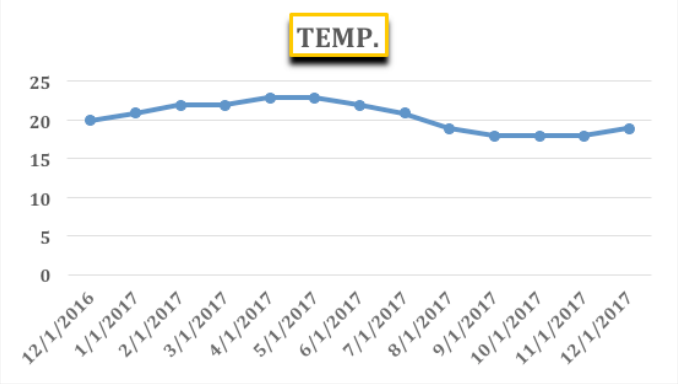


Figure 21.1.2.1. Temperature at 2 m depth at CANEXMAR SA during the experimental period.

Results

Fish transport scheme from the lab to the sea was developed according to following protocol: A track was prepared with 500L fish transport boxes; Fish density during transport was around 20-22 kg/m^3 and oxygen level maintained close to 6.5 using an oxygen bottle; Timing: initial to final fish picking from the tanks at the FCPCT (11:00-13:00) and arriving to the port (13:30), total 2:30 hours fish management. During the growth in the cages, a sampling schedule for proximately every 90 days was agreed with the company, although it was determined by sea overview and water current:1) Wt 3 batches of fish at the cages and determine medium weight & size to determine/adjust fish feeding; 2) Take 15-20 fish to the FCPCT laboratories- for the individual fish sampling; 3) Weight; length; observations & photos; 4) Parasites observations; 5) Eviscerate & wt again the fish; 6) Dissect the 2 fillets and weight (1 by 1) and stored 1 whole fillet for biochemical analysis; 7) Remain fillet and the rest of the whole fish for health analysis.

Along the trial fish were fed a daily evening meal during 30 min proximately with a commercial high protein diet. Fish responses during meals were normal and no important mortalities observed along the assay. The sea overview and the current water conditions were defined as those for the medium levels according to company daily record scales. Fish growth along the trial showed a slow increase during the first period with a higher slope response after April (**Fig 21.1.2.2**), which means that fish need an

acclimation period after stocking in the sea, while moreover cage was better covered to avoid too much light incidence and daily feeding properly adjusted and managed. Recorded water temperature did also start increasing after April. **Table 21.1.2.1** shows the results summary of the individual fish sampled at the FPCT at the different samplings along the trial.

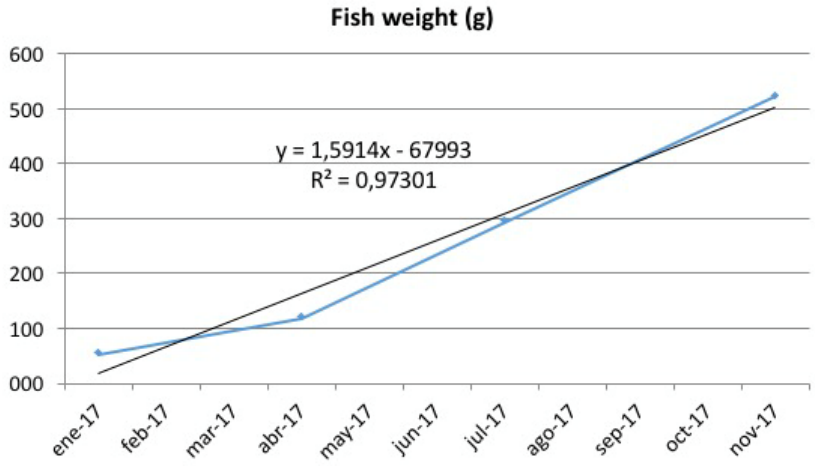


Figure 21.1.2.2. Fish weight (g) along the whole cage trial at CANEXMAR.

Table 21.1.2.1 Different measure parameters along the trial at the CANEXMAR experimental cages.

Sampling	**Temperature (ºC)**	**weight (g)**	**Growth (g from the initial)**	**Growth (% initial)**
Jan-17	18	52.91±23.86	-	-
April-17	19	119.00±25.44	66.09±25.44	124.91±48.08
July-17	21	293.70±57.35	240.79±57.35	455.10±108.40
Nov-17	23	521.82±103.73	468.91±103.73	886.24±196.05
Sampling	**Total length (cm)**	**K**	**Evisc weight (g)**	**VSI**
April-17	21.01±1.46	1.27±0.07	105.83±15.04	11.33±0.94
July-17	29.66±1.72	1.11±0.07	275.70±55.72	9.19±2.47
Nov-17	35.31±2.68	1.16±0.08	546.75±91.22	7.14±1.65
Sampling	**Right fillet (g)**	**Left fillet (g)**	**Fillet (%)**	**HSI**
April-17	26.33±6.09	25.94±4.74	55.77±3.75	1.82±0.41
July-17	48.92±11.01	56.20±14.73	65.80±3.00	1.11±0.42
Nov-17				1.55±0.35

Task 21.2 Development of feeding methods (led by IEO, Salvador Jerez).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D21.1 Definition of optimum feeding methods for greater amberjack grow out***.

Task 21.3 Development of appropriate husbandry practise (led by HCMR, Nikos Papandroulakis)

The work performed in the task is fully presented in ***Deliverable 21.1***. Below a summary of the work performed during the period is presented.

21.3.1 Temperature tolerance

For large size individuals, semi-closed recirculation system tanks (6 tanks, 10m3 each) were used. Triplicate groups of 160 fish from 203.18±20.70g where submitted to 23ºC and 26ºC during 105 days, while in the 2nd trial fish from 450-550g were acclimated and feeding at 20ºC and 23ºC (FCPCT facilities). In both cases fish where manually fed to apparent satiation 2 times per day (08:30 and 14:30), from Monday to Saturday, with a commercial high protein diet (51% protein, 20% lipid). The effect of the diet quality on fish growth response at higher temperatures was moreover monitored during last part of the first trial, being fish changed to feed a commercial lower protein diet (40% protein, 20% lipid).

Results

A summary of the growth results from both trials is shown in **Fig. 21.3.1.1**

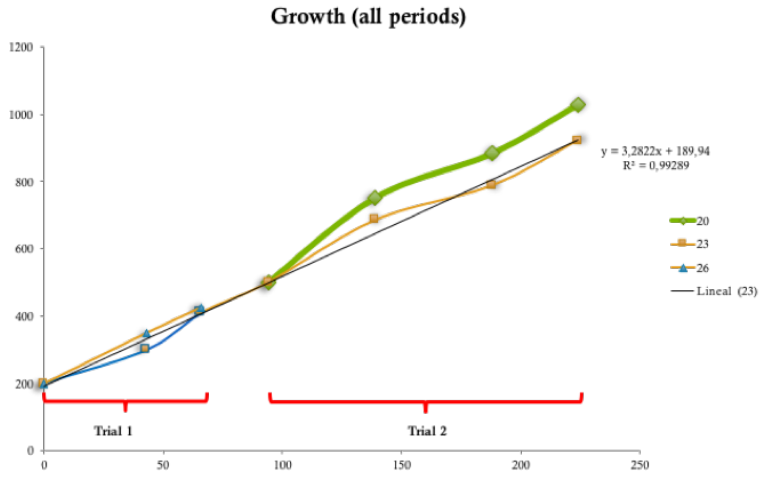


Figure 21.3.1.1 Growth curve for the amberjack growth at 20ºC, 23ºC & 26ºC from around 200g to 1000g in the 2 consecutive trials (X/Y axis =days of feeding/fish weight in g).

Survival rates were high, around 95%, being not significantly influenced by temperature over the duration of the trials. For similar feed intake (1.43 % day^{-1}), higher growth (but not significant) in weight and length were observed after 43 days at the temperature of 26°C (346.48 ± 44.99g) in respect to 23ºC (308.53 ± 39.82g). Better SGR and lower FCR were obtained for 26°C (SGR=1.24±0.15 % day^{-1}; FCR=1.30±0.21), in respect to 23ºC (SGR=0.97± 0.11 % day-1; FCR=1.94 ±0.25).

For the digestion studies, the effect of temperature and total reaction time on protein digestion is shown in **Fig. 21.3.1.2**.

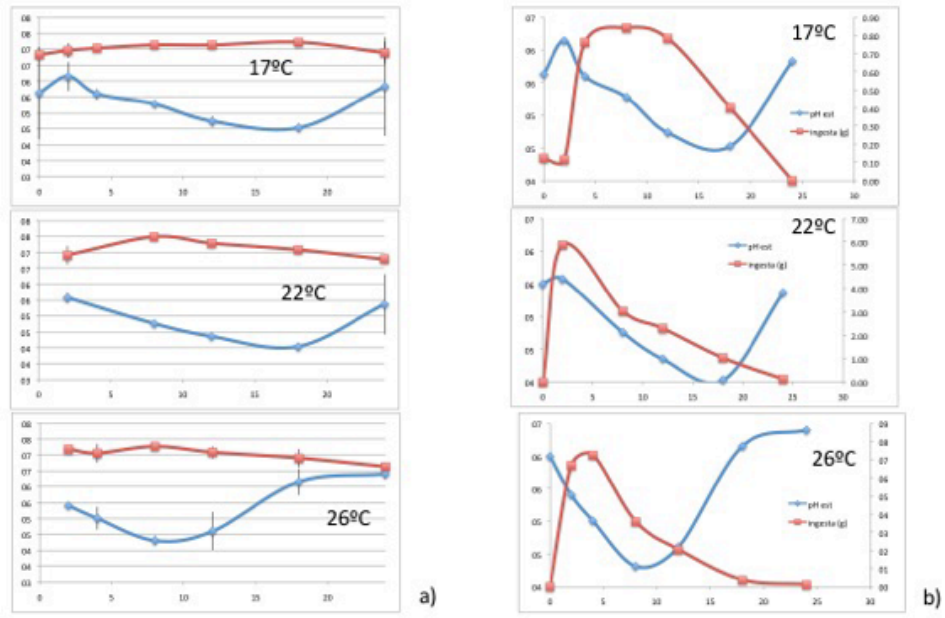


Figure 21.3.1.2. a) Intestine (red) and stomach (blue) pH values at the 3 temperatures; b) Stomach pH (blue) versus gastric evacuation.

Results showed no significant variation pattern post intake in the intestine, not also been affected by temperatures.

21.3.2 Stocking density

A 4 months rearing trial was performed, with 4 different stocking densities by triplicate (12 groups) with fish initial size of 150 g reared in 4000 l tanks**,** during 2017. A total of 480 juveniles born in captivity (average weight of 175.7±56.4g and size 20.2±2.3cm) were divided into 4 homogeneous groups, by triplicate, stocked at four different initial densities of 1.3, 1.7, 2.4 and 3.2 kg m^3 for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively. The final stocking density reached for the different treatments at the end of assay (120 days) were 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively.

Fish maintained in indoor tanks with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu) and temperature. They were fed daily with a commercial pellet for turbot (Skretting Ltd, Norway), supplied *ad libitum* at a feeding frequency accordingly to fish size. Feed left uneaten was recovered from the bottom of the tank 30 minutes after its administration to quantify the daily feed intake (FI).

Dead fish during the trial were recorded daily, measured and observed to check the presence of parasites or other pathologies.

At the beginning (day 0), and at 30, 60, 90 and 120 days, all fish in each tank were measured for weight and length. At 0, 60 and 120 days, five fish per tank were then selected randomly for blood collection from the caudal vessels using heparinized syringes.

A total of five fish at the beginning (0 day) and six fish per treatment at the end of the trial (120 days), were sampled to determine biometric parameters (viscerosomatic and hepatosomatic indexes) and to obtain samples of muscle and liver. Tissue samples were frozen in liquid nitrogen and stored at -80°C until analysis.

During the study, specific growth rate (SGR, % day^{-1}), condition factor (CF, g cm^{-3}), Viscerosomatic index (VSI, % body weight), Hepatosomatic index (HSI, % body weight), survival (S, %) and feed intake (FI, % body weight) were calculated as below:

- SGR=100 × (ln final Body weight (g) - ln initial Body weight (g)) × $days^{-1}$
- CF=100 × (Body weight (g) × Total $length^{-3}$ (cm))
- VSI=100 × Visceral weight (g) × Body $weight^{-1}$ (g)
- HSI=100 × Liver weight (g) × Body $weight^{-1}$ (g)
- S=100 × final fish number × initial fish $number^{-1}$
- FI=100 × feed consumption (g) × average $biomass^{-1}$ (g) × $days^{-1}$

Results

The Specific Growth Rate (SGR) of the fish stocked at HD was significantly higher in the periods 30-60 and 60-90 days (P<0.05). In the period 90-120 the tendency changed and the SGR decreased with the increasing of the fish density. Thus, although the SGR tended to rise with the increasing of the fish density, not significant differences were observed in the overall period (0-120 days) **(Fig. 21.3.2.1).**

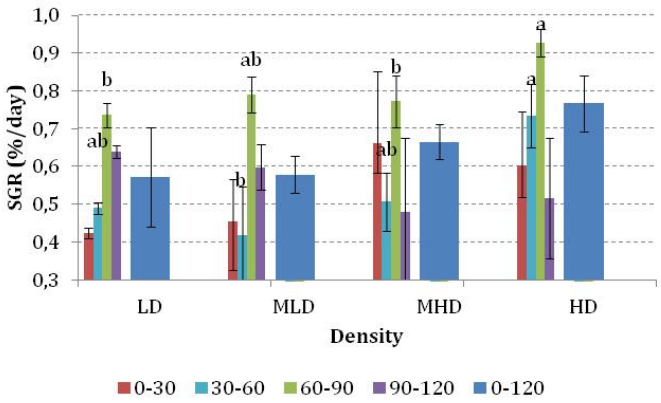


Figure 21.3.2.1. Specific growth rate (SGR, % day^{-1}) at the different periods and overall trial (120 days) of fish stocked at different densities (kg m^{-3}). Different letter indicates significant differences among treatments (P<0.05).

Feed intake (% body weight day^{-1}) decreased significantly during the first three months of experimental period in all stocking density assayed (**Fig. 21.3.2.2)**. Results of two-way ANOVA showed that both factors time (month) and stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four months during which the feed intake decreasing with the increase of density, although no significantly.

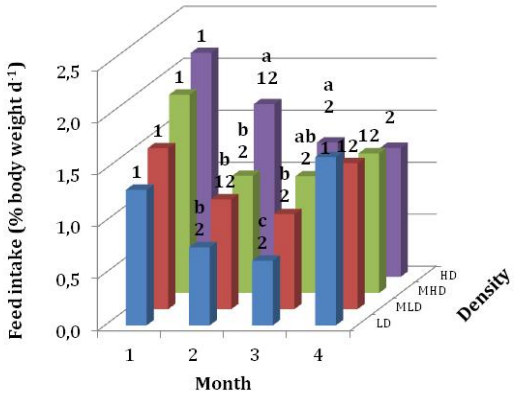


Figure 21.3.2.2. Feed intake (% body weight day^{-1}) of fish stocked at Low (LD), Medium low (MLD), Medium high (MHD) and High (HD) density during the trial. Different letter indicates significant differences among different treatments at each period. Different number indicates significant differences among each period at the different treatment (P<0.05).

In this study the SGR of 150 g initial fish tended to decrease only in the last period of the trial (90-120 days). This supports the view that increasing stocking densities have a negative impact on growth rates of greater amberjack.

Deviations from Annex I and their impact:
Project Coordinators comment: This WP was supposed to examine also the effect of floating vs submersible cage on greater amberjack performance (WP 21, Action 21.1.2, lead by P28. CANEXMAR). The experiments planned in the DOW described the use of a floating and a submersible cage for two (2) consecutive growing periods (years). However, the work has not been undertaken in its full, and only a floating cage was employed and only for a single rearing period (January to November).

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

A. Fernández Montero, M. J. Caballero, S. Torrecillas, V. M. Tuset, A. Lombarte, R. Ruiz, M. Izquierdo, L. Robaina, D. Montero. 2018. Effect of temperature on growth performance of greater amberjack (*SERIOLA DUMERILI* Risso 1810) Juveniles. Aquaculture Research, 49, 908-918.

The effect of feeding frequency on growth performance, food consumption and welfare of juvenile greater amberjack, *Seriola dumerili*. (In preparation)

Effects of stocking density and body weight on growth, feed intake, and health status parameters of greater amberjack, *Seriola dumerili* (In preparation)

WP 22 Grow out husbandry – pikeperch

WP No:	22	**WP Lead beneficiary:**		P16. FUNDP
WP Title (from DOW):	Grow out husbandry – pikeperch			
Other beneficiaries (from DOW):	P9. UL	P21. DTU	P29. ASIALOR	
Lead Scientist preparing the Report (WP leader):	Patrick Kestemont (P16)			
Other Scientists participating:	Mandiki Robert (P16), Baekelandt Sébastien (P16), Fontaine Pascal (P9), Ledoré Yannick (P9), Ivar Lund (P21), Jiri Bossuyt (P39)			

Objectives

1. Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status,
2. Characterization of pikeperch growth, immune and physiological status in farm conditions,
3. Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity.

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

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):

During the second reporting period, the aim was to characterize the effects of multiple variables on stress, immune response and growth performance by a multifactorial experiment. We then performed a fractional multifactorial trial testing 8 relevant husbandry and environmental factors. Each experimental unit represented a combination of 8 factors in two modalities including grading, stocking density (15 *vs* 30 kg/m^3), feed type (sinking *vs* mid-floating), light intensity (10 *vs* 100 lux), light spectrum (red *vs* white), photoperiod (long *vs* short), dissolved oxygen (60 *vs* 90 %) and temperature (21 *vs* 26 °C).

The results showed a clear effect of the feed type and light intensity on husbandry variables. Furthermore, stress and immune markers were affected by several interactions between feed type, light spectrum, temperature, photoperiod and oxygen saturation. Combining the results on husbandry performances and on stress and immune status, three combinations of modalities were selected as suitable for improving performances of pikeperch in intensive culture. Apart from the sinking feed, these experimental conditions were mainly characterized by their light characteristics.

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

The first objective (Task 22.2) during the third period was to characterize growth, immune and physiological status of pikeperch in order to validate the effects of the best-identified variables in farm

conditions. The ongrowing experiment from juveniles to marketable size started in April and finished around January 2018. Since light characteristics may be an important factor in pikeperch culture (see Task 22.1), it was decided to maintain fish under the two, defined as, optimal experimental modalities but testing only red vs. white light spectrum, since other factors modalities induced less variability. Several samplings of organs were done along this *in vivo* experiment. The analyses for stress and immune markers were performed from February to April 2018. A complementary *in vivo* experiment was conducted in FUNDP facilities in order to further understand some effects of the light intensity (10 vs 100 lux) and two light spectra (white and red) on stress status, humoral innate immune response and expression profile of immune-relevant genes in pikeperch. While light spectrum had little influence on tested variables, the use of a high light intensity was followed by long-term stress associated to an immune suppression. Several immune variables also followed a day-night variation. Since the secretion of the melatonin hormone by the pineal gland follows a circadian rhythm by being produced only during the dark phase of the photoperiod, it is thought to be a crucial immunomodulatory component. However, this hypothesis needs further investigations.

The second objective (Task 22.3) during the third periodic report was to assess the effects of pikeperch domestication level and geographical origin on growth and stress sensitivity (Task 22.3). For this task, the *in vivo* experiment conducted in the URAFPA facilities (Nancy, France) started in October 2017 and lasted 3 months. Fish were examined for physiological stress responses and immune competence. This task will allow establishing basis knowledge for future selection studies of pikeperch strains according to the rearing conditions of commercial fish farms.

Summary of progress towards objectives (49-60 Mo):

The objective (for the Task 22.2) was to validate in commercial farm conditions identified as optimal for pikeperch rearing by assessing growth related parameters and physio-immunological status of pikeperch at different developmental stages (from 10 g to 500 g). Light spectrum (red or white) did not significantly affect the growth parameters or stress level measured through plasma cortisol and glucose levels in plasma. However, red light improved lysozyme activity while the industrial white light increased peroxidase activity, suggesting that the two tested rearing conditions acted differently to immune functions, but it is not clear if they could induce a different level of immunocompetence. In the present experiment, we also tested the stress sensitivity of fish under red or white light conditions to grading manipulations that are unavoidable practices in pikeperch rearing. Increase in plasma cortisol and glucose was clearly observed 30 min post-stress with no statistical differences in red and white rearing conditions. These results better defined than those from the multifactorial study in Task 22.1 that pikeperch juveniles are highly sensitive to emersion stress. Therefore, the stress responsiveness to frequent manipulations in pikeperch juveniles reared in farm intensive conditions may be the major factor affecting their immunocompetence since a relationship between stress response and immune status has been established in other percid fish.

The objective (for Task 22.3) was to study of the effects of the domestication level and geographical origin on physiological stress response and immune status of pikeperch. While basal stress level did not differ between Czech F0 and F4 populations, the response to the net chasing stress was higher in the Czech F4 juveniles than the Czech and French F0 juveniles, indicating that domestication level increased stress response to the net chasing stress in pikeperch. Moreover, the highest stress sensitivity of F4 populations observed in the current study was associated to the best immune status as evidenced by immune bactericidal markers.

Details for each Task

Task 22.1 Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status (led by FUNDP, Patrick Kestemont).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 22.1. Effects of multiple variables on stress, immune response and growth performances and recommendations of optimal conditions for pikeperch grow out.***

Task 22.2 Characterization of pikeperch growth, immune and physiological status in farm conditions (led by F2B, Jiri Bossuyt).

Objectives

Based on the results from the multifactorial experiment (see Task 22.1), growth and physio-immunological status of pikeperch at different developmental stages (from 10 g to about 500 g) are compared, in farm conditions (Fish2Be), between standard husbandry conditions usually applied in routine by the SME. From this task, the expected results will help to recommend the best conditions applicable in pike perch farming for reducing stress level and supporting maximal growth performances.

Experimental design

For this experiment, a stock of early 8100 pikeperch juveniles was produced by Fish2Be farm (Belgium). Once they reached 11 g, fish were randomly distributed into 6 indoor 2,000 L-tanks. After a 2-week acclimation, the industrial white light spectrum was replaced by a red spectrum (610 nm) for half of the tanks. Since the objective was to validate the effects of the light spectrum in farm conditions, the fish farmer used the same rearing methods as usually followed in commercial farms. Light intensity (10 lux at water surface), photoperiod (12 L:12 D daily cycle) and temperature (21 °C) were maintained constant. Grading process was applied on days 49, 83, 133, 186 and 291 in order to reduce size heterogeneity, then dividing the experiment in 5 periods. With the increase in individual body weight, the stocking density is usually reduced in the commercial production system of pikeperch. So on day 83 (beginning of the third period), 500 fish from each tank were transferred into 1,500 L- tanks, at Inagro's facilities (Belgium) and the same rearing conditions were applied.

In order to assess the effects of grading procedures on stress and immune status, we sampled fish 2 hr before and 30 min after the grading manipulations at days 49, 83, 133, 186 and 291. The grading process consisted in chasing, emersion, and manipulations in order to reduce size heterogeneity by discarding smallest and biggest fish. Since high size heterogeneity observed in pikeperch culture may highly influence the physiological stress response, each sampling consisted in capturing 4 of the smallest and 4 of the biggest fish.

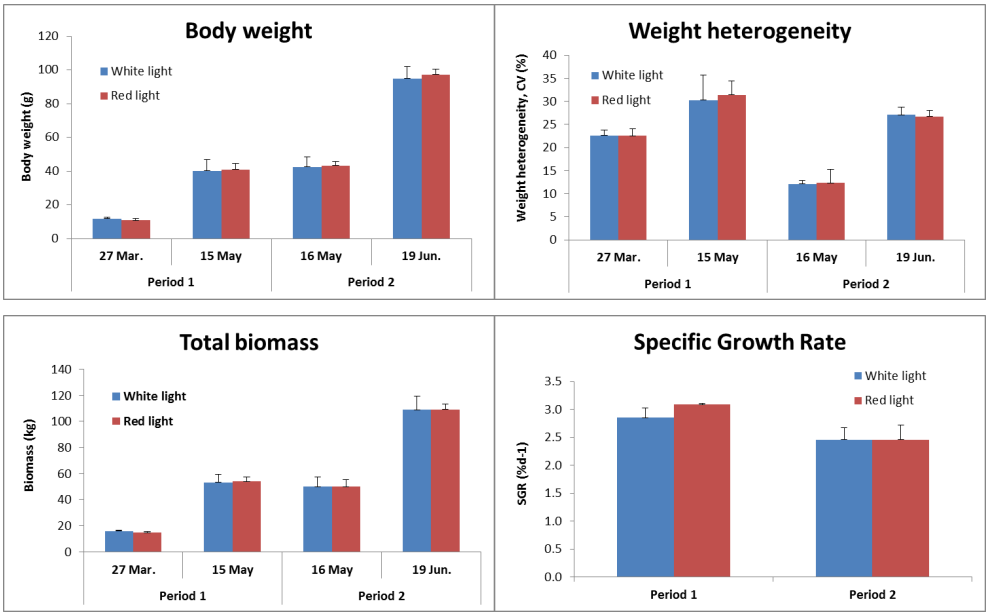


Figure. 22.2.1: Growth performances (mean ± s.d. of final body weight, weight heterogeneity, biomass and SGR) of pikeperch reared under a white (W) or a red (R) spectrum for periods 1 (27 Mar. to 15 May) and 2 (16 May to 19 Jun.). N = 3. Lowercase letters indicate a significant difference between light spectra at $p < 0.05$.

Due to poor growth parameters (strong heterogeneity and low gains of weight and biomass) after the tank transfer (periods 3 to 5), it was decided to focus on the 2 first samplings (days 49 and 83) to evaluate the physio-immunological status of pikeperch.

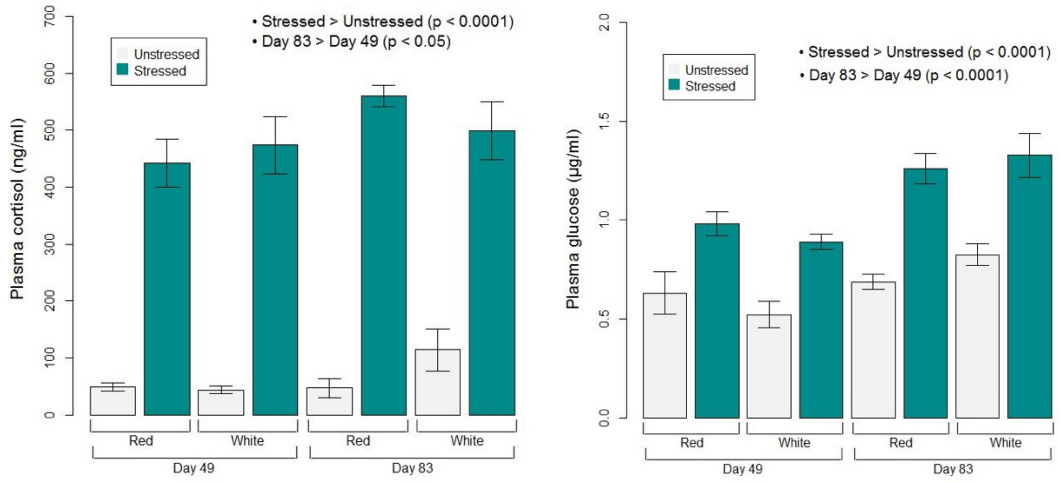


Figure. 22.2.2: Mean (± SEM) of plasma cortisol (left) and glucose (right) levels of pikeperch juveniles submitted or not to grading process (unstressed vs stressed fish), and reared under a red or a white light spectrum, at days 49 and 83. N = 24. All significant results are indicated on the graphs.

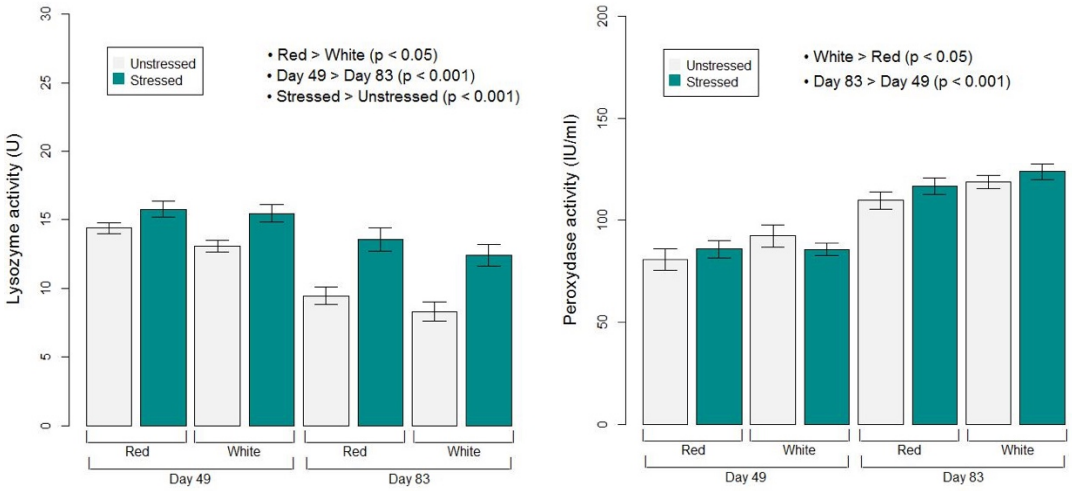


Figure. 22.2.3: Mean (± SEM) of lysozyme (left) and peroxydase (right) activities in plasma of pikeperch juveniles submitted or not to grading process (unstressed vs stressed fish), and reared under a red or a white light spectrum, at days 49 and 83. N = 21. All significant results are indicated on the graphs.

Results and discussion

During the first ongrowing phase (until day 83), good SGR results (**Fig. 22.2.1**) were observed for both experimental conditions, and values (around 3.0 and 2.5 %.day^{-1} for periods 1 and 2, respectively) were

higher than to those previously reported in lab conditions facilities for pikeperch juveniles at comparable developmental stages (Wang et al., 2009, Baekelandt et al., 2018). Unfortunately, poor SGR values were recorded afterwards due perhaps to the tank changing even if the same rearing conditions were maintained. Considering these poor results, the validation comparison of white and red light conditions was based on the results from the first ongrowing phase. During this phase of juvenile developmental stages, all husbandry parameters were statistically comparable between the experimental conditions confirming that light characteristics act mainly through light intensity.

Light spectrum (red or white) did not significantly affect the growth parameters or stress level measured through plasma cortisol and glucose levels in plasma (**Fig. 22.2.2**). However, red light improved lysozyme activity while the industrial white light increased peroxidase activity (**Fig. 22.2.3**), suggesting that the two tested rearing conditions acts differently to immune functions, but it is not clear if they could induce a different level of immunocompetence. The only study found on immune regulation by the light spectrum in teleosteans focuses in goldfish (*Carassius auratus*) (Eslamloo et al, 2013). This study revealed that a red or a blue environmental lights are chronically stressful and immunosuppressive with increase in plasma cortisol and decreases in lysozyme and plasma antiprotease. The fish immune responses might be indirectly altered by different background colors through effects on stress-induced hormones (Eslamloo et al, 2013).

In the present experiment, we also test the stress sensitivity of fish under red or white light conditions to grading manipulations that are unavoidable practices in pikeperch rearing. Increase in plasma cortisol and glucose was clearly observed 30 min post-stress with no statistical differences in red and white rearing conditions (**Fig. 22.2.2**). These results precise better than those from the multifactorial study in Task 22.1 that pikeperch juveniles are highly sensitive to emersion stress. In aquaculture, prolonged, repeated and/or unavoidable other stressors are largely associated to maladaptive physiological effects including failures in immune functions and disease resistance (Fast et al, 2008; Douxfils et al, 2011; Tort, 2011). It has also been demonstrated that percid fish such as Eurasian perch (*Perca fluviatilis*) are more sensitive to some aquaculture stressors such as emersion and handling (Jentoft et al, 2005; Douxfils et al., 2014). Therefore, the stress responsiveness to frequent manipulations in pikeperch juveniles reared in farm intensive conditions may the major factor affecting their immunocompetence since a relationship between stress response and immune status has been established in other percid fish (Milla et al., 2010; Mathieu et al., 2013).

The full description of the work and results is provided in ***Deliverable 22.2. Characterization of pikeperch growth, immune and physiological status in farm conditions.***

Task 22.3 Effect of pikeperch domestication level and geographical origin on growth and stress sensitivity (led by FUNDP, Patrick Kestemont).

Objectives

The main objective was to study the effects of the domestication level and geographical origin on physiological stress response and immune status of pikeperch. Selection of the experimental pikeperch populations was based on the Deliverable 4.2 indicating two main genetically differentiated groups that are actually available in European pikeperch farms, namely northern European populations differed to those from central Europe.

Experimental design

The selection of the experimental pikeperch populations was based on the Deliverable 4.2 indicating two main genetically differentiated groups that are actually available in European pikeperch farms, namely northern European populations differed to those from central Europe. Therefore, 3 batches of pikeperch larvae were transferred to the URAFPA facilities at the University of Lorraine, France, in order to test juveniles acclimatized to the same rearing conditions. These 3 batches included a wild French F0 strain (Lindre River, France) and two Czech strains with a wild (F0) and a domesticated (F4) batches. During the

acclimation period, fish were maintained in the same rearing conditions optimized according to the results from the multifactorial experiment (Deliverable 22.1).

It was planned that the experimental comparison would start when fish reached 20 g body weight, but due to high variations in feeding behavior and growth between the Czech populations and the French one, fish were randomly distributed into 9 indoor 800 L tanks (3 tank units per strain) on the basis of comparable biomasses (5 $kg.m^{-3}$), but with different initial body weight. Therefore, only parameters of stress and immune status were considered. To characterize the stress response of the different batches, we exposed pikeperch to chasing of 30 s. Samples were collected both before and after the application of this stress event.

Results and discussion

Effect of strain on stress responsiveness and immune status

The results from the current experiment confirmed the findings from the multifactorial study (see Deliverable 22.1) that rearing conditions with low light intensity sustained a basal stress physiological status for pikeperch juveniles as evidenced by basal plasma levels of cortisol and glucose. That basal physiological stress status did not differ between wild Czech and French populations F0. Moreover, the two wild pikeperch juvenile populations exhibited a comparable high stress response to the application of stress net chasing in terms of plasma cortisol level. This finding indicated that there are no marked differences in stress responsiveness between the Northern and Central pikeperch populations despite the differences in their geographical origin or genetic differences (see Deliverable 4.2). There is no information about the stress sensitivity among the strain populations of percid fish. As found in the current study, previous studies have reported a high stress response in other percid fish, such as Eurasian perch submitted to a single or repeated netting stressor for 0.5-1 minute (Milla et al, 2010; Douxfils et al, 2014). It was also shown that the latter species as a newly aquaculture species is more sensitive to aquaculture stressors than some salmonid aquaculture species (Jentoft et al, 2005), and this may be the case for pikeperch. As for stress parameters, levels for the tested immune markers at day 60 were comparable before and after stress application, indicating comparable immune status between the wild Czech and French populations F0. This is likely because stress sensitivity and immune status are often associated, but such relationship is not yet characterized for pikeperch populations.

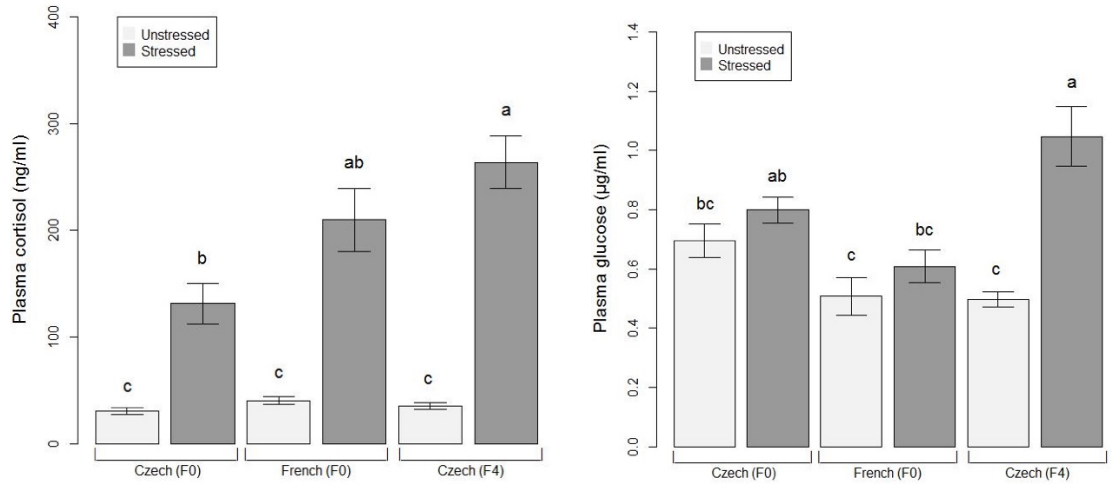


Figure. 22.3.1: Plasma cortisol (left) and plasma glucose (right) (Mean ± 1 SEM) of pikeperch juveniles (n = 12) exposed or not to chasing of 30 s. Tested batches included a wild French F0 strain and two Czech strains with wild (F0) and domesticated (F4) fishes. Significant results are indicated by different letters.

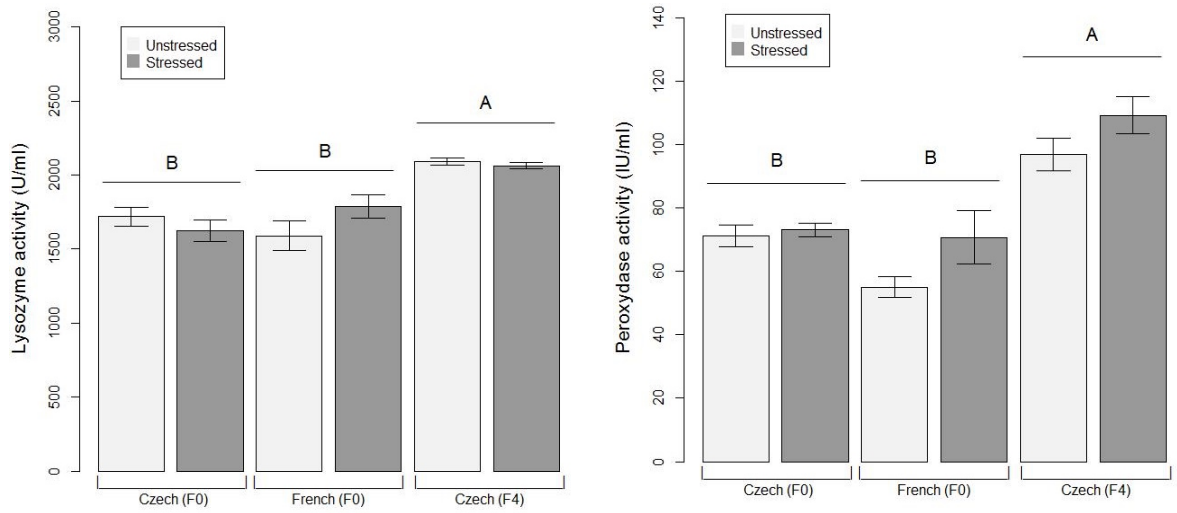


Figure. 22.3.2: Plasma lysozyme (left) and peroxidase (right) activities (Mean ± 1 SEM) of pikeperch juveniles (n = 12) exposed or not to chasing of 30 s. Tested batches included a wild French F0 strain and two Czech strains with wild (F0) and domesticated (F4) fishes. Significant results are indicated by different letters.

Effect of domestication on stress responsiveness and immune status

While basal stress level did not differ between Czech F0 and F4 populations, the response to the net chasing stress was higher in the Czech F4 juveniles than the Czech and French F0 juveniles, indicating that domestication level increased stress response to the net chasing stress in pikeperch. In other percid species, such as in Eurasian perch, it has been shown that the effect of domestication level on stress response varies depending to the type of aquaculture stressor. For example, when exposing Eurasian perch juveniles to single and repeated emersion or hypoxia stress manipulations, it was observed that both F1 and F5 generations displayed similar stress responsiveness (Douxfils et al, 2012; 2014); while chronic confinement induced an increased sensitivity of the hypothalamic-pituitary-interrenal axis and a significant cellular stress response (HSP70) in F4 individuals than in F1 juveniles (Douxfils et al, 2011). Such differential effect of domestication regarding the stress sensitivity may depend to how the induced stress intensity threatens the internal homeostasis. Indeed, stress response is first an adaptive mechanism to promote the best chance of survival towards deleterious environmental changes. So in the current study, the higher stress sensitivity of F4 populations than F0 observed in the current study may be an adaptive feature to the net chasing stressor driven along the domestication process of pikeperch.

As a fact of matter, the highest stress sensitivity of F4 populations observed in the current study was associated to the best immune status as evidenced by immune bactericidal markers. Such modulation of the association between the stress responsiveness and the immune status has been reported in other percid fish. Indeed, previous authors observed a decrease in both innate and specific immune functions in F1 exposed to various aquaculture stressors (confinement, hypoxia and emersion) fish, while a stimulation of immunity occurred in F4 ones of Eurasian perch juveniles, suggesting a rapid adjustment of the immune system to the stress event as an improvement of immune competence trough the domestication process (Douxfils et al, 2011; 2012; 2014).

The full description of the work and results is provided in ***Deliverable 22.3. Effects of husbandry practices and environmental factors on pikeperch growth, immune and physiological status.***

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Baekelandt, S., Redivo, B., Mandiki, S.N.M., Bournonville, T., Houndji, A., Bernard, B., El Kertaoui, N., Schmitz, M., Fontaine, P., Gardeur, J.N., Ledore, Y., Kestemont, P., 2018. Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pikeperch *Sander lucioperca*. Gen Comp Endocrinol 258, 194-204.

Baekelandt, S., Mandiki, S.N.M., Schmitz, M., Kestemont, P. (2019). Influence of the light spectrum on the daily rhythms of stress and humoral innate immune markers in pikeperch *Sander lucioperca*. Aquaculture, 499, 358-363.

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Deviations from Annex I and their impact:

The objective for the Task 22.2 was to confirm in farm conditions some previous results by following growth and physio-immunological status of pikeperch at different developmental stages (from 10 g to about 500 g). Unfortunately, once they reached 100 g, poor SGR values were recorded due perhaps to the tank changing even if the same rearing conditions were maintained. Thus, we focused only on first on growing phase.

The experiment for the Task 22.3 was delayed because it was not possible to get juveniles of different geographic origins and domestication levels due to a total loss of larvae by a Rhabdovirus occurrence in April 2016 in the URAFPA facilities. Therefore, the *in vivo* experiment for this task started in October 2017.

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

1. Evaluating the geographic range for grow-out of grey mullet in the Mediterranean basin,
2. 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 are the study of some parameters during the grow-out of grey mullet. The first study is related to the definition of an optimal weaning diet. The second study is a multifactorial comparison of different stocking densities and rearing systems with individuals of different origin (wild VS F1) fed an improved diet. During the period preliminary actions took place related to the collection of the required wild juveniles but also the definition of the optimal diet that will be tested. It included the collection of wild grey mullet post-larvae (about 300 mg) which in September 2014, in order to carry out Task 23.3.

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

In the grey mullet, studies determined the cost-benefit of different weaning diets on juvenile grey mullet performance as well as evaluating the effect of stocking density on the grow out of grey mullet as a function of geographic region. Task 23.1 concluded that it is possible to replace at least 75% of the fishmeal dietary component with plant-based meals without compromising growth, survival or body composition. Although the feeding trials are on-going in Israel (Task 23.2), Greece (Task 23.3) and Spain (Task 23.4), the picture emerging is that increasing stocking density markedly reduces average fish weight while having little effect on survival resulting in a skewed size distribution to smaller fish.

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

P4. IOLR in Israel compared the densities of 4 and 6 mullet/m^2 that were fed the P4.IOLR mullet grow out diet and demonstrated that the average wet weight (WW) gains in these two density treatments were not significantly different from each other. On the other hand, the effect of higher stocking densities (10 and 12 fish/m^2), which were fed the P31.IRIDA extruded diet, did have an effect on average wet weight (WW) and size distribution where the lower density demonstrated significantly higher weight gain. In addition, the FCR was improved suggesting that the extruded P31.IRIDA diet, which also replaced poultry meal with

fishmeal in the diet, was superior to the P4. IOLR pelleted diet. Moreover, the fish at the higher density (12 fish/m^2) revealed an FCR of 3.5 while the FCR for fish in the 10 fish/m^2 treatment was 3.0. P18.CTAQUA in Spain conducted a pond trial that also demonstrated the effect of density on the final wet weight of the fish. SGRs for the 0.5 and 1.0 fish/m^2 were 0.83% and 0.73%/d, respectively, after a growing period of 533 days. A greater percentage of smaller fish was found in the 1.0 fish/m^2 treatment compared to 0.5 fish/m^2 while a higher percentage of larger fish was found in the 0.5 fish/m^2 treatment compared to 1.0 fish/m^2. There was a generalized lymphocyte infiltrate in mucosa and lamina propria of the intestine of grey mullet reared at low density while moderate congestion of blood vessels was found in 50% of the samples from the high-density treatment. P1. HCMR in Greece tested the density effect of stocking wild fry at 4 and 6 fish/m^2 and that weighed *ca* 21 g per fish. This group found no significant differences between treatments in survival, growth performance and size distribution. Although these results largely agreed with P4. IOLR trial of the same densities, the Greek fish exhibited only a 30 g/fish gain over the 14 month feeding period.

Summary of progress towards objectives (49-60 Mo):

Task 23.3 compared the grow-out of grey mullet at different densities in commercial ponds in Greece (**P1. HCMR**), Spain (**P18. CTAQUA**) and Israel (**P4. IOLR**), which generally represent the range of different environmental and growing conditions existing in the Mediterranean basin. The results of Greece and Spain were reported in the 3rd periodic report. During the 4th period, the Israel growth trial was conducted. In the 144 m^2 plastic lined ponds, the density effect was more muted and there was only a slight growth advantage in 1 fish per m^2 (153.9 g) treatment over the 2 fish per m^2 (145.3 g) treatment. On the other hand, survival in the lower density fish (80.4%) was considerably higher than the 61.1% found in the higher density treatment. However, there was a major problem with herons consuming large amounts of fish, possibly more in the 2 fish per m^2 pond. The generally poor growth performance of the grey mullet in the Greek, Spanish and Israeli trials can be due to a number of factors. Certainly attempting to grow mullet in full strength seawater (40 ‰), which was the case in the Israeli trial, would be problematic as this species demonstrates the best growth in brackish or low salinity water, where energy channeled to osmoregulation is considerably less. However, a major impediment common to all the growth trials was likely the extruded diet, which remains not sufficiently attractive to the fish as they appear to prefer the detritus and primary productivity of the pond over the more nutrient dense feed.

Details for each Task

Task 23.1. Determine the cost-benefit of different weaning diets on the performance and health status of wild juveniles (led by IRTA, Enric Gisbert).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable D23.1 Cost effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status***.

Task 23.2 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of F1 juveniles stocked at two different densities in cement and earthen ponds (led by IOLR, Bill Koven).

The objectives of **Task 23.2** were to (1) perform a number of studies on various tank types and stocking densities using an improved **P4.IOLR** mullet feed and no pond fertilization with the aim to increase stocking density and improve production. (2) Compare the effect of feeding an improved grey mullet extruded feed (**P31.IRIDA**) on the grow-out in monoculture of F1 juveniles stocked at different densities in two earthen 6,000 m^2 ponds at **P25.DOR** in Israel. The results of objective 1 of **Task 23.2** contributed to deliverable ***D23.2 Stocking protocols for pond monoculture grow out of F1 and wild caught grey mullet***, which have been completed in the 4th reporting period and reported. Results from objective 2, which are reported here, contributed to deliverable ***D23.3 Comparison of the project's improved grey mullet grow-***

out feed under the different environmental and water conditions in Israel, Greece and Spain, which has been completed and reported during the 4th reporting period.

After metamorphosing from larvae to juveniles in seawater, grey mullet move to the less saline waters of estuaries and river mouths. Many countries, such as Israel, Greece and Spain, stock mullet fry and fingerlings in inland freshwater lakes, ponds and reservoirs, as they grow faster in low salinity, for commercial rearing and as a form of fisheries enhancement (culture-based fisheries). In aquaculture, grey mullet is generally grown in polyculture in semi-intensive ponds and netted enclosures in shallow coastal waters with common carp, grass carp, silver carp and Nile tilapia in brackish or freshwater. In monoculture, fish will consume the natural productivity, detritus as well as extruded feeds. However, the order of preference for these nutrient sources is unclear. It is becoming increasingly apparent that if the intensive farming of grey mullet is to succeed, then extruded diets, that are nutritionally dense and satisfy the nutrient requirements of this species, must be selectively consumed over naturally available food. In tropical areas, the growing season will last 7-8 months where the mullet will reach 0.75-1 kg. However, if kept for two on- growing seasons, the fish will reach 1.5-1.75 kg.

The study to address objective 2 in task **23.2** could not be carried out as described in the DOW since the SME **P25.DOR** closed their operation and did not participate further in the project. **P4.IOLR** attempted to carry out this experiment in two 144 m^2 plastic lined ponds but to prepare the ponds, in terms of installing new piping, plastic lining and anti-predator nets was very time consuming as well as finding sufficient numbers of fish to stock the ponds.

Methods and Materials

F2 grey mullet juveniles were stocked at two different densities (1 and 2 fish per m^2) in 2 plastic lined earthen ponds (each 144 m^2) (**Figure 23.2.1**). All fish were weighed individually at the beginning of the study and the average weight for the 1 and 2 fish per m^2 treatments were 20.72 g and 20.51 g, respectively. The ponds were fed with Red sea ambient seawater (40 ‰) at an exchange rate of 2 pond volumes day^{-1} where the temperature ranged from 18°C in winter to 30°C in summer. The fish were fed the 1.5 mm **P31.IRIDA** extruded pellets at ration level of 3% of pond fish biomass distributed over two daily feedings and the duration of the trial was 18 months.

Results and Discussion

The weight distribution as a percent of the population at the beginning of the study is shown in **Fig. 23.2.2a, which** demonstrates that the majority of the mullet population in both ponds (ca. 60%) were around 10 g. At the end of the study, there was no clear difference in size distribution in the two density treatments (**Fig. 23.2.2b**). In fact, the plastic-lined earthen pond trial in Israel exhibited only a muted density effect on growth of 1 fish per m^2 (153.9 g) treatment over the 2 fish per m^2 (145.3 g) treatment. On the other hand, growth was very similar in the two densities (**Fig. 23.2.3a**) while survival in the lower density fish (80.4%) was considerably higher than the 61.1% found in the higher density treatment (**Fig. 23.2.2b**). The generally poor growth performance of the grey mullet in the Israeli trial can be due to a number of factors. Although there was no choice, attempting to grow mullet in full strength seawater (40 ‰) was not going to deliver the best growth as a significant amount of energy will be channeled into osmoregulation instead of building tissue. A major impediment is likely the extruded diet, which remains not sufficiently attractive to the fish as they appear to prefer the detritus and primary productivity of the pond over the more nutrient dense feed. Moreover, in earthen ponds the mullet is likely using sediment to aid mashing of the plant material in the gizzard for better digestion and absorption. In order to improve the feasibility of intensive monoculture of this species, the dietary formula of the current grey mullet feed must be improved.

Figure 23.2.1 Harvesting and live weighing of the mullet from the plastic lined 144 m^2 grow-out ponds at **P4.IOLR** in Eilat

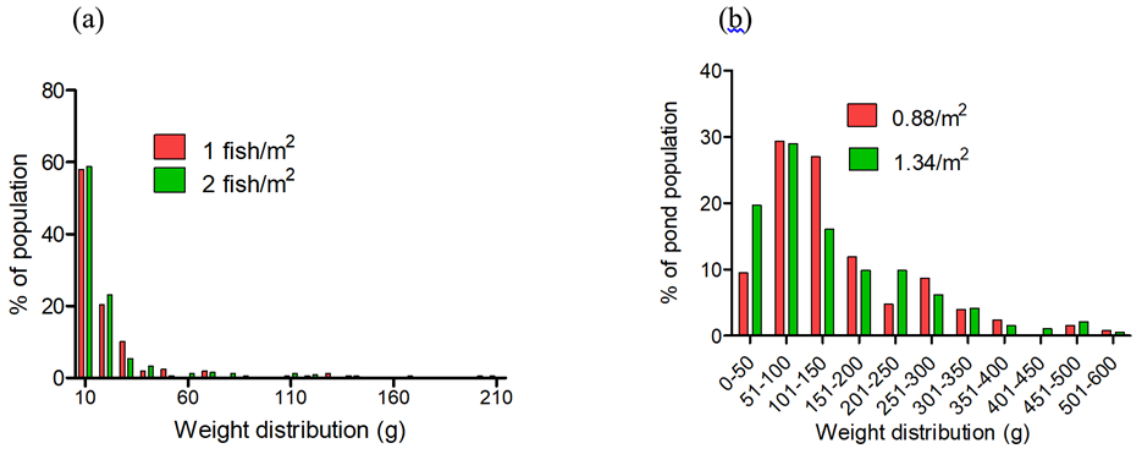


Figure 23.2.2 The weight distribution as a percent (%) of the population at (a) stocking and at (b) end of the experiment in Israel.

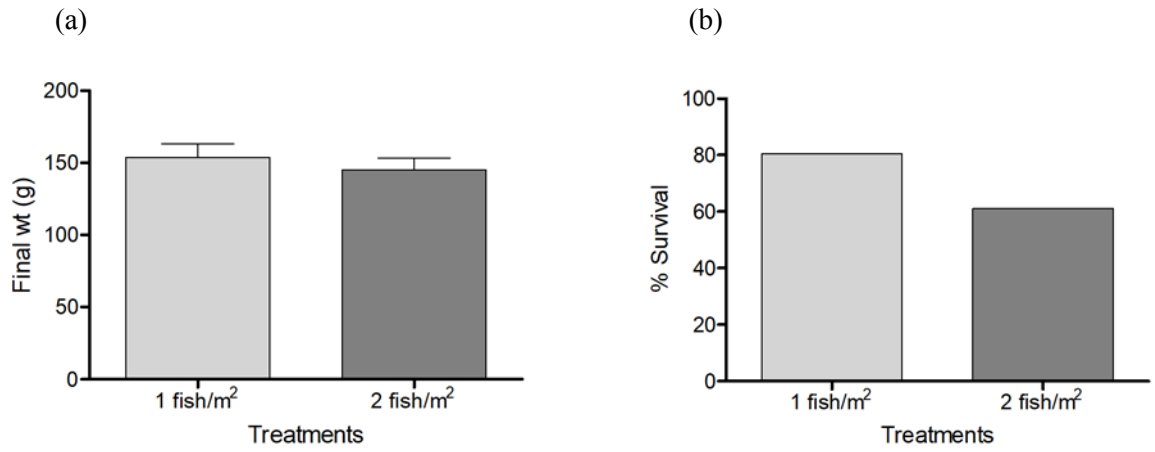


Figure 23.2.3 Final weight (wt) and survival in each of the two treatment ponds at the end of growth trial in Israel.

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 Kotzamani, and Evangelos Geitonas (P26. GEI).

This task has been completed during the previous reporting period and reported. The full description of the work and results have been provided in ***Deliverable D23.3 Comparison of the project's improved grey mullet grow-out feed under the different environmental and water conditions in Israel, Greece and Spain***.

A feeding trial was contacted in GEI's farm under HCMR's supervision employing wild-caught mullets weighing 21 ± 1.4 g which were distributed into six 14 m^3 grow-out rectangular cement ponds at the predefined densities (4 and 6 individuals per m^2, 80 and 120 fish per pond, respectively) using three replications for each density in order to investigate the effect of the two stocking treatments on fish growth and health.

Methods and Materials

Fish recruitment

The collection of the grey mullet fry was held under a license issued by the Decentralized Administration of Peloponnese-Western Greece & Ionion, General Directorate of Forest and Agriculture Affairs, Department of Agricultural Exploitations & Fisheries (Greece), during the period of September 10 through October 23, 2014. Mr. Brezas Dimitrios was responsible for the implementation of the task and has a long experience of mullet farming including also the collection of this specific species. The collection was performed in North - West region of Peloponnese (Greece) with the use of a specialized net with a mesh size of 2 mm (the collection was performed to minimize the negative impact on survival) (**Fig. 23.3.1**). After the collection of the necessary number of fish (5,000), the individuals were acclimatized to the minimum salinity conditions (the farm is operating with freshwater) by dropping the salinity of the transportation tank 2‰ every 1 hour. That was achieved by using farm water until the salinity reached levels of 8-9 ‰ then the fish were finally transported in a plastic tank.

On November 25th, 2014 all recruited fish were transferred to the facilities of VAS. GEITONAS & CO LTD (**P26.GEI**) farm in Psathochori, Arta, Greece and were assigned in two cement ponds of 10 m^3 each one, supplied with fresh water. Total weight of recruited fish was about 2,200 kilograms and the average fish body weight was 0.28 g. The water temperature was 21.5 ^{0}C and oxygen content at 8ppm. During the 10 days' adaptation to captivity/acclimatization period fish losses were reached up to 7%. The fish were started to feed daily at 3% of their body weight with a commercial feed PERLA 4-0, Skretting.

Adaptation period

For a period of 8 months the fish were reared under common field conditions and fed a commercial diet (Elite 1, Veronesi, Italy; 1.2-1.4 mm, 50% protein, 21% fat) routinely used by the farm. Furthermore, during that period the water dissolved oxygen levels and temperatures as well as the mortality were checked and recorded in daily basis. The percentage of mortalities recorded during the acclimatization period (first 10 days) was circa 7%. After that period the survival was 100%. During the acclimatization period a sample from the population was obtained to serve for species identification. The identification was performed at HCMR by specialized personnel. The species identification was based on the otoliths shape and outline according to Tuset et al., 2008 (**Fig. 23.3.2**). Furthermore, the same individuals were dissected, and the pyloric caeca were counted as *Mugil cephalus* is the only species having only 2 according to FAO (http://www.fao.org/fishery/culturedspecies/Mugil_cephalus/en) (**Fig. 23.3.3**). The identification results confirmed the species to be *Mugil cephalus*.

Figure 23.3.1. Wild caught *mugil cephalus* fish.

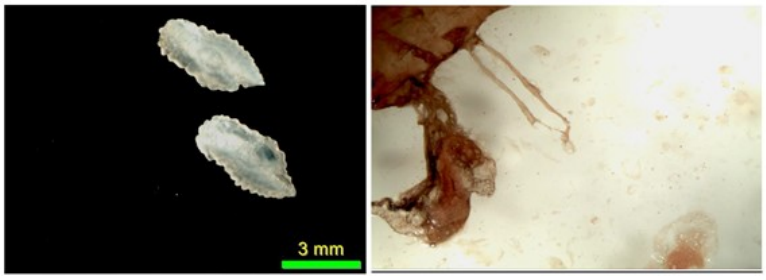


Fig. 23.3.2. Otoliths shape and outline (left) and pyloric caeca (right) of flathead grey mullet (*Mugil cephalus*)

On July 27, 2015 the fish were sorted for weight (circa 21g ± 1.4) and distributed into six grow-out rectangular cement tanks (14 m^3 volume of each one) at the predefined densities (4 and 6 individuals per m^2) using three replications for each density (**Fig. 23.3.3 & 23.3.4**). Before weighing, the fish were anaesthetized using phenoxyethanol (0.25 mg/L) and a sample of 15 fish was randomly sampled from initial population for analysing the whole-body composition. The cement ponds were continuously supplied with artesian bore water with a dissolved oxygen level around 8 ppm, which is considered optimum and water temperature at 18-22°C. The photoperiod followed the natural cycle of the season.

Figure 23.3.3. Cement ponds in the facilities of GEI in which wild fish were assigned and its headquarters (left below).

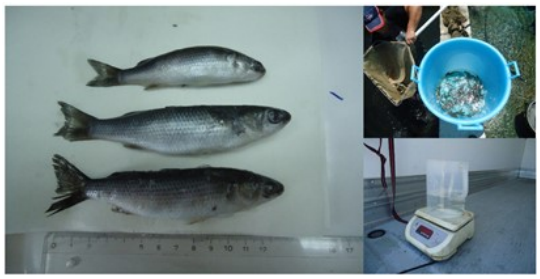


Figure 23.3.4. Initial weighing of grey mullet fry in GEI facility on July 27, 2015**.**

The feeding trial started using the experimental extruded feed (IRIDA mullet 1.5mm) provided by **P31.IRIDA** SA (**Fig. 23.3.5**). 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. Fish in all ponds were fed the same amount of feed, which was represented a ration at 2% of pond biomass that was divided into two daily feedings. Feeding was not performed when the farm staff observed feed wastes at the bottom of pond from the previous feeding. Monitoring of fish health and feed consumption as well as a recording of water physicochemical parameters were performed daily.

Figure 23.3.5. Extruded feed (1.5mm) for feeding flathead grey mullet provided from IRIDA SA.

On May 30, 2016 an intermediate monitoring of fish growth was carried out in GEI farm by HCMR's staff. Appoximately15 fish were randomly sampled from each tank, anaesthetized using phenoxyethanol (0.25 mg/L) and fish weights were recorded (**Fig. 23.3.6**).

Figure 23.3.6. Intermediate weighing of flathead grey mullet fry in GEI facility on May 30, 2016.

The feeding trial was completed on November 4, 2016, and it lasted in total 15 months. At the end of the trial, all fish from each pond were anesthetized and individually weighed after being deprived of feed for one day. Six fish from each cement pond were sampled at random, sacrificed using an overdose of anesthetic (MS-222, Pharmaqua, Athens, Greece) then pooled, minced, freeze-dried and ground to be analysed for initial whole-body composition. In addition, muscle and liver tissues from five fish from each pond were sampled for analysing their lipid and fatty acid composition.

Figure 23.3.7. Final weighing and sampling of mullet fish by HCMR's staff in GEI fish farm on 4th of November 2016.

Chemical analyses

Samples of diet and fish whole bodies, from each cement pond were analysed for dry matter and ash according to AOAC (1995). Moisture content was measured after drying the samples at 105°C for 24 h, ash was determined after ignition at 500°C for 12 h, crude protein content was analysed by using the Kjeldahl method (N × 6.25) (Kjeltec 8100, FOSS, Denmark) and total fat was estimated gravimetrically by using Soxtec™ SoxCap™ extraction (2050 automated analyser, FOSS, Denmark) with petroleum ether following acid hydrolysis (only in feeds). Gross energy of the diets was determined by an adiabatic bomb calorimeter (IKA, Werke GmbH, Staufen, Germany).

The determination of fatty acid methyl esters (FAMEs) in muscle and experimental diets were carried out by a modification of direct transesterification method proposed by (Lepage and Roy, 1984). 300-400 mg of sample were weighted in tubes and then 5ml of methanol/toluene (3/2 v/v) and 5 ml of freshly prepared acetylochloride/methanol (1/20 v/v), were added. The tubes containing this mixture were incubated at 100ºC for 60 min, allowed to cool at room temperature. Then 5 ml of water and 5 ml of hexane were added, the tubes were shaken, centrifuged and the hexane layer was taken for the FAMEs analysis. FAMEs were then analyzed using an Agilent GC-7890 B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a flame-ionization detector (GC-FID) and a DB-23 capillary column (60 m x 0.25 mm i.d. x 0.15 µm film thickness) (Agilent, Santa Clara, CA, USA). Helium was used as carrier gas at 2 ml/min constant flow, the split ratio was 1:50 and the injected volume 1.0 µL. The thermal gradient was 50ºC for 1 min, 50ºC to 175ºC at 25ºC/min, 175 ºC to 230ºC at 4ºC/min and held at 230ºC for 15 min. The injector and detector temperature were maintained at 250 and 280ºC, respectively. Fatty acids were identified by comparison with known standard mixture (Supelco 37 Component FAME Mix). Fatty acid methyl ester contents in feed and muscle tissue were expressed on the % of total FAMEs basis.

Survival and growth performance

Fish growth performance and feed consumption indexes were calculated according to the following equations:

- Survival %
- Specific growth rate, (SGR) (%/d) = 100 × [(ln FBW – ln IBW)/feeding days], where FBW and IBW are final and initial body weight, respectively.
- Total feed intake, (TFI) per fish= g DM feed/fish, where DM is the dry matter of the mean feed consumption per fish.
- Feed intake, (FI) (%/d) of initial body weight = 100 x (TFI x IBW^{-1}),
- Feed conversion ratio (FCR)= dry feed consumed / weight gain
- Protein efficiency ratio (PER)= weight gain / protein intake

Statistical analyses

Cement ponds were considered as experimental units and fish represented the sample units. All data from the individual observations were tested for normality and homogeneity of variance prior to be subjected to one–way ANOVA using Kolmogorov- Smirnov and Levene tests, respectively. Ponds means were used for comparisons. Significant differences between means were determined by Tukey's test. The level of significance was set at $P < 0.05$. All statistical tests were performed using the General Linear Model (STATISTICA version 12.0, StatSoft, USA).

Results and discussion

The chemical composition of analysed feed produced by IRIDA SA is presented in **Table 23.3.1**. Crude protein was about 34%, crude fat 14.5%, carbohydrate 37% and gross energy 20 MJ/kg. The fatty acid analysis of the feed revealed high levels of 18:1n-9 and 18:2n-6 (32.5% and 14.5%, respectively) and low levels of EPA and DHA (1.61% and 1.69%) (**Table 23.3.2**). The whole- body composition of mullet was changed as the fish were growing, protein and lipid were increased at the expense of water compared to their initial body composition (**Tables 23.3.3 and 23.3.4**).

Table 23.3.1. Chemical composition of the experimental diet IRIDA mullet 1.5mm produced by IRIDA S.A *(% or specified)*.

Moisture	6.38 ± 0.04
Crude Protein	33.94 ± 0.03
Crude Fat	14.48 ± 0.27
Ash	8.27 ± 0.02
Carbohydrate*	36.93 ± 0.02
Gross energy (MJ kg^{-1})	20.12

Data are mean ± SD.

*Calculated by difference: 100 - (%protein + %fat + %ash + %moisture) (i.e. N-free extractives + crude fiber).

Table 23.3.2. Fatty acids analysis of the experimental diet (% of total identified FA) as determined in HCMR's Nutrition lab. *Data are means, n=6 ± SD.*

FAs	Average	SD
C14:0	4.05	0.01
C15:0	0.30	0.00
C16:0	14.48	0.01
C16:1n-7	3.55	0.02
C17:0	0.17	0.00
C18:0	3.01	0.00
C18:1n-9 cis	32.50	0.06
C18:1n-7	3.22	0.01
C18:2n-6 cis	14.46	0.04
C18:3n-3	2.63	0.01
C18:4n-3	0.87	0.01
C20:0	0.31	0.01
C20:1n-9	4.74	0.03
C20:2n-6	0.53	0.01
C20:3n-6	0.1	0.01
C20:4n-6	0.16	0.01
C20:3n-3	0.19	0.00
C20:4n-3	0.30	0.01
C20:5n-3	1.61	0.01
C22:1n-11	4.90	0.04
C22:1n-9	0.40	0.01
C22:6n-3	1.69	0.02
C24:1n-9	0.47	0.01
Σ PUFA n-3	7.28	0.01
Σ PUFA n-6	15.31	0.07
n-3/n-6	0.48	0.00
Σ Saturates	22.86	0.03
Σ Monoenes	49.77	0.13
EPA+DHA	3.30	0.01

Table 23.3.3. Initial whole-body composition (% wet weight) of flathead grey mullet fry.

Moisture (%)	64.9 ± 0.4
Crude Protein	13.8 ± 0.1
Crude Fat	17.0 ± 0.2
Ash	3.0 ± 0.2

Data are mean ± SD.

Table 23.3.4. Whole body composition (% fresh weight) of grey mullet at the predefined densities (4 and 6 individuals per m^2) fed the experimental diet at the end of the trial.

	4 fish/m^2	**6 fish/m^2**
Moisture (%)	60.30 ± 0.96	61.27 ± 1.41
Crude Protein (%)	15.91 ± 0.83	16.17 ± 0.59
Crude Lipid (%)	20.04 ± 1.41	19.38 ± 1.69
Ash (%)	3.75 ± 0.29	3.52 ± 0.27

Data are presented as means ± SD. Different superscripts in row means indicate statistically significant differences (Tukey's HSD. P<0.05).

There were no significant differences in survival and growth performance of wild fry between the two densities treatments at the end of the experiment. The survival of fish was similar in both density treatments, with values of 72.1 ± 11.3% and 75 ± 5.3% for 4 and 6 fish/m^2 treatments, respectively At the completion of feeding trial, fish that were stocked at 4 individuals/m^2 had an average weight of 51.6 ±11.2 g, which was the same with that (51.2 ± 8.5 g) of fish from the 6 individuals/ m^2 treatment and represented a growth increase of 143% in both treatments (**Figure 23.3.8**). The frequency analysis showed that the weight range of 20-80 g represented the dominant weights of the fish population in both density treatments (**Figure 23.3.9**). On the other hand, larger fish (80-180 g) from the 6 fish/m^2 treatment represented a bit larger part of the population (14 %) than the lower density of 4 individuals/m^2, which was 12 % of the population.

At both density levels a very poor growth of fish was observed (only ~30 g weight gain) over the 14-month feeding period. It is remarkable that fish failed to grow significantly following the intermediate sampling which was contacted on May 30, 2016 (Table 23.3.5). For this reason, the growth performances were calculated only for the period from July 27, 2015 until May 30, 2016 in which the fish gained weight. Fatty acids analysis of fish muscle and liver did not reveal any significant difference between the two densities treatments at the end of the feeding trial (**Tables23.3.7 & 23.3.8**).

Table 23.3.5. Growth performance indices for grey mullet fed the experimental diet from July 27, 2015 until May 30, 2016.

	Stocking treatment	
	4 fish /m^2	**6 fish/m^2**
Initial body weight (g)	20.50 ± 2.01	21.07 ± 0.12
Body weight (g) (until May 30, 2016)	49.99 ± 10.32	42.15 ± 6.04
WG	29.49 ± 8.62	21.08 ± 6.15
DGI %	0.30 ± 0.06	0.23 ±0.06
TFI	134.51 ± 2.59	125.35 ± 3.43
FCR	4.93 ± 1.91	4.61 ±1.45
PER	0.70 ± 0.23	0.72 ± 0.19
SGR	0.28 ± 0.05	0.22 ± 0.05

Data are presented as means ± SD (n=3). Row means that have no superscript in common are significantly different from each other (Tukey's HSD. P<0.05).

WG: weight gain (g/fish); DGI: Daily growth index; TFI: Total feed intake (g) per fish; DFC: Daily feed consumption (%); FCR: Feed conversion ratio; PER: Protein efficiency ratio; SGR: Specific growth rate; TGC: Thermal growth coefficient.

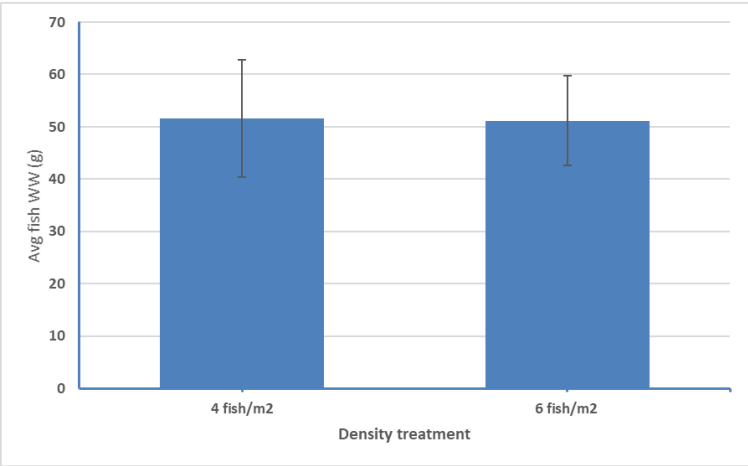


Figure 23.3.8. The effect of two stocking treatments (4 & 6 fish/m^2) on the average fish weight at the end of the trial. N=80. 120 of the 4 and 6 fish/m^2 treatments, respectively. ANOVA of values was found not significant (P>0.05).

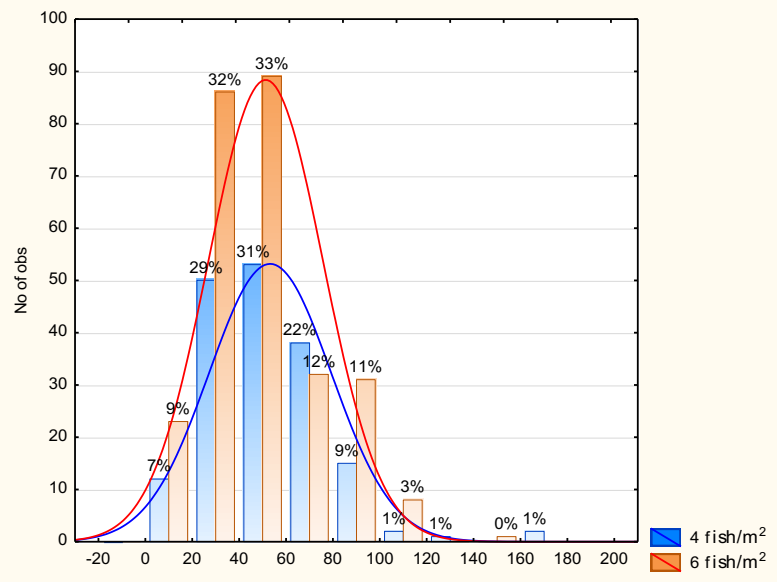


Figure 23.3.9. The effect of the two stocking treatments (4 and 6 fish/m^2) on the weight distribution in the population.

Table 23.3.6. Fatty acids analysis of muscle from grey mullet at the end of the trial (% of total identified FA). *Data are means, n=6 ± SD.*

	4 fish/m2		6 fish/m2	
FAs	**Average**	**SD**	**Average**	**SD**
C14:0	2.70	0.37	2.71	0.33
C15:0	0.27	0.01	0.27	0.03
C16:0	17.20	0.44	16.19	0.66
C16:1n-7	6.16	0.78	5.92	1.41
C17:0	0.26	0.03	0.24	0.03
C16:4 n1	0.36	0.09	0.33	0.01
C18:0	3.80	0.68	3.63	0.29
C18:1n-9 cis	19.15	2.15	19.54	1.75
C18:1n-7	3.26	0.17	3.10	0.26
C18:2n-6 cis	12.76	1.07	12.67	0.66
C18:3n-3	2.25	0.17	2.58	0.10
C20:0	0.18	0.02	0.17	0.04
C20:1n-9	2.13	0.23	1.78	0.43
C20:2n-6	0.94	0.42	0.67	0.06
C20:3n-6	0.25	0.02	0.22	0.02
C20:4n-6	1.61	0.44	1.47	0.07
C20:3n-3	0.24	0.01	0.24	0.01
C20:4n-3	0.50	0.03	0.52	0.03
C20:5n-3	4.28	0.45	4.80	0.57
C22:1n-11	0.86	0.14	1.41	1.25
C24:0	3.51	0.28	3.37	0.10
C22:6n-3	10.90	1.63	11.14	0.99

C24:1n-9	0.29	0.04	0.26	0.13
Σ PUFA n-3	18.50	2.01	19.28	0.50
Σ PUFA n-6	15.19	0.34	15.52	0.59
n-3/n-6	1.22	0.16	1.25	0.07
Σ Saturates	27.65	1.49	26.73	0.83
Σ Monoenes	32.53	3.21	32.66	0.63
EPA+DHA	15.18	2.07	15.94	0.55

Table 23.3.7. Fatty acids analysis of liver from grey mullet at the end of the trial (% of total identified FA). *Data are means, n=6 ± SD.*

	4 fish/m^2		6 fish/m^2	
FAs	***Average***	***SD***	***Average***	***SD***
C14:0	2.01	0.32	1.91	0.38
C15:0	0.25	0.02	0.28	0.02
C16:0	12.23	0.84	12.12	0.22
C16:1n-7	8.89	1.58	8.00	2.15
C17:0	0.16	0.01	0.23	0.04
C18:0	1.99	0.13	2.49	0.63
C18:1n-9 cis	22.82	1.00	18.64	0.84
C18:1n-7	5.60	0.53	5.35	0.34
C18:2n-6 cis	12.58	0.90	11.90	0.39
C18:3n-3	2.19	0.25	2.24	0.31
C20:0	0.11	0.02	0.10	0.01
C20:1n-9	2.48	0.34	1.95	0.21
C20:2 n-6	1.37	0.22	1.26	0.12
C20:3n-6	0.40	0.03	0.39	0.02
C20:4n-6	1.38	0.35	1.72	0.15
C20:3n-3	0.49	0.05	0.50	0.08
C20:4n-3	0.79	0.07	0.79	0.05
C20:5n-3	2.13	0.40	3.26	0.24
C22:1n-11	0.21	0.09	0.13	0.01
C24:0	3.97	0.77	4.51	0.33
C22:6n-3	10.17	1.40	12.76	0.99
C24:1n-9	0.15	0.03	0.18	0.03
Σ PUFA n-3	15.78	1.85	19.56	1.57
Σ PUFA n-6	16.58	1.16	16.01	0.51
n-3/n-6	0.95	0.06	1.23	0.06
Σ Saturates	20.78	0.34	21.99	0.28
Σ Monoenes	40.64	2.55	34.75	2.47
EPA+DHA	12.30	1.71	16.03	1.16

Conclusions

The results from the present study did not indicate any clear effect of stocking density on mullet growth. However, poor growth of grey mullet reared in cement tanks under GEI's rearing conditions was found after a period of 14 months. Several reasons may have negatively affected the fish growth such as the unsuitability of cement ponds for mullet culture, which was resulted to improperly feeding of fish, the lack of natural food, palatability and nutritious issues of feed, which didn't meet fish requirements or perhaps husbandry practices as well.

Task 23.4 Compare the effect of feeding an improved grey mullet diet on the grow-out in monoculture of wild juveniles at two different densities in ponds in Spain (led by CTAQUA, Rocio Robles).

This task has been completed during the previous reporting period and reported. The full description of the work and results have been provided in ***Deliverable D23.3 Comparison of the project's improved grey mullet grow-out feed under the different environmental and water conditions in Israel, Greece and Spain*** and is provided here in brief.

A farm trial has been conducted with a fish farm company (not part of the Diversify consortium) producing seabass in earthen ponds. A batch of wild grey mullet fingerlings of 1 g average body weight were received at the facilities of Ctaqua and were acclimatized from 10 ppt salinity (water salinity from origin) to the 35 ppt of the recirculation aquaculture system (RAS) water where they were first grown until they reached the right size to be seeded in the two earthen ponds at the farm were the field trial has been carried out (Trebujena, province of Cádiz (South Spain)).

The fish were grown in the RAS system, during 4 months until they reached 3.5 ± 0.4 g average body weight. During the two weeks before moving them to the ponds, grey mullet fingerlings were acclimatized to the lower salinity of the farm pond water (12 ppt) and to the feed they would receive at the farm. The feed was provided by P31.IRIDA and it is a diet specifically formulated for grey mullet (based on P4. IOLR formula). Two feed sizes were provided: 1.5 mm diameter and 3 mm for the last part of the growing period.

Materials and Methods

Wild grey mullet fingerlings were purchased to a supplier specialized in harvesting and delivering wild specimens in the Northwest of Spain (Delta del Ebro). Fingerlings were transported by the company till the premises of CTAQUA.

The fish arrived to the facilities of P18.Ctaqua in February 2015. A total of 1500 grey mullet fingerling of 1 g average body weight, were stocked in a recirculation system (RAS) with 15 tanks of 125 l each. The RAS is comprised of units for mechanical filtration, biofiltration, protein skimmer and UV treatment (**Fig. 23.4.1)**. 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.

Figure 23.4.1 RAS system used to acclimatize and grow the grey mullet fingerlings before seeding in the farm ponds.

Water quality parameters were controlled twice per week, except temperature and dissolved oxygen that have been checked daily, as well as mortality and fish welfare. Fingerlings remained in the RAS during 4 months until they reached an average body weight of 3.5 g. During the last two weeks of June, grey mullet fingerlings were acclimatized to the lower salinity in the farm (12 ppt) and to the extruded feed they would receive in the field. This feed was provided by P31.IRIDA, a diet specifically formulated for grey mullet (based on IOLR formula) of 1.5 mm diameter (see Task 23.2; P4.IOLR/P31.IRIDA; Tables 2.1.1 and 2.3.1), although natural feeding was also available in the ponds.

A total of 1.344 grey mullet fingerlings of 3.5 ± 0.4 g were moved to the farm and seed in two different ponds named L3 and L4 following the density stablished in the DOW:

- L3: 1100 m^2; in this pond the density of 0,5 indiv per m^2 was used; 544 fingerlings were seeded.
- L4: 800 m^2; in this pond the density used was 1 indiv per m^2; 800 fingerlings were seeded.

The trial lasted 18 months (from July 2015 till December 2016). During the trial the grey mullet have been fed the extruded diet provided by the **P31.IRIDA**. Feed has been provided in two pellet size: 1.5mm diameter for the first growing period and 3 mm for the second growing period. Fish have been fed manually once per day at the first time of the morning to check fish feeding behavior and with automatic belt feeders for the rest of the day (**Fig. 23.4.2)**. The photoperiod and temperature followed the natural cycle of the season.

Figure 23.4.1 View of the two ponds L4 (top) and L3 (bottom) used for the trial.

Four samplings have been performed: at stocking (July 2015), two intermediate samplings, February 2016 and June 2016, and the final sampling in December 2016, where all harvested fish were individually live weighed and measured.

Although it was planned after the sampling of the summer 2016 to modify the culture conditions and collect all the fish from pond L4 to move it to the pond L3, it was not possible to harvest completely the pond L4. In this situation, the culture continued in the two ponds and a final sampling was performed in December 2016. It has to be considered the difficulty of this type of field samplings since it is very difficult to congregate all the fish in the net without causing high stress (**Fig.23.4.3)**. Likewise, in the final sampling it was not possible to harvest all the fish from the ponds since the pond cannot be completely emptied. For all the samplings, fish were collected in the net and transfer immediately to a bath with clove oil in order to tranquilize them to proceed to the weight sampling (**Fig. 23.4.4).** Since the earthen ponds are systems with high productivity it was always necessary to count with extra oxygen supply to perform the samplings.

Figure 23.4.2 Final sampling in the farm (top pictures). It is a labor-intensive task and it is very difficult to avoid that fish do not escape from the net (especially grey mullet which is known for its capacity to jump quite high from water surface; see red circle).

Figure 23.4.3 Set-up for the field sampling including extra oxygen supply.

To further document the culture of grey mullet in earthen ponds, at the moment of the final sampling, 10 individuals from each pond were dissected and individual samples of distal intestines were preserved in buffered formalin to evaluate the histological status of the epithelium (task not included in the DOW). Samples of fillet from 6 specimens were frozen (-80ºC) and sent to **P15. ULL** for proximate analyses and fatty acid profile (in connection with **WP28**).

Results

A summary with the results of the four sampling of the trial is included in the **Table 23.4.1.** The results obtained during the pond trial reflect the effect of density on the final wet weight of the fish from both density conditions. Pond L3 (0.5 indiv per m^2) yielded fish with an average final body weight of 294.02 ± 138.89 g and in the pond L4 (1 indiv per m^2), fish had an average final body weight of 174.48 ± 55.36 g. These results are in accordance with the findings of **P4.IOLR**. **Fig. 23.4.5** presents the final average body weight of the grey mullet reared at the two densities. Condition index was similar for both culture densities as it is presented in **Fig. 23.4.6,** which it is an indication of no differences in feeding conditions between the two ponds.

Final specific growth rate (SGR) values from the two culture conditions were 0.83 % day^{-1} for the lowest density pond and 0.73% day^{-1} for the highest density, for a total culture period of 533 days. Concerning FCR, no reliable data is available since the farm staff did not always load the automatic feeders everyday based on their observation of the fish reaction when feeding manually at the first time in the morning and did not really follow the advice of the scientific staff. Taking this into account and considering an estimate of 30% mortality (survival cannot be better estimated since the ponds were not fully harvested), the available data for calculation provides an overall FCR of 0.46 which is not considered reliable. A better management of the feed supply could provide a more reliable results in this type of trials. In any case, this indicates that the grey mullet grown in earthen ponds is able to feed on the food sources present in this type of ecosystem which is their natural environment. These results and assumptions are in line with the analytical data, which indicate that the fillet composition of the grey mullet resembles the fillet composition of a wild grey mullet, with low fat content.

Table 23.4.1 Data on grey mullet samplings done during the pond trial.

DATE	POND	INITIAL FISH NUMBER	N (sampled fish)	ABW (g)
01/07/2015	L3	544	-	3,6
	L4	800	-	3,6
04/02/2016	L3	544	57	92,65
	L4	800	88	33,17
21/06/2016	L3	544	28	163,55 ± 28,99
	L4	800	183	62,94 ± 30,78
15/12/2016	L3	544	157	294,02 ± 138,89
	L4	800	97	174,48 ± 55,36

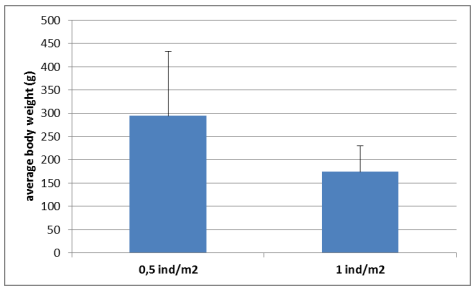


Figure 23.4.4 Final average body weight of the grey mullet reared at the two densities.

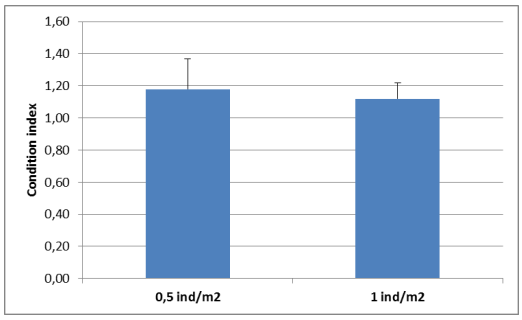


Figure 23.4.5 Condition index of the fish at the final sampling moment.

However, the weight range distribution of the grey mullet at the final sampling moment (December 2016) shows a skewed distribution of fish size (**Fig. 23.4.7**). There is an effect of the culture density on the weight range of the grey mullet. In the higher density treatment, 61.85% of the population is in the range of 150-200 g and only 29.30% of the lower density pond is in that weight range. Moreover, in the high-density treatment only the 2.06% of the population is above 400 g average body weight, much lower than the 22.93% of the population of the lower density treatment that is above the 400 g of average body weight. Additionally, from this 22.93%, there is a 11.46% of the fish population above 500 g average body weight.

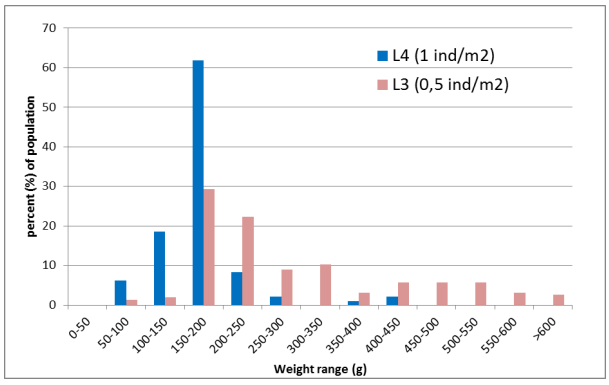


Figure 23.4.6 Weight range distribution of grey mullet reared at two different density in the earthen pond trial.

Proximate composition and fatty acid profile of the filet

Data on somatic indexes, fileting yield and proximate composition of the samples taken at the end of the farm trial are included in **Table 23.4.2.** Somatometric measurements were appropriate for the size of

fish (see more detail comparison between farmed and wild grey mullet in **D28.7**). Filleting yield of the reared fish was good and indicate the potential of using the species for elaborating processed fish products such as smoked fish fillet. On the other hand, the condition index is low which probably correlates with the not appropriate feeding management in the farm.

Table 23.4.2 Average somatic indexes, technical yields and proximate composition of grey mullet (*Mugil cephalus*) reared in earthen ponds with extruded diets and natural temperature in Cádiz (Spain).

	Farm trial
Body weight (g)	200.3 ± 69.9
CI	0.96 ± 0.08
Dressing yield (%)	90.43 ± 1.47
Filleting yield (%)	46.84 ± 1.17
HSI	0.84 ± 0.17
VSI	4.12 ± 0.51
VFI	------
Moisture (%)	77.98 ± 0.70
Fat (% ww)	0.86 ± 0.17
Protein (% ww)	19.33 ± 0.80
Ash (% ww)	1.21 ± 0.08

Data are means ± SD (n=6). CI: condition index; HSI: hepatosomatic index; VSI: viscerosomatic index; VFI: visceral fat index.

Considering that the fat content of the extruded diet (IRIDA) was 15.09%, it is surprising that the grey mullet flesh reflects exactly the opposite trend, however this is consistent with the low values of HSI and VSI (**Table 23.4.2**). It is well documented that aquafeeds provide high contents of total fats compared to what the species naturally consume in the wild. According to the fat contents, the farmed grey mullet of this farm trial, better resembles a wild specimen fat content than a reared one (see D28.7).

In spite of the improved and more sustainable formula produced by IRIDA for Batch 2, the fish seemed not to easily get use to this experimental diet, completing their daily feeding intake with the natural food items available in the earthen ponds (CTAQUA, personal communication). Therefore, the above-mentioned difficulties to accept the experimental diet, together with the partial naturally feeding, may explain the lower flesh fat contents and HSI and VSI found in these individuals. Concerning the total fatty acid content and main fatty acid composition of the grey mullet fillets, the results are shown in **Table 24.4.3**. Comparing these results with the ones presented in **Task 28.3.2** (**D28.7**), they seem to confirm that the grey mullet from this farm trial were partially eating the naturally abundant food and not exclusively the experimental extruded diet. These fish display a fat profile with a very healthy and more balanced lipid profile than fish fed the other extruded commercial diets.

Table 23.4.3 Total fatty acid content (g 100 g fillet^{-1}) and main fatty acid composition (% total fatty acids) of fillets of grey mullet reared in earthen ponds with IRIDA extruded diet and natural temperature in Cádiz (Spain).

	Batch 2
Total FA	0.52 ± 0.11
16:0	21.16 ± 1.01
18:0	5.95 ± 0.42
Total SFA	33.96 ± 1.37
16:1[1]	4.97 ± 1.03
18:1[2]	7.13 ± 0.80
Total MUFA	14.91 ± 1.62
18:2n-6	2.00 ± 0.24
20:4n-6	4.22 ± 0.95
Total n-6 PUFA	8.73 ± 2.00
18:3n-3	3.31 ± 1.40
20:5n-3	8.80 ± 1.40
22:6n-3	22.17 ± 2.29
Total n-3 PUFA	40.82 ± 1.20
DHA/EPA	2.58 ± 0.54
ARA/EPA	0.48 ± 0.08
n-3/n-6	4.90 ± 1.22
IA	0.55 ± 0.05
IT	0.21 ± 0.01

Data are means ± SD (Batch 2, n=6; Batch 3, n=4). [1] mainly n-7 isomer; [2] mainly n-9 isomer; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. IA, Index of atherogenicity; IT, Index of thrombogenicity.

As it is mentioned in Task **28.3.2**, there is increasing interest in the culture of the omnivorous grey mullet as a high quality source of protein and as a species that requires little or no dietary fishmeal (FM), in addition, a selection of mullet products that has been sensory-tested in five countries among regular fish consumers including: 1) fresh fillet with healthy seasoning, 2) thin smoked fillet and 3) fish fillets in olive oil prepared by the DIVERSIFY partner **P18. CTAQUA** showed that the tested products from grey mullet were well accepted (Deliverable **D29.4**). Furthermore, since products with a lower degree of processing generated higher expected scores and higher acceptability in the blind test, further insights are necessary to perform mullet grow out trials with sustainable diets able to provide optimum quality fillets in terms of both sensorial and proximate and fatty acid attributes.

Sensorial analysis

Table 23.4.4 shows the mean values obtained for each sensory descriptor of grey mullet specimens reared in earthen ponds with IRIDA extruded diet at natural temperature. Grey mullet from the field trial had a considerably low-fat content if we compare it with the grey mullet fed commercial extruded diet

as it is presented in the **Task 28.3.2** (0.86% vs 3.23%). The fillet sample presented fewer fat droplets in the exudate, lower brightness and a firmer and less juicy texture. Adhesion between the teeth is in accordance with the lower fat content as well as butter flavours. A more detailed and complete description and comparison of the sensory descriptors of grey mullet filet reared in earthen ponds with different extruded diets is included in the full report of **Task 28.3.2**.

Table 23.4.4 Mean values for each sensory descriptor of fillets of grey mullet reared in earthen ponds with IRIDA extruded diet and natural temperature in Cádiz (Spain).

Sensory descriptor	Grey mullet farm trial (PISTRESA)
O_Sardine	5.2
O_Ammonia	1.6
O_Earthy	2.0
O_Butter	2.0
O_Sea food	0.3
O_Acid	0.5
O_Boiled vegetables	0.2
O_Cheese	0.0
O_Rancid	0.1
Colour white to brown	5.9
Colour uniformity	4.8
White spots	2.6
Laminar structure	2.9
Exudates quantity	2.9
Exudates turbidity	3.4
Fat droplets	0.8
Exudate particles	4.1
Exudate proteins	2.2
Black lines in the flesh	1.4
Brightness	4.0
Yellowness	2.2
F_Sweet	1.1
F_Acid	0.5
F_Bitter	3.2
F_Earthy	3.3
F_Sardine	5.4
F_Butter	1.1
F_Sea food	0.2
F_Boiled vegetables	0.1
T_Firmness	5.3
T_Crumbliness	5.5
T_Juiciness	3.4
T_Chewiness	4.8
T_Pastiness	3.8
T_Teeth adherence	3.0

O: odour; F: flavor; T: taste.

Histological analyses

With regard to the histological analyses of the intestines from the grey mullet reared in earthen ponds, the samples from the high-density pond were not well preserved during the transport from the farm and could not be sent for histological evaluation due to tissue deterioration. Only the intestines from the lower density treatment were processed and histologically evaluated.

All the intestines samples presented a marked and generalized lymphocyte infiltrate **(Fig. 23.4.8)** in mucosa and lamina propria; 50% of the samples showed mild to moderate number of intraepithelial degenerated forms with occasional impact of hydropic degeneration and vacuolation of the mucosal epithelium and few infiltrated eosinophilic granular cells (EGC). Intestinal sub-mucosa displayed mild to moderate mixed cellular infiltrate including mononuclear and EGC infiltrate with focal marked EGC infiltrate and moderate congestion of blood vessels in 50% of the samples (**Fig. 23.4.8)**. One of the samples presented in the muscle intestinal layer a mild impact of Myxosporean aggregates (Sphaerospora-like) which is a normal condition in fish cultured in earthen ponds (**Fig. 23.4.9)**.

Examined intestines presented marked cellular infiltrate in intestinal mucosa and submucosa. These signs are compatible with chronic enteritis with multifocal degenerative signs. No widespread bacteria, parasite or fungal forms were detected in the examined sections.

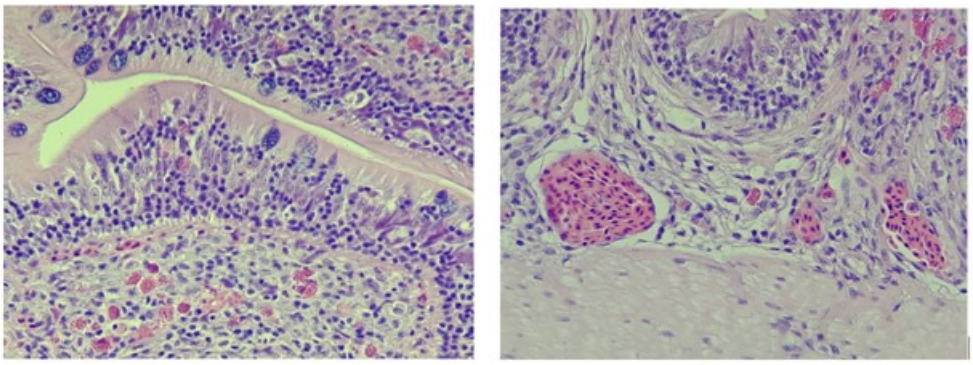


Figure 23.4.7 Left: Generalized lymphocyte infiltrate in mucosa and lamina propria of the intestine of grey mullet reared at low density. Right: Image of moderate congestion of blood vessels found in 50% of the samples.

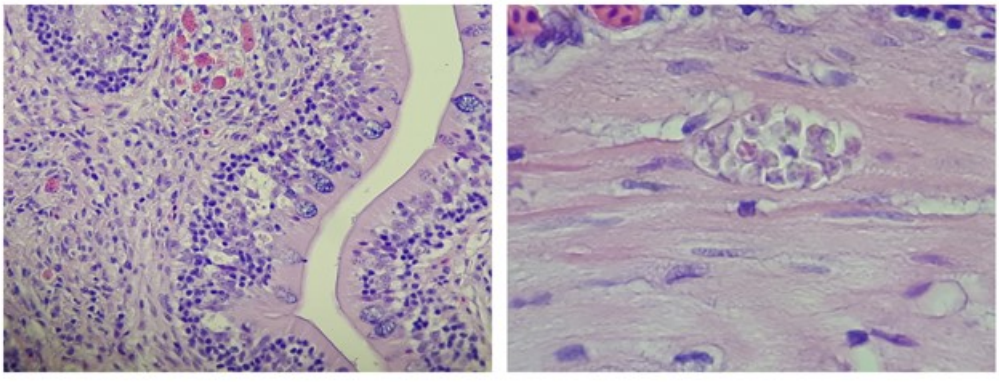


Figure 23.4.8 Left: hydropic degeneration and vacuolation of the mucosal epithelium. Right: Myxosporean aggregates (compatible with Sphaerospora-like parasite) detected in one fish (magnification x100).

Discussion

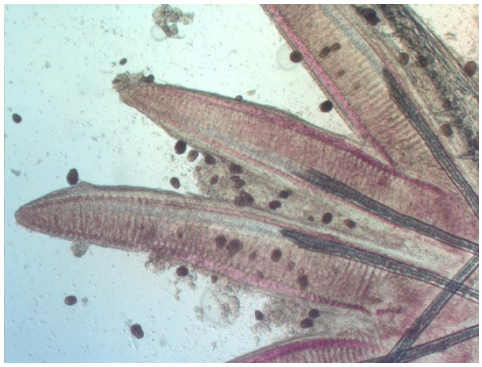
While the trial has been performed with one single replicate of each density condition, the presented results clearly indicate a growth advantage in the lower stocking density (0.5 fish per m^2) and it is consistent with the results from the **P4.IOLR** in Israel, which also showed a density effect (**Task 23.2**).

Deviations from Annex I and their impact:
The Israeli growth trial (Task 23.2) was originally planned to be carried out in the large brackish ponds at **P25. DOR** DGAY YAM near Haifa. However, this company closed down after the first year in the project. Consequently, smaller and seawater fed ponds at **P4.IOLR** in Eilat, Israel had to be prepared (netting, piping, plastic covering etc.) in order to attempt to carry out this task. These preparations, the delayed arrival of the IRIDA diet due to custom's regulations, collecting sufficient numbers of juvenile mullet and the lack of suitable personnel to carry out this growth trial with periodic weighings were the main reasons for the delay of the performance of this study. Moreover, due to the present lack of suitable technical staff at the **P4.IOLR** to carry out lipid class and fatty acid analyses, the poor growth and the high heron predation mortality of the grow-out mullet and the delayed termination of the experiment near the end of the project made it difficult to complete any meaningful analyses of FCR, PER, SGR that are mentioned in the DOW.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Group Work Packages

Fish health



Excellent progress has been made over the last year resulting in completion of all remaining tasks and submission of all remaining deliverable reports. The final feeding trials to mitigate the effects of Systemic Granulomatosis (SG) in meagre were completed in **Task 24.1**. **Task 24.2,** dedicated to chronic ulcerative dermatopathy, was also completed successfully and the results submitted as Deliverables D24.7 and D24.8. In **Task 24.3** two alternative infestation models were tested for the development of a challenge method for *Scianocotyle pancerii*, while another experiment was tested the efficiency of cinnamon as an antiparasitic agent for meagre infested with *S. panceri*. The main target of **Task 24.4** was to identify and isolate *Nocardia* sp. from SG-affected meagre. Following extensive samplings, only a single case of nocardiosis was found in cultured meagre in West Greece. The conclusion from this task is that nocardiosis is present in Greece but does not cause SG. In the same task it was proposed to produce an autogenous vaccine based on *Nocardia* isolates but as none were found until after the 2nd periodic report a commercially available trivalent vaccine was trialled. The vaccine conferred good protection to juvenile meagre following challenge with *Vibrio anguillarum*, with additional analysis of the immune response undertaken. In **Task 24.7** specific challenges with bacterial pathogens were performed, with *Photobacterium damselae* subsp. *piscicida* and *V. anguillarum*. Finally, in **Task 24.8**, a health manual for meagre was compiled and uploaded onto the project website.

In greater amberjack the effect of varying temperature and photoperiod on the behaviour and reproductive success of *Neobenedenia melleni* in greater amberjack was studied, to inform alternative control methods in farm facilities. In addition, methods based on the specificity of binding between the parasite and the host fish (using anti-attachment substances such as mannose), or for reinforcement of the immune system of amberjack through faecal microbiota transplantation (FMT), were tested. Two weeks after reinfection fish kept in continuous dark showed the highest number of eggs released per day, whilst fish in continuous light had the lowest. Bath treatments with different concentrations of mannose resulted at day 5 in the number of eggs recorded to be significantly lower than those at day 0 and 1. Lastly, the number of *N. melleni* eggs released per day following the FMT treatments was not different among groups, although fish given a transfection with seabream faeces after a previous dose of antibiotic treatment had significantly lower number of parasites per fish surface than treatment without antibiotic supplementation. During these studies the use of mesh dish devices for evaluating the level of infestation of amberjack with *N. melleni* gave highly satisfactory results, with weekly monitoring of egg number shown to be sufficient to estimate the level of parasitosis. In **Task 25.5** diagnosis and treatment of bacterial/viral infections of amberjack were reported, with *P. damselae* sub. *piscicida* isolated from an outbreak in this project period. Both *P. damselae* and *V. anguillarum* were used to establish challenge tests and, in addition, the minimum inhibitory concentration (MIC) of a variety of antibiotics (Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline) were determined for these bacteria, as well as for *Bacillus sp.* and *Vibrio algynolyticus*. Finally in Task 25.6 a diagnostic manual for greater amberjack health was collated, and contains two sections; the first, containing the bacterial diseases that have been studied within the DIVERSIFY project, and the second, parasitic diseases including *Zeuxapta seriolae* and *Neobenedenia girellae*. The manual has been uploaded to the project website.

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

4. Identify the causes of systemic granulomatosis (SG), and chronic ulcerative dermatopathy,
5. Investigate anti-parasite treatments in juvenile meagre,
6. Undertake preliminary characterisation of immune genes and study specific immune responses post-vaccination,
7. Evaluate the occurrence of *Nocardia* infections in meagre and develop an autogenous vaccine,
8. 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 on going. 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 on going.

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

The progress of the specific WP is in accordance with the provisions of the DoW. **Task 24.1** is dedicated to the study of Systemic Granulomatosis (SG) of meagre. In this task we have foreseen 5 feeding trials where we will test different diets in relation to the development of the disease. Three trials have already finished, two of which submitted in the form of Deliverable. The last two trials are scheduled for this year. From the above task we have obtained significant insights concerning the development of the disease and its pathobiology. In addition, we have seen that both high inclusions of Phosphorus and astaxanthin have beneficial effects concerning the severity of the disease. **Task 24.2** is related to the chronic ulcerative dermatopathy. We have finished the rearing trials in this reporting period and have already obtained the samples to be analysed. Analysis is still in progress; however preliminary results confirm the hypothesis that the disease is related to the use of borehole water. Further, qPCR analysis has indicated that there is

overexpression of the genes, which are connected with the specific osteolytic enzymes showing that the mechanism of the disease involves the activation of the osteoclasts by the increased CO_2 in borehole water. In **Task 24.3** we investigated various antiparasitic drugs against the most significant parasites of meagre. In the reporting period an experiment was performed in order to assess the acceptability of medicated feeds by juvenile meagre. In **Task 24.4** we have made extensive samplings for the isolation of *Nocardia* spp or related bacterial strains. We have not been able to isolate this pathogen even from severely affected fish using selective microbiological media specific for Acid-fast bacteria. However, this task provides significant information concerning the bacterial pathogens of meagre. In the same task we have foreseen to produce an autogenous vaccine based on the *Nocardia* isolates. Since this has not been achieved we have decided to change the direction and use a commercially available *Vibrio anguillarum* vaccine. **Task 24.5** is dedicated to the characterisation of the immune system. The task has been completed successfully and the relative information has been submitted as a deliverable. The results of this task are of great importance not only for the progress of this WP, but also for future studies that will require molecular markers of the immune system. **Task 24.6** is directly linked to the isolation of *Nocardia* and the production of the autogenous vaccine. Since this is going to change, the task will start this year with a *Vibrio anguillarum* vaccine. In **Task 24.7** we have planned specific challenges with bacterial and viral pathogens. In this period the P1. FCPCT team who are engaged in this task have started the fine-tuning of the challenge experiments using juvenile meagre and the analytical techniques that will be used. Finally, in **Task 24.8** we have been recording diseases occurring in our stocks but also in stocks of collaborating fish farms to develop a diagnostic manual for the diseases of the species. Several incidences have been recorded with an outbreak of monogeneans in broodstock and of mycobacteriosis in cage cultured fish being the most significant.

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

Task 24.1 is related to the study of Systemic Granulomatosis (SG) of meagre. All of the 5 feeding trials have already finished, three of which are submitted in the form of a Deliverable. From the above task we have seen that both high inclusions of phosphorus, vitamin C and astaxanthin have beneficial effects concerning the severity of the disease, while plant proteins in the diets of meagre negatively affects SG. **Task 24.2** is dedicated to chronic ulcerative dermatopathy. In this reporting period we have finished the analysis of samples obtained from the rearing trials in borehole water and natural sea water. The results indicate that the disease can be induced with the use of borehole water. Histology and SEM analysis confirmed that the lesions were limited to the lateral line organ mainly in the head while, qPCR analysis has indicated that there is overexpression of the genes, which are connected with the specific osteolytic enzymes. In **Task 24.3** two alternative infestation models were devised for the development of a challenge method for *Scianocotyle pancerii* while another experiment was set up to test the efficiency of cinnamon as an antiparasitic agent for meagre infested with *S. panceri*. The results are currently under analysis. The main target of **Task 24.4** was to identify and subsequently isolate *Nocardia* sp. from SG-affected meagre. Following extensive samplings, we have identified only one single case of nocardiosis in cultured meagre from a fish farm in West Greece. The conclusions of this task are that nocardiosis is present in Greece; however, it is not the cause of SG. In the same task we had proposed to produce an autogenous vaccine based on the *Nocardia* isolates. Since none were found until after the 2nd periodic report we decided to change direction and use a commercially available *Vibrio anguillarum* vaccine. The trials for this task are scheduled for this year**. Task 24.5** is dedicated to the characterisation of the immune system. The task has been completed successfully and the information has been submitted as a Deliverable. Furthermore, manuscripts have been published in peer-reviewed journals covering description of gene expression analysis of several of the identified immune transcripts from meagre. **Task 24.6** has been completed and the results of the analysis of antibody and cytokine kinetics post stimulation with PAMPs, has been reported in the Deliverable. In **Task 24.7** we have planned specific challenges with bacterial and viral pathogens. During the reporting period, two disease outbreaks were recorded and a challenge test was conducted with the isolated *Nocardia*. Finally, **Task 24.8** is still in progress and runs throughout the lifespan of the WP. Several bacterial strains have been isolated; however, none of these can be considered a primary pathogen and are probably environmental opportunists.

Summary of progress towards objectives (49-60 Mo):

Task 24.1 is related to the study of Systemic Granulomatosis (SG) of meagre. All of the 5 feeding trials have already finished and the results have been submitted as Deliverables (D24.1, D24.2, D24.5, D24.11, D24.14). **Task 24.2** is dedicated to chronic ulcerative dermatopathy. The task has been completed successfully and the results have been submitted as Deliverables (D24.7 and D24.8). In **Task 24.3** two alternative infestation models were devised for the development of a challenge method for *Scianocotyle pancerii* while another experiment was set up to test the efficiency of cinnamon as an antiparasitic agent for meagre infested with *S. panceri*. The results have been submitted as a Deliverable (D24.9). The main target of **Task 24.4** was to identify and subsequently isolate *Nocardia* sp. from SG-affected meagre. Following extensive sampling, we have identified only one single case of nocardiosis in cultured meagre from a fish farm in West Greece. The conclusions of this task are that nocardiosis is present in Greece; however, it is not the cause of SG. These results have been submitted as a Deliverable (D24.4). In the same task we had proposed to produce an autogenous vaccine based on the *Nocardia* isolates. Since none were found until after the 2nd periodic report it was decided to change direction and use a commercially available *Vibrio anguillarum* vaccine. The trials for this task were completed in the current reporting period and have been submitted as a deliverable (D24.12). Briefly, a commercial trivalent vaccine containing *V. anguillarum* was tested and it was shown to confer protection to juvenile meagre following challenge using a strain of *V. anguillarum*. **Task 24.5** is dedicated to the characterisation of the immune system. The task has been completed successfully and the information has been submitted as a Deliverable. Furthermore, manuscripts have been published in peer-reviewed journals covering description of gene expression analysis of several of the identified immune transcripts from meagre. **Task 24.6** has been completed and the results of the analysis of antibody and cytokine kinetics post stimulation with PAMPs, has been reported in the Deliverable. In **Task 24.7** specific challenges with bacterial pathogens were performed. Following the challenge using Nocardia that was completed in a previous reporting period, challenges with *Photobacterium damselae* subsp. *piscicida* and *V. anguillarum* were performed. These results together with the description of the bacterial outbreaks in FCPCT facilities were submitted as a Deliverable (D24.12). Finally, in **Task 24.8** performed in this reporting period, a health manual for meagre was compiled and submitted as a Deliverable (D24.17).

Details for each Task

Task 24.1. Systemic Granulomatosis (led by HCMR, Pantelis Katharios).

Sub-task 24.1.1. Feeding trials (HCMR, Pantelis Katharios)

Trial 1. (HCMR) The effect of 3 levels of dietary vitamin D in the development of SG

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.1 The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre***.

Trial 2. (HCMR) The effect of various dietary Ca/P ratios in the development of SG

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.2 The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre.***

Trial 3. (HCMR)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided ***Deliverable 24.5 The effect of high plant protein diets in the development of Systemic Granulomatosis in meagre.***

Trial 4. (FCPCT-Daniel Montero) Effects of vitamins E, C, plus astaxanthin.

This task has been completed during the previous reporting periods and the full description of the work and results have been provided ***Deliverable 24.11 Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis***.

Trial 5 (FCPCT-Daniel Montero) The effect of Se, Mn and Fe will be examined in SG prevention

This task has been completed during the previous reporting periods and the full description of the work and results have been provided ***Deliverable 24.11 Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis***.

Sub-task 24.1.2. Health and pathological assessment (led by HCMR, Pantelis Katharios).

This subtask included all the information regarding the description and the development of Systemic Granulomatosis following all the feeding trials that were performed in the frame of the DIVERSIFY project. It resulted in the description of the disease, the time of first appearance of the granulomas and the relationship to the various diets included in the Diagnostics protocol for Systemic Granulomatosis in meagre (D24.14) and aetiological factors and D24.15 Report for the prevention/treatment of Systemic Granulomatosis in meagre.

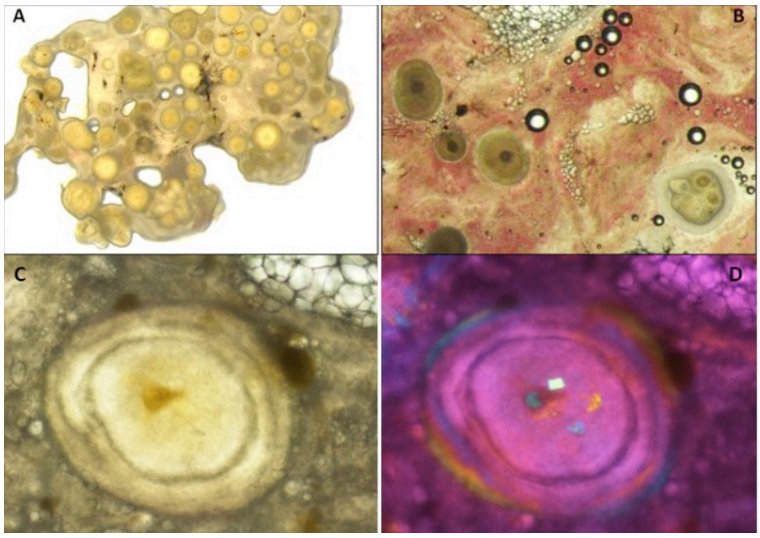


Figure 24.1.1. Multiple granulomas and high magnification of a granuloma as seen in fresh squash preparations (A-C) and using a polarized lens (D)

The conclusions and recommendations regarding SG are:

- Nocardiosis is present in Greece, most probably in a confined geographical region; however it is not the cause of SG.
- Vitamin D3 supplementation did not affect the development of the SG.
- High P content in the diet seems to improve the condition.
- Plant protein replacement affects negatively the progression of the SG.
- High dietary content of the antioxidants vitamin E and C ameliorates the severity of SG, decreasingthe incidence and number of fish with higher severity of SG.
- The addition of Zn, Mn and Se did not ameliorate the granuloma incidence or severity.

Taken together, the improvement of SG by a change in the diet together with the absence of pathogens inSG-affected population suggests that the metabolic hypothesis is more probable. The occurrence of only a single case of nocardiosis with different characteristics enforces this hypothesis. However, the aetiology is still unknown and other nutritional metabolic factors have to be tested.
Taking into account all the above results our recommendations for prevention of SG in meagre are:

- A combined diet with high percentage of fishmeal (60%), high dietary content of P (15 g kg-1) and high content of antioxidants vitamins E and C.
- Since there is no data available about the reversibility of SG, we recommend to start feeding with this diet when the fish weight is about 2 g.

A full description of the results of this subtask can be found in ***Deliverables D24.14*** and ***D24.15***.

Task 24.2. Chronic Ulcerative Dermatopathy (led by HCMR, Pantelis Katharios).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.7 Diagnostics protocol for Chronic Ulcerative Dermatopathy in meagre and aetiological factors*** and ***Deliverable 24.8 Report on the prevention/treatment of chronic ulcerative dermatopathy in meagre***

Task 24.3. Anti-parasitic treatments (led by IRTA, Ana Roque).

Parasites such as *Sciaenacotyle panceri*, a monogenean found on the gills of meagre, are also known to cause mortality in farms in the Mediterranean and require development of appropriate treatments. A test was performed to evaluate the tolerance of the fish to each chemical product that was considered to be a potential treatment. At the end of each test, fish were sacrificed humanely and samples of the gills and blood taken. Gills were fixed and analysed by histology in order to evaluate any damage caused at the tissue level either by the potential treatment or by the parasite should they be encountered in the test population during the course of the treatment trials. Plasma was collected from the blood and general stress indicators were measured, including cortisol, glucose and lactate. Potential treatments were repeated. Five trials using essential oils as in-feed antiparasitic treatments against monogeneans were conducted at IRTA and cinnamon was shown great potential in reducing the infestation. The results have been presented in **Deliverable 24.9 Determination of effective treatments for common monogenean parasites in meagre.**

Task 24.4. *Nocardia* infection in meagre (led by HCMR, Pantelis Katharios).

Sub-task 24.4.1 Isolation and characterization of the pathogen (led by HCMR, Pantelis Katharios).

This task has been completed during the previous reporting periods and the full description of the work has been provided in ***Deliverable 24.4 Isolation and characterization of Nocardia from infected meagre.***

Sub-task 24.4.2. Evaluation of a commercial *Vibrio anguillarum* vaccine (led by IRTA, Karl Andre).

The objective of this sub-task was to document the efficacy of a vaccine against pathogens of significance for commercial aquaculture of meagre *Argyrosomus regius*. The original bacterium for this study was a *Nocardia* species that was hypothesized to be relevant for the systemic granulomatosis affecting meagre, but since the association of *Nocardia* to this disease has not been proven during the progress of this project we have chosen and had approved a different pathogen for study: *Vibrio anguillarum*. There are reports of nodavirus and monogeneans infecting meagre, but among bacteria *V. anguillarum* and *Photobacterium damselae* are the pathogens that have been reported. This work has focused on *V. anguillarum* as it is a pathogen with broad host-range and likely to be of concern for intensive rearing facilities of meagre. The vaccine administered during this Task was provided by Acuipharma. It is a trivalent vaccine effective against *V. anguillarum, V. harveyi* and *P. damselae*. Since meagre represents a species relatively new to aquaculture no vaccine preparations have been formally approved and licensed for use with this species. As a multivalent vaccine it will also have broader applicability in a commercial production setting. During the course of this work tissues relevant to the immune response were collected for analysis of gene expression. The tissue samples were collected from fish pre- and post-vaccination and injection with *V. anguillarum* and immune gene expression were evaluated for select target genes (provided in ***Deliverable 24.13. Description of immune gene expression pre- and post-immunization of meagre***). Following vaccination and subsequent challenge test with *V. anguillarum*, the vaccine protected the vaccinated fish as shown in **Table 1**.

Table 1. Total mortality recorded during ten days post-injection using 3.3 X 10^8 CFU/mL of *V. anguillarum*. Mean weight of those that died is shown.

Treatment	Total Mortality	Mean Weight (SD)
PBS-PBS	0%	-
PBS-VB	17.5%	27.01 (4.8)
VAC-VB	0%	-
VAC-PBS	0%	-

The full description of the work and results has been provided in ***Deliverable 24.12*** **Determination of Efficacy of Vaccination of Meagre against *Vibrio anguillarum*.**

Task 24.5. First characterisation of the immune system (led by UNIABDN, Chris Secombes).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.3 Cloning of key marker genes of innate and adaptive immune responses in meagre.***

Task 24.6. Monitor specific immune responses (led by UNIABDN, Chris Secombes and IRTA, Karl Andre).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 24.10 Kinetics of antibody and cytokine production established post-pathogen exposure or stimulation with PAMPs.***

Task 24.7. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in meagre (led by FCPCT, Daniel Montero).

The objective of the task was to report the major bacterial and viral diseases found in meagre, and where useful to provide information and recommendations about treatments that have been developed.

Within the different years, only few pathology incidences were detected in meagre and are listed in Table 2. All samples were seeded in (BHIB) supplemented with 1.5% NaCl at 25ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl and the bacteria grown were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16S RNA.

Table 2. Occurrence of different pathogens within the project period.

Year	Pathogen species	Outbreaks	
2014	*Nocardia sp.*	No Outbreaks	routine isolation
2015	*Vibrio algynoliticus*	No outbreaks	Routine isolation
2016	*V. algynoliticus*	September 2016	3 broodstock died
	Bacillus sp.	November 2016	mortality occurrence in 23g juveniles
2017	*V. algynoliticus*	No outbreaks	routine isolation
2018	*Photobacterium damselae* subsp. *piscicida*	August 2018	massive mortality in fry (0.2 g body weight)

Following the bacterial challenges using Nocardia that were reported in the previous period, new challenges were conducted.

Challenge test against Photobacterium damselae and Vibrio anguillarum. Forty meagre juveniles (average 40 g body weight) obtained from FCPCT fish stock, were maintained in a closed seawater flow circuit with water at a temperature of 22ºC and a salinity of 37‰ under a 12-h light/12-h dark cycle. The fish were intraperitoneally inoculated with 100 µl of the different bacterial strain *Photobacterium damselae subsp. piscicida* ME-1 or *Vibrio anguillarum* at 10^5 colony-forming units/fish. Those bacteria were isolated from clinical outbreaks registered in Canary Islands. Inoculated and control fish were monitored for clinical disease and mortalities for the duration of the study (15 days). Mortality was attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs (**Fig. 2**). Only *P. damselae* subsp. *piscicida* produced mortality in meagre juveniles.

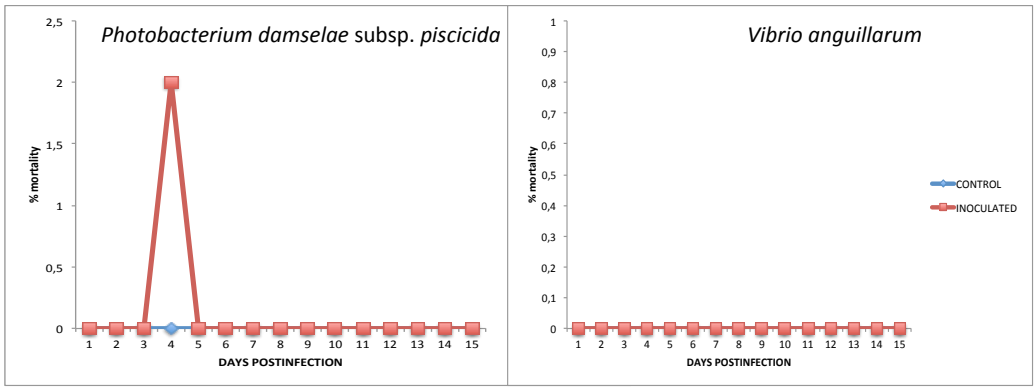


Figure 24.1.2. Results of challenge test against different bacteria isolated from outbreaks registered in farms from Canary Islands.

In addition the Minimum Inhibitory Concentrations of various antibiotics were assessed for several bacterial pathogens. Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline (Sigma-Aldrich) were the tested antibiotics. The full description of the work and results have been provided in ***Deliverable 24.16*** **Report of the major bacterial and viral diseases found in meagre, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided.**

Task 24.8 Diagnostic-recommendation manual for meager health (led by HCMR, Pantelis Katharios).

A diagnostic manual for meagre health has been created. The manual is divided in two sections; the first, containing the non-infectious diseases that have been studied within DIVERSIFY project, Systemic Granulomatosis and Chronic Ulcerative Dermatopathy and the second, infectious diseases including Nocardia infection and other bacteria as well as parasites such as *Sciaenacotyle panceri* and *Diplectanum sciaenae*. The manual is based on the findings obtained during the course of the project and is compiled from input from all participating partners. The manual is an appendix in this Deliverable report and has been uploaded at the website of the project as a pdf that can be downloaded freely.

The full description of the work and results has been provided in ***Deliverable 24.17*** **Diagnostic-recommendation manual for meagre fish health and in the appended Technical Manual for Meagre health.**

Deviations from Annex I and their impact:
None.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Milne, D.J., Campoverde, C., Andree, K.B., Zou, J., Secombes, C.J., 2017. Two types of TNFalpha in meagre (*Argyrosomus regius*): Discovery, distribution and expression modulation. Mol Immunol 92, 136-145.

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Ruiz García, M.Á., Hernández-Cruz, C.M., Caballero, M.J., Fernández-Palacios, H., Saleh, R., Izquierdo, M., Betancor Quintana, M.B., 2018. Incidence of systemic granulomatosis is modulated by the feeding sequence and type of enrichment in meagre (*Argyrosomus regius*) larvae. Aquaculture Research 000, 1-12.

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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), Salvador Jerez (P8), Virginia Martín (P8), Covadonga Rodríguez (P15), Jose Pérez (P15), Pilar Foronda (P15).			

Objectives

1. Provide early diagnosis tools for Epitheliocystis,
2. Develop "antiparasite diets" to be used prior to sea cage culture,
3. Begin characterisation of the immune system, with a focus on mucosal (skin/gill) defences,
4. Develop anti-monogenean parasites infection rearing protocols.
5. 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 are also reported.
- Task 25.5 – Studies of the seasonality of potential diseases of amberjacks was started. Several strains of bacteria were isolated from skin ulcers, including *Vibrios* of the *harveyi* clade, and *Staphylococcus epidermidis*. A challenge test with *Photobacterium* subsp. *piscicida* was performed by ip injection but the fish were not susceptible to this species at the dose used (10^3cfu/fish).
- Task 25.6 – Various Greek fish farms were visited for a health status survey. Monogenean and digenean gill parasites were found and analysis of the associated pathology was begun. The anthelmintic praziquantel appeared an effective treatment.

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

In the 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.

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

In the current reporting period progress has been made against all tasks and is outlined in detail below. This included progress in identifying the causative agent of epitheliocystis. Whilst the mesocosm studies gave no clear results, samples collected from collaborating fish farms in Greece revealed that, in contrast to the prevalent belief that Epitheliocystis is caused by Chlamydia, at least in Greece the main pathogens causing Epitheliocystis disease are bacteria that belong in the β- or γ-proteobacteria. These bacteria have a mainly intracellular life cycle and their cultivation in vitro has not yet been accomplished. Studies to promote resistance to parasitic incidence on greater amberjack trialled two different prebiotics, namely MOS and cMOS. Positive effects were found for the cMOS and prebiotic combination (MOS + cMOS) following challenge with *N. girellae*, in terms of lower infestation levels and lower numbers of parasites per cm^2 of skin. Immune gene expression analysis of skin and gills also showed positive effects with cMOS. Whether cMOS could impact on bacterial load was also studied. After feeding for 90 days the fish were stressed by crowding and prevalence of opportunistic bacteria detected in samples from liver and spleen. Whilst crowding resulted in 100% prevalence for opportunistic bacteria in both tissues, twice the number of bacterial species (Vibrio's) were present in the control diet fed fish vs cMOS fed fish. Further immune gene analysis was undertaken using cell suspensions from kidney and spleen, and showed these cells are highly responsive to PAMPS, with differences in kinetics and magnitude of increases seen dependent upon the stimulant. Studies of one of the antimicrobial molecules in greater amberjack, piscidin, showed good bacterial growth inhibition against two fish pathogens, *Vibrio anguillarum* and *Yersinia ruckeri*. The effect of stocking density on parasite (*Neobenedenia melleni*) egg production was studied and revealed that egg number tended to decrease (P=0.08) with increased culture density. Several anti-attachment factors were also trialed and two treatments (cumin and mannose) showed a reduction in egg number over the following 2-8 days. These effects were compared to traditional anti-parasite chemical treatments, including copper sulphate, formaldehyde and hydrogen peroxide. Copper sulphate and hydrogen peroxide had no effect on egg number bur formaldehyde was effective, especially when used on several occasions. Nevertheless the potential of mannose in particular was apparent and might be improved by further optimisation of treatment dose and frequency of application. These practical applications will ultimately be incorporated into a diagnostic recommendation manual, to be published at the end of the DIVERSIFY programme.

Summary of progress towards objectives (49-60 Mo):

During this final reporting period, all remaining tasks were completed as outlined below. In Task 25.4 two environmental parameters, the effect of varying temperature and photoperiod, on the behaviour and success of the reproductive strategy of *N. melleni* in greater amberjack was studied, to inform alternative control methods in farming facilities. In addition, methods based on the specificity of binding between the parasite and the host fish (using anti-attachment substances such as mannose), or for reinforcement of the immune system of *S. dumerili* through faecal microbiota transplantation (FMT), were also tested. The number of eggs of *N. melleni* was significantly lower in fish maintained in 24L:0D compared to 0L:24D. Two weeks after reinfection fish maintained in continuous darkness showed the highest number of eggs released per day, and fish maintained in continuous light the lowest, to the end of the trial. Bath treatments with different concentrations of mannose (30 mM, 50 mM, 70 mM) did not cause detachment of *N. melleni* adults from greater amberjack or a reduction in the number of eggs emitted per day by the parasite. However, in all treatments, the number of eggs recorded at day 5 was significantly lower than those at day 0 and 1, with the 30 mM treatment having the largest decrease. Lastly, the number of *N. melleni* eggs released per day following the FMT treatments was not different among groups, although fish given a transfection with seabream faeces after a previous dose of antibiotic treatment had a significantly lower number of parasites per fish surface than treatment without antibiotic supplementation. During these studies the use of mesh dish devices for evaluation of the level of infestation and reinfection of *S. dumerili* with *N. melleni* gave highly satisfactory results, with weekly monitoring of the number of eggs of the parasites shown to be sufficient to estimate the level of parasitosis. In Task 25.5 diagnosis and treatment of bacterial/viral infections of amberjack were to be reported but over the project period few disease incidences were detected in greater amberjack. However, during this reporting period, *Photobacterium damselae* sub. *piscicida* was isolated in an outbreak, and was used together with *Vibrio anguillarum* to establish challenge tests. In addition, the minimum inhibitory concentration (MIC) of a variety of antibiotics (Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline) were determined for P. damselae and V. anguillarum, as well as *Bacillus sp*. and *Vibrio algynolyticus*. Finally in Task 25.6 a diagnostic manual for greater amberjack health was created. The manual is divided in two sections; the first, containing the bacterial diseases that have been studied within DIVERSIFY project, and the second, parasitic diseases including *Zeuxapta seriolae* and *Neobenedenia girellae*. The manual is based on the findings obtained during the course of the project, compiled from input from all participating partners, and has been uploaded to the project website as a pdf that can be downloaded freely.

Details for each Task

Task 25.1. Study of Epitheliocystis during larval rearing (led by HCMR, Pantelis Katharios).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 2.5.2 Mucus defences of greater amberjack analysed and immune potential characterised,*** and ***Deliverable 2.5.4 Protocol for early diagnosis of epitheliocystis during early stages of greater amberjack juveniles***.

Task 25.2. Promoting resistance to parasitic incidence on greater amberjack (led by FCPCT, Daniel Montero).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 2.5.2 Impact of dietary regime on parasite resistance and mucosal defences of greater amberjack juveniles*** and ***Deliverable 2.5.5 Impact of oral administration of greater amberjack with mucus stimulation products on immune resistance to parasite infections and development of molecular markers for its evaluation.***

Task 25.3. Identification of immune markers (led by UNIABDN, Chris Secombes).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 25.1 Marker genes of mucosal immunity in grater amberjack cloned and ways to increase their expression level determined*** and ***Deliverable 25.2 Mucus defences of greater amberjack analysed and immune potential characterised.***

Task 25.4. Effectiveness of stocking density and anti-oncomiracidia attaching substances in the control of monogenean parasites (led by IEO).

This task included studies designed to evaluate the effects of two environmental parameters such as temperature and photoperiod, on behaviour and success of the reproductive strategy of *N. melleni* in greater amberjack to develop alternative control methods against its infection in farming facilities. In addition, some methods based on the mechanisms responsible for the specificity of the binding between the parasite and the host fish, and others, focused on testing methods for reinforcement of the immune system of *S. dumerili*, through fecal microbiota transplantation (FMT), were also tested. The influence of some of these treatments were evaluated on growth, survival, plasma metabolites, humoral immune parameters, hepatic oxidative status and on gut and gill osmoregulatory epithelium integrity, to give an overall picture of the main physiological processes affected by the parasite infection.

Some preliminary experiments, summarized in previous reports, were performed and useful information was attained concerning the efficacy of baths with anti-oncomiracidia substances and the stocking density of greater amberjack juveniles. Based on these results, new trials were developed in two ways:

1. Effects of environmental parameters on the biological cycle of *Neobenedenia melleni* and degree of *Seriola dumerili* infestation.

Experimental conditions

A total of 81 juveniles of greater amberjack hatched in the IEO-COC facilities were distributed in 9 groups of 9 fish each. Fish were maintained under three photoperiod regimens for 68 days as follows: Treatment Control (natural photoperiod and maximal light intensity of 200 lux), Treatment 24L:0D (continuous artificial light for 24 h at 1000 lux) and Treatment 0-3L:24-21D (dark (< 2 lux) for 24 h from day 0 to 34 and light (1000 lux) for 3 h and dark for 21 h from day 34 to 68). The level of parasitosis by monogeneans was monitored by dish traps placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014). The rhythm of eggs released by adults of *N. melleni* parasitizing fish maintained at different photoperiod conditions was monitored over 24 h using mesh dishes submerged for 3 h in each tank and the number of eggs entangled counted.

Results

The Specific Growth Rate (SGR) and Condition Factor (CF) of fish maintained in continuous darkness (0L:24D) were significantly lower ($P<0.05$) during the period 0-34, while no differences were observed in the period 34-68, and in the overall period (0-68) between treatments. The feed intake was significantly lower ($P<0.05$) in the fish maintained in continuous darkness during the period 0-34 days and 3L:21D in the period 34-68, and in the overall period. No fish mortality was recorded in the period 0-34 days in any of the different photoperiod treatments, but the fish maintained in 3L:21D from 34 to 68 days, previously maintained from 0 to 34 days under continuous darkness, exhibited the lowest survival rate ($P<0.05$).

Regarding parasite behaviour, the number of eggs of *N. melleni* during the first 3 weeks was significantly ($P<0.05$) lower in fish maintained in 24L:0D (41±45 eggs d^{-1}) than 0L:24D (179±73 eggs d^{-1}). The Control group showed an intermediate number of eggs (107±37 eggs d^{-1}). Two weeks after reinfection fish maintained in continuous darkness (0L:24D) showed the highest number of eggs per day, and fish maintained in continuous light (24L:0D) the lowest, to the end of the trial. The mean number of *N. melleni* eggs per day after reinfection to the end of the trial (68 days) was significantly higher in fish maintained in photoperiod treatments of 0-3L:24-21D than in fish under continuous light (24L:0D) (**Fig. 25.4.1**).

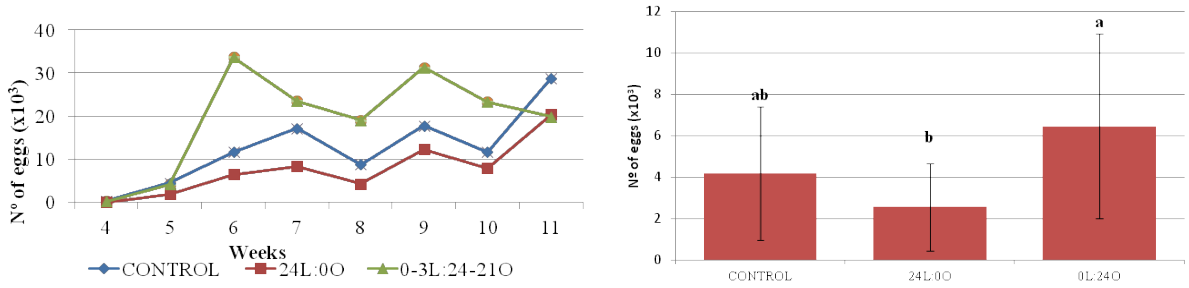

Figure 25.4.1. Number of eggs of *N. melleni* collected from greater amberjack maintained in different photoperiod treatments following reinfection (week 3) to the end of the trial (68 days) (a), and mean number of eggs over the whole period (b). Different letters indicate significant differences ($P<0.05$).

2. Effects of anti-attachment substances and novel methods to reinforce the immune system of *Seriola dumerili.*

A set of experiments were carried out in order to test the effects of anti-attachment substances such as mannose and a novel method of Faecal Microbiota Transplantation (FMT) to reinforce the immune system of *S. dumerili*, in order to improve their defences against infection by *N. melleni*.

Experimental conditions

A total of 96 juveniles of greater amberjack hatched in the IEO-COC facilities and parasitized by *N. melleni*, were randomly distributed in 12 homogeneous groups. The groups were maintained in fiberglass tanks for 4 months with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu), temperature (19.0 ± 0.3ºC) and oxygen saturation (91.2 ± 1.1%).

The level of parasitism by monogeneans was monitored by dish traps placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014) for 4 months (January-May).

*Mannose treatment***.** The immersion or "baths" of the fish in mannose solutions was carried out in three increasing doses (30, 50 and 70 mM), and in sea water (control), resulting in 4 treatments in triplicate (M0, M30, M50 and M70), for 5 min.

Faecal microbiota transplantation, FMT. The Faecal Microbiota Transplantation (FMT) treatment was performed as a method for the reinforcement of the immune system of fish. Four treatments were applied in triplicate: Control (buffer PBS); transfection with warthog faeces (*Phacochoerus africanus*) provided by IRTA (FMTP), and transfection with faeces of gilthead seabream (*Sparus aurata*) maintained at the IEO-COC facilities, with a previous dose of antibiotic (single dose of sulphanilamide, 125 mg kg^{-1}) (FMTGS) and without a previous dose of antibiotic (FMTG). Inoculation of the faecal transplant was carried out orally by syringe for 3 consecutive days. The dose was 0.3 ml of stool suspension per fish per day. At the beginning and at 7, 15, 21 and 34 days post inoculation of faeces, all fish in each tank were anesthetized and measured for biometric parameters, and samples of faeces of all fish of each tank obtained by cannulation. At each sampling time, 2 fish per tank were then selected randomly for blood collection to determine haematological and plasmatic parameters. At the end of the trial, a total of two fish per tank were sampled to obtain samples of gill and gut for analysis of digestive enzymes, and to isolate gill, and hepatic (hepatocytes) and intestinal (enterocytes) epithelial cells for the evaluation of their viability and functional integrity (viability test with Trypan blue exclusion and ATPase activity).

In order to evaluate the intensity and sites of infection preference and to quantify the number of adult parasites of *N. melleni*, the sacrificed fish were submerged in fresh water to cause the detachment of adult parasites attached to the skin. The surface area of each *S. dumerili* fish was estimated (Ohno *et al.*, 2008) and the number of parasites per fish surface determined.

Results

Bath treatments with different concentrations of mannose (30 mM, 50 mM and 70 mM) did not cause a significant detachment of adults of *N. melleni* from greater amberjack. The increase in mannose concentration did not affect ($p>0.05$) the number of eggs emitted per day by the parasite. However, in all tested treatments, the number of eggs recorded at day 5 was significantly lower ($p<0.05$) than those registered at day 0 and 1, showing the M30 treatment had the largest decrease followed by the M50 treatment. From day 6, the number of eggs collected was stable for all tested mannose concentrations.

The number of eggs per day collected increased significantly three weeks after reinfection in all groups of fish, showing this was a successful method to infect fish (**Fig. 25.4.2**)

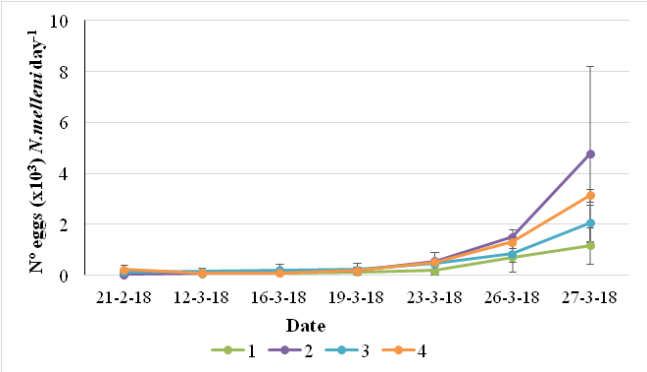

Figure 25.4.2. Number of *N. melleni* eggs per day collected after the reinfection experiment in all groups of fish. C, control treatment; M30, bath with 30 mM of mannose; M50, bath with 50 mM of mannose; M70, bath with 70 mM of mannose.

The number of *N. melleni* eggs per day released after the application of transfection treatments was not different among treatments ($p>0.05$). At 7 day post-transfection, the number of eggs collected was similar to that recorded at the beginning in all tested treatments. However, at days 15 and 21, the eggs per day collected decreased significantly ($p<0.05$), reaching values at the end of the trial (day 34) that were similar to those recorded at the beginning. The SGR and CF of greater amberjack after the transfection did not change significantly during the experimental period for any of the tested treatments. Feed intake ratio also did not show differences ($P>0.05$) during the overall experimental period (0-34 day) between different treatments.

The number of erythrocytes increased, but not significantly, during the experimental period in the Control (T1) and T2 treatments with respect to the initial values (0 day). In contrast, treatments with transfection of seabream faeces without (T3) and with a previous dose of antibiotic (T4) showed final values similar to those recorded at day 0. MCV decreased in control (T1) and the transfection with warthog faeces treatment group (T2) at day 21 compared with values at day 0, with the MCV significantly higher in Treatments T3 and T4. There were significant differences in cholesterol and triglyceride plasma levels during the experimental period. Cholesterol was higher at day 15 post-transfection than day 0, and triglyceride levels were significantly higher at days 15 and 34, than day 7, regardless of the treatment.

The fish given a transfection with seabream faeces after a previous dose of antibiotic treatment (T4) showed a higher number of parasites detached from fish, and had a significantly lower number of parasites per fish surface than treatment without antibiotic supplementation (T3). The preferred sites of parasite attachment on greater amberjack were the eyes and the area located above the lateral line. Detached adult parasites measured 2.52 to 2.61 mm in length and were 1.20 to 1.23 mm wide. There were no significantly differences between treatments.

Viability of isolated enterocytes and gill cells was not significantly different between the two treatments, with more than 91 % viability achieved even under severe *N. melleni* infections. ATPase activity in gill cells was not affected by any transfection treatment whereas this activity was significantly reduced in

enterocytes when this transfection was based upon *S. aurata* microbiota. Regarding digestive enzyme activities, results showed that transplantation with *P. africanus and S. aurata* induced a significant change - higher activity for the tissue alkaline protease.

Conclusions

The continuous light conditions (24L:0D) did not improve the growth performance of greater amberjack, but negatively affected the number of eggs produced by *N. melleni*, which was correlated to a lower fish parasite load. However, the difference in the photoperiod is not the only factor that seems to influence the rhythm of egg release of the *N. melleni* parasite. The embryo development and hatching of *N. melleni* was increased in dark conditions and high temperature, increasing the reinfection success of the parasite. A concentration of 30 mM mannose was the most effective dose tested in the "baths" applied to *S. dumerili* parasitized with *N. melleni*, in try to reduce the number of eggs emitted by the parasite. The use of mesh dish devices for evaluation of the level of infestation and reinfection of *S. dumerili* with *N. melleni* gave highly satisfactory results. This device allowed the application of control measures or to increase the number of eggs emitted per day by more than 20 times over a period of two weeks, in the challenge trials.

Faecal microbiota transplantation (FMT) as a method of improving the immune system of *S. dumerili* had no immediate effects on the number of eggs emitted by *N. melleni* in any of the treatments tested. However, the increase of the mean corpuscular volume (VCM) in the treatment group undergoing transfection with gilthead seabream faeces with previous dose of antibiotic, and the gut alkaline protease activity could indicate a decrease in the anaemic state of the parasitized fish and modulation of gut microbiota. The high levels of lactate, triglycerides and plasma cholesterol obtained regardless of the treatment used, suggest a possible activation of lipid metabolism in infected specimens as a consequence of the stress associated with parasitosis by *N. melleni*.

General considerations and recommendations

The results from this task can aid management practices for monitoring parasite prevalence in the following ways:

- To detect the number of eggs of *N. melleni* and *Z. seriolae* and to estimate the level of parasitism of reared fish, a simple submerged mesh disc system to which the eggs adhere, can be used. The weekly monitoring of the number of eggs of the parasites with this system is sufficient to estimate the level of parasitosis.
- Taking into account the time necessary between the release of the egg and the sexual maturation of the parasite, it would be possible that monitoring every two or three weeks will provide reliable information.
- In *N. melleni*, the embryo development of the egg and the percentage of hatching are favored by high temperatures and photoperiod of a few hours of light. The periods of the year in which this occurs would be the most critical times for a more exhaustive control.
- The time of immersion of the egg collector device must be at least 24 h, since the rate of egg emission can be modified with environmental conditions. If the surface of the culture facilities is covered by natural or artificial materials that favor the adhesion of the eggs, the estimation of the population of parasites with the discs is completely different to a situation where the surface is clear.

The full description of the work and results is provided in ***Deliverable 25.6 Rearing protocol against monogenean parasites***.

Task 25.5. Description, diagnosis and treatment of other bacterial/viral infectious diseases occurring in amberjack (led by FCPCT, Daniel Montero).

Within the different years, only few disease incidences were detected in greater amberjack. During this reporting period, *Photobacterium damselae* sub. *piscicida* was isolated in an outbreak that occurred in August 2018, with fry massive mortalities. All samples were seeded in BHIB supplemented with 1.5% NaCl at 25 ºC or in blood agar base (BAB, Cultimed) supplemented with 5% sheep blood and 1.5% NaCl.

The bacteria grown were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests. For final identification, strains were sent to the Spanish Type Culture Collection for sequencing of 16S RNA. Regarding Nodavirus, 3 routine samples per year were conducted within the P2 facilities, and Nodavirus determination on amberjack broodstock within facilities was also conducted, with negative records of Nodavirus in the whole population.

Experimental challenge tests

Taking into account the outbreaks/occurrence of pathogens seen, different challenge tests were conducted at the Marine Biosecurity Station from FCPCT (University of Las Palmas de Gran Canaria).

Challenge test against Photobacterium damselae and Vibrio anguillarum.

Fifty juveniles (average 90 g body weight) obtained from FCPCT were maintained in a closed seawater flow circuit with water at a temperature of 22ºC and a salinity of 37‰ under a 12-h light/12-h dark cycle. The fish were inoculated intraperitoneally with 100 µl of the different bacterial species - *Photobacterium damselae subsp. piscicida* ME-1 or *Vibrio anguillarum* at 10^5 colony-forming units/fish. Those bacteria were isolated from greater amberjack individuals. Inoculated and control fish were monitored for clinical disease and mortalities for the duration of the study (15 days). Mortality was attributed to the inoculated bacterium if the injected organism was recovered in pure culture from the internal organs (**Fig. 25.5.1)**.

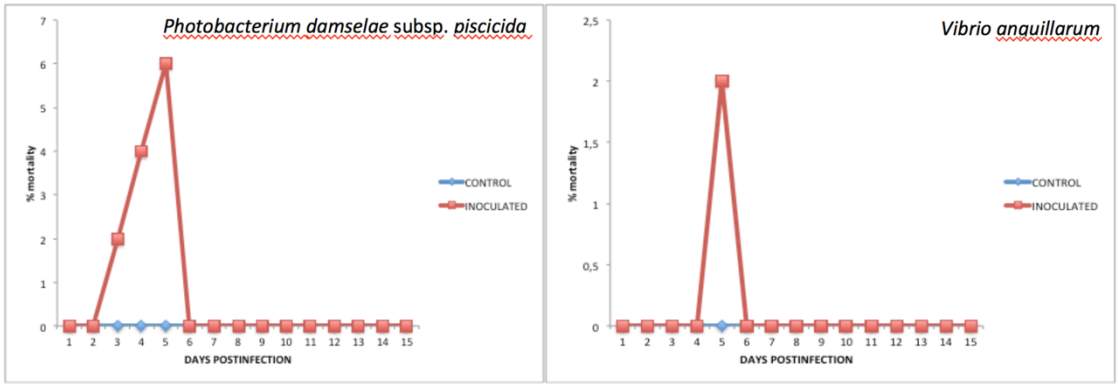

Figure 25.5.1. Results of challenge test against different bacteria isolated from individuals from the Canary Islands.

Minimum inhibitory concentration (MIC) of different antibiotics for the major bacteria in greater amberjack.

Florfenicol, Erythromycin, Sarafloxacin, and Oxytetracycline (Sigma-Aldrich) were the tested antibiotics. The initial concentrations used ranged from 1280µg/ml to 10µg/ml for Erythromycin, Sarafloxacin, from 800µg/ml to 6.25µg/ml, and Oxytetracylcine, from 1000µg/ml to 7.8125µg/ml, in serial dilutions with a dilution factor of 1:2. The antibiotics were diluted in TSB when used for *Vibrio anguillarum*, *Bacillus sp.*, *Vibrio alginolyticus* and *Photobacterium damselae subsp. piscicida*. The total volume used for the incubation in the microtiter plate was 100µl, 50µl from bacteria and 50µl from the antibiotic solution. The experiment was repeated x3 for every bacteria and antibiotic. The turbidity of the suspensions on the

microtiter plate was measured by spectrophotometry. The measurements were made at a wavelength of 600nm.

Results of MIC for *Vibrio anguillarum* (**Fig. 25.5.2**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 80µg/ml, for florfenicol of 1000 µg/ml, for oxytetracycline of 250 µg/ml and for sarafloxacin of 156 µg/ml.

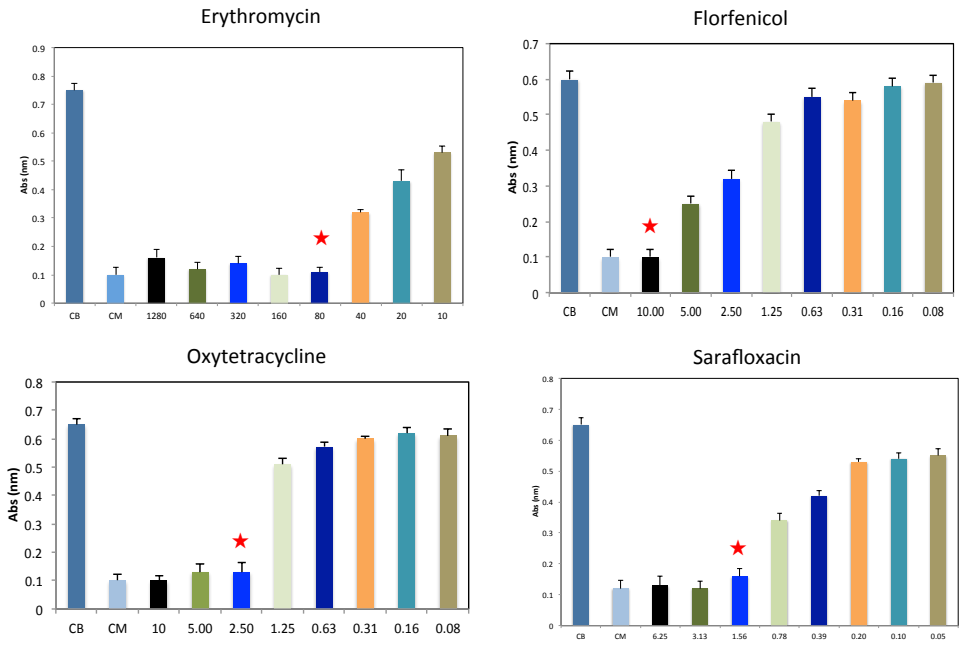

Figure 25.5.2. Absorbance obtained with respect to the concentration of antibiotic used for *Vibrio anguillarum*. The samples marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Bacillus sp.* (**Fig. 25.5.3**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 160µg/ml, for florfenicol of 1000µg/ml, for oxytetracycline of 125µg/ml and for sarafloxacin of 156 µg/ml.

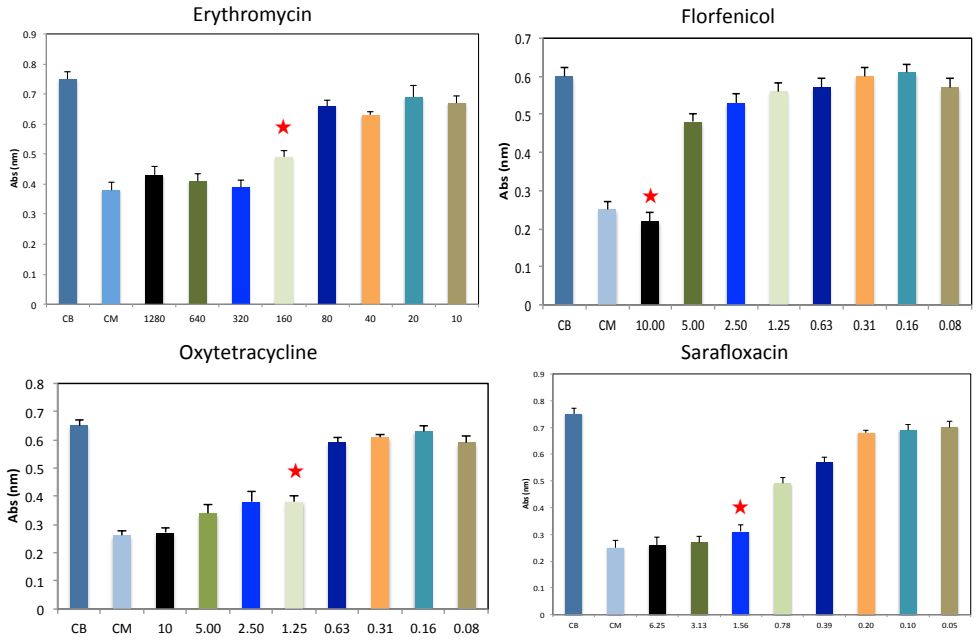

Figure 25.5.3. Absorbance obtained with respect to the concentration of antibiotic used for *Bacillum sp*. The samples marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Vibrio algynolyticus* (**Fig. 25.5.4**) showed that the isolated strain from amberjack presents a MIC for oxytetracycline of 1000 μg/ml. This strain was resistant for the rest of the antibiotics tested.

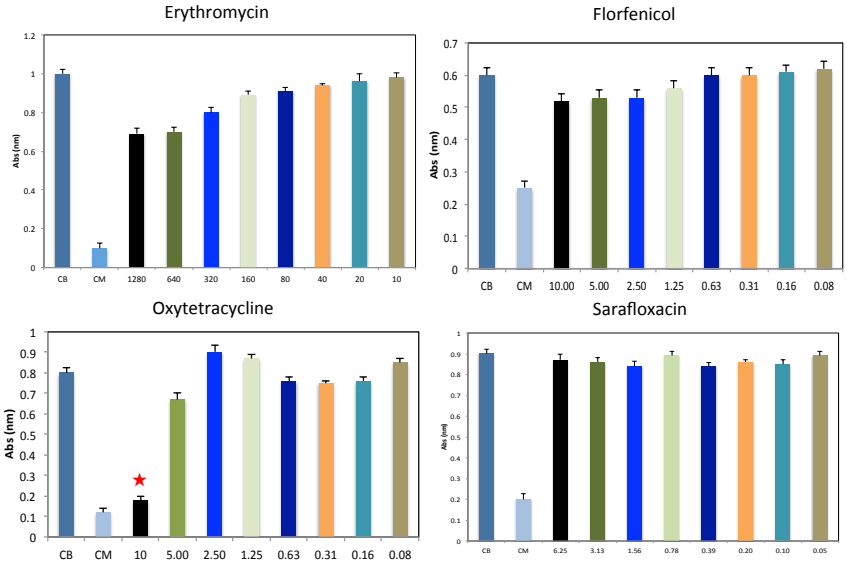

Figure 25.5.4. Absorbance obtained with respect to the concentration of antibiotic used for *Vibrio algynolyticus*. The sample marked with the star represents the MIC for the tested antibiotic.

Results of MIC for *Photobacterium damselae subsp. piscicida* (**Fig. 25.5.5**) showed that the isolated strain from greater amberjack presents MICs for erythromycin of 80µg/ml, for florfenicol of 1000 µg/ml, for oxytetracycline of 250µg/ml and the strain was resistant for sarafloxacin.

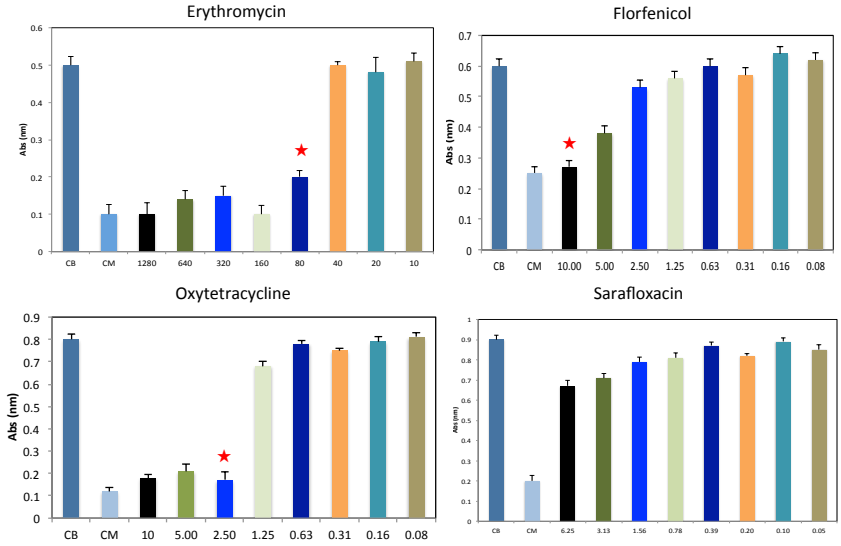

Figure 25.5.5. Absorbance obtained with respect to the concentration of antibiotic used for *Photobacterium damselae* sub. *piscicida*. The sample marked with the star represents the MIC for the tested antibiotic.

The full description of the work and results is provided in ***Deliverable 25.7 Report on the major bacterial and viral diseases found in greater amberjack, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided.***

Task 25.6 Diagnostic-recommendation manual for greater amberjack health (led by HCMR, Pantelis Katharios).

A diagnostic manual for greater amberjack health has been created. The manual is divided in two sections; the first, containing the bacterial diseases that have been studied within DIVERSIFY project, and the second, parasitic diseases including *Zeuxapta seriolae* and *Neobenedenia girellae*. The manual is based on the findings obtained during the course of the project and is compiled from input from all participating partners. The manual is an appendix in this Deliverable report and has been uploaded at the website of the project as a pdf that can be downloaded freely. A copy of the table of contents is given below (**Fig. 25.6.1**).

Contents

Figure 25.6.1. Table of contents of the greater amberjack health manual.

The full description of the work and results is provided in ***Deliverable 25.8 Diagnostic-recommendation manual for greater amberjack fish health*** and at the appended **Health Manual,** which is uploaded on the DIVERSIFY website.

Deviations from Annex I and their impact:
None.

Manuscripts that resulted from this Task

Fernández-Montero, A., Torrecillas, S., Izquierdo, M., Caballero, M.J., Milne, D.J., Secombes, C.J., Sweetman, J., Da Silva, P., Acosta, F. & Montero, D. (2019). Increased parasite resistance of greater amberjack (*Seriola dumerili* Risso 1810) juveniles fed a cMOS supplemented diet is associated with upregulation of a discrete set of immune genes in mucosal tissues. Fish & Shellfish Immunol. **86**: 35-45.

WP 26 Fish health – Atlantic halibut

WP No:	26	**WP Lead beneficiary:**		P7. IMR
WP Title (from DOW):	Fish Health – Atlantic halibut			
Other beneficiaries (from DOW):				
Lead Scientist preparing the Report (WP leader):	Sonal Patel			
Other Scientists participating:	Audun Helge Nerland			

Objectives

1. Determine the effect of delivering recombinant capsid protein during late larval stages on protection to nodavirus (Viral Neural Necrosis, VNN).

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

During the first period we focused on assessment of the use of several expression systems for production of nodavirus capsid protein. The goal was to assess two eukaryotic expression systems; microalgae and a protozoan (*Leishmania tarentolae*), in addition to *E. coli* and in tobacco plant. Apart from microalgae, all other three systems were assessed.

Expression of the nodavirus capsid protein in all three systems could be achieved. However, it was only in the *E. coli* system that we achieved sufficient and high expression for further use of the protein as antigen for vaccination purposes. Further optimisation for sufficient expression in plant and protozoan systems and a method for purification of the recombinant protein was achieved by the previous reporting period.

There has also been liaison with Targetfish, EU project to consider if amongst the VNN expressed by various systems in their project, the scientist involved could suggest a candidate that can be included in the testing in task 26.2.

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

Multiple expression systems were tested for production of the capsid protein of VNN during period 1 and 2. There had been a few delays getting the recombinant capsid protein made in tobacco leaves. In addition, issues with wetlab challenge facilities aquarium led to DL26.2 and DL26.3 being pushed back to month 48. Researchers in the TargetFish project were contacted and it was agreed that the best candidate that has shown promising results in sea bass will be possibly delivered to IMR for inclusion in a halibut trial during spring 2017. Since the production of halibut is only once a year, the vaccination and challenge trial was delayed and started in late spring 2017.

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

VNN capsid protein expressed by varying expression systems in the previous reporting time were delivered to halibut larvae 100 dph either through i.p. injection or through *Artemia*. The juveniles were transported to

challenge facility 10 weeks after vaccination, and challenged with NNV. 8 weeks post challenge, the juveniles will be sampled to assess for effect of vaccination and possible adaptive immune response.

Summary of progress towards objectives (49-60 Mo):

All juveniles that were challenged in the previous period were sampled for brain and spleen. Brain samples were analysed for Nodavirus using a RNA2 specific real time rt-PCR assay (Korsnes et al, 2005) to assess the effect of different vaccination treatment. The individuals within each treatment group and between treatment groups were in varying developmental phases and accordingly were expected to have different weights at the start of the experiment. During the experiment weight and development of the individuals continued to differ and this was reflected in the large individual differences seen at the end of the experimental period. Several individuals weighing below 7-10 g still had the appearance of late larval stages, while individuals above 15-20 g showed signs of successful metamorphosis and migration of eyes. The treatment groups showed no difference in protection. The treatment groups that were non-vaccinated and not treated, together with the one that was injected with PBS formulated with adjuvant, showed almost similar amounts of viral RNA2 compared to the vaccinated groups. When comparing vaccine delivery systems oral to injection and with adjuvant-injected groups, the adjuvant-injected groups showed slightly lower amounts of virus (1-3 Ct values), and a single individual in most of the injected groups had very little virus.

The same formulation that was used as a positive control in this study did not give any protection to larvae, leading us to speculate that the amount of antigen that was delivered could have been too low. Testing these antigens in larvae that are sorted such that all individuals are in the same developmental phase or by delivering the antigens along with dry pellets rather than through *Artemia* would reveal if the antigens can give protection at a stage earlier than at 25 g.

Details for each Task

Task 26.1 Production of VNN capsid protein (led by IMR).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 26.1 Assess the use of two eukaryotic expression systems; microalgae and a protozoan (Leishmania tarentolae) for production of nodavirus capsid protein.***

Task 26.2 Monitor and assess immune response and protection (led by IMR).

This task has been completed during the fourth reporting period and the full description of the work and results have been provided in ***Deliverable 26.2 Testing of the delivery of vaccine candidates through Artemia to Atlantic halibut larvae*** and ***Deliverable 26.3 Determine immune response and effectiveness of orally delivered nodavirus capsid protein on protection of Atlantic halibut larvae.***

Uptake of *microorganisms expressing NNV antigen and the purified antigen*

Both GFP *E.coli* and GFP *L. tarantolae* were taken up very efficiently by the *Artemia*. As the *Artemia* (as well as the microbes) were inspected live, it was observed that the *Artemia* filtered and accumulated both type of microbes. To examine the presence of the recombinant antigen in the *Artemia* after being fed by the different formulations, and later the presence of recombinant antigens in the larvae after having been fed by the *Artemia*, samples were applied on a SDS-PAGE and analysed be immunoblotting. The results showed

that the recombinant capsid proteins were present but were degraded by the *Artemia*. The capsid antigen fed to the *Artemia* as inclusion bodies was degraded from the original 43 kD protein to one of around 17 kD. For *Artemia* fed with *E. coli* expressing recombinant antigen there were positive signals on the blot, but the size indicates a partial degradation of the protein. For the recombinant protein expressed in *Pichia* the signals indicate proteins larger than 43 kD which might be due to overloading of the total amount of protein on the gel. For *Artemia* fed with *L. tarentolae* expressing the recombinant protein it was difficult to see any signal at all. However, these results are qualitative, and not quantitative. *Leishmania* gives less expression of recombinant protein than the other host systems, and it might be that the amount of protein applied to the gel is lower that the detection limit. For days 1 and 2 the pictures were quite similar, while for day 3 the signals were weaker, but still with the same tendency.

In conclusion, it was shown that both *E. coli* and *L. tarentolae* are readily filtered from the surroundings and taken up by *Artemia*. It is difficult to conclude from this experiment, however, the extent to which the microbe or its harboring proteins are degraded in the intestine of the *Artemia*. Presumably this will also be dependent on the different types of proteins. For example, GFP might be more stable than the recombinant capsid protein.

SDS-PAGE and immune-blotting reveal that *Artemia* will digest the recombinant protein, but the degradation depends on the expressing host. The recombinant antigen could be detected in the intestine of the larvae only at day 1, post end of the feeding and in just one out of six larvae examined.

Oral delivery to halibut larvae and assessment of efficacy

To test whether the antigen delivered to the larvae can offer any protection, halibut larvae at 100 days post-hatch, just before they were weaned to commercial dry pellets, were chosen for use. *Artemia* were produced according to the standard protocol used at IMR and used for this purpose.

The treatment groups were as follows:

At the experiment start each treatment group had 50 larvae/juveniles.

1. Pichia extract expressing Nodavirus capsid protein – oral delivery through *Artemia*
2. Pichia extract with empty vector, with no antigen - oral delivery through *Artemia*
3. Purified inclusion bodies of Nodavirus capsid protein from *E. coli* with mineral oil adjuvant – *i.p.* injection
4. Purified VLPs from *Pichia* – *i.p.* injection
5. Purified VLPs from *Pichia* with adjuvant – *i.p.* injection
6. Purified Nodavirus capsid protein expressed in tobacco leaves with mineral oil adjuvant – *i.p.* injection
7. Live *L. tarantolae* expressing Nodavirus capsid protein - oral delivery through *Artemia*
8. Live *E. coli* expressing Nodavirus capsid protein - oral delivery through *Artemia*
9. Purified inclusion bodies of Nodavirus capsid protein from *E. coli* - oral delivery through *Artemia*
10. PBS with mineral oil adjuvant – *i.p.* injection
11. Negative control – non-treated

The larvae/juveniles used for vaccination were in varying developmental phases, and this combined with the treatment and handling, resulted in several larvae/juveniles dying during the first few days. At the end of 10 weeks post vaccination, the juveniles that had survived within each treatment were transferred to the wetlab challenge facility, IMR, Bergen, acclimatized for 10 days, and challenged with Nodavirus by *i.p.* injection of 50 µl at $1x10^{7.5}$ $TCID_{50}$ /ml. The experiment was terminated in week 50, 2017, and all fish were

sampled for brain and spleen. Brain samples were analysed for Nodavirus using a RNA2 specific real time rt-PCR assay to assess the effect of different vaccination treatment.

The treatment groups showed no difference in protection. The treatment groups that were non-vaccinated and not treated, together with the one that was injected with PBS formulated with adjuvant, showed almost similar amounts of viral RNA2 compared to the vaccinated groups. When comparing vaccine delivery systems oral to injection and with adjuvant-injected groups, the adjuvant-injected groups showed slightly lower amounts of virus (1-3 Ct values), and a single individual in most of the injected groups had very little virus.

In conclusion, although it has been shown that *Artemia* will take up and accumulate the various forms of recombinant Nodavirus capsid proteins and act as a vector for oral delivery to larvae of Atlantic halibuts, the challenge experiments indicate that this strategy of antigen delivery does not induce protection against Nodavirus infection, at least under the conditions used in this study.

Deviations from Annex I and their impact:

Since none of the vaccination treatments showed any protection, the spleen was not analysed to assess the immune genes involved in adaptive immunity. This analysis would not have given any further understanding to the results obtained and would simply have reconfirmed that all groups had little or no specific immune response. Also, the brain samples that were collected for histology and IHC were not analysed for the same reason.

Manuscripts that resulted from this Task

None

Group Work Packages

Socioeconomics

In this period, activities have been done in:

- New product development (WP28)
- Business model and marketing strategy development (WP 30)

Work package 27 had been finished in an earlier reporting periods. However in this reporting period the team of WP27 papers has been working on scientific papers on basis of the outcomes of the deliverables. These papers have not been submitted to peer reviewed journals yet.

The work in WP28 that started in the previous period in work package 28 has been finished on before mentioned objectives resulted in Deliverables 28.5, 28.6, 28.7 and 28.8. These deliverables learn that each of the new species has unique sensory profiles and that the processing has a direct effect on the technical characteristics and nutritional value. Next to that filleting yields and protein contents did not seem to be influenced significantly by fish size or rearing and dietary histories at grow out stage. Regarding the sensory properties, diet had only an important effect on the sensory characteristics of the grey mullet, especially in aspects related to the fillet fat content and its oxidative stability. The production in industrial scales of the developed products from the DIVERSIFY fish species can be a feasible task, subject to the application of certain principles and conditions. The technical yields that can be achieved are very satisfying for all products, thus providing high profit margins. The duration of high quality life ranges widely depending on the product nature but also on the optimization of processing and preservation procedures. Frozen products and sterilized (oil-preserved fish fillet and fish pate) have long high quality shelf life, expanded in months, while fresh products have a high quality shelf life of few days, varying with the ingredients included (most sensitive ingredient is the limiting factor), the manufacturing process and the packaging type.

All earlier work in WP has been performed according to the DOW. The consumer study and the product samples developed in this work package have been used in a funnelling approach in WP 29 and 30. By the end of Reporting Period 3, all activities in WP29 have been finished. However in this 4th Reporting Period, the team of WP29 has worked on scientific papers on basis of the deliverables of WP29.

In work package 30, in this reporting period work has been done on Task 30.2 and 30.3. Task 30.2 was about a real market test for greater amberjack has been performed, that learned that:

- Consumers need help in positioning of the new species. Some species are well known as wild catch in some regions of the EU. In all other regions consumers need a reference product.
- Innovative consumers try new products first. Traditional consumers need recipes and more information before they try a new species
- Communicate that the product is environmentally friendly helps in some countries.
- Only in Spain price promotion helps, in other countries it works out negative for a new species
- Southern European consumers are more open for cultured greater amberjack
- Market penetration should be done country by country instead of pan-European

WP 27 Socioeconomics – Institutional and organization context

WP No:	27	**WP Lead beneficiary:**		P6. DLO
WP Title (from DOW):	Socioeconomics – Institutional and organizational context			
Other beneficiaries (from DOW):	P6. DLO	P10. TU/e	P11. AU	P12.APROMAR
Lead Scientist preparing the Report (WP leader):	Gemma Tacken (DLO)			
Other Scientists participating:	Victor Immink (P6), Machiel Reinders (P6), Olga vd Valk (P6), Athanasios Krystallis (P11), Javier Ojeda (P12), K. Grigorakis (P1), M. Keller (P34)			

Objectives

1. To give insight in the competitive field of and market developments in the European aquaculture market with a focus on the species selected in DIVERSIFY (meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet),
2. To assess the obstacles for growth in the current aquaculture production chains and for these selected species,
3. To identify market opportunities for future growth of the European aquaculture sector for the selected species.
4. Propose a certification framework for the species addressed in DIVERSIFY

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

The first year of the project the activities in WP27 have 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 work reported in the previous Reporting Period (13-30 Mo):

In Period 2, a Porter analysis showed that the 6 DIVERSIFY species are unknown in the market and that some species have specific markets with high brand awareness and recognition, and others where they are completely unknown. The trend mapping learned that there will be an increasing competition on the EU market for animal proteins on the long run. Another important trend is increasing requirements with respect to quality, traceability, sustainability and animal welfare. The success failure study identified key aspects

for a successful introduction in the market and shows that the EU market is very diverse with great variations in market preferences. Before introduction in the market, per product the advantages that meet consumers' needs have to be identified and communicated. Involvement of the industry and the retail is very important as well as a positioning on environmental and ethical issues in the UK and Germany or convenience in Spain and France.

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

No work was planned during this period, and all activities were completed in the previous Reporting Periods.

Summary of progress towards objectives (49-60 Mo):

No work was planned during this period, and all activities were completed in the previous Reporting Periods.

Details for each Task

Task 27.1 External environmental analysis (led by DLO, Gemma Tacken)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.1 Report on external environmental factors that affect or will affect the production chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet***, and ***Deliverable 27.2 Report on current certification schemes and standards and their business***

Task 27.2 Competitive analysis (led by DLO, Gemma Tacken)

Sub-task 27.2.1 Competitive analysis (prepared by Victor Immink (P6. DLO) and Javier Ojeda P12. APROMAR)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.3 Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet.***

Sub-task 27.2.2 Trend mapping

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.4 Report on trend mapping for the European aquaculture and fisheries sector, and protein market in the (near) future***.

Sub-task 27.2.3 International survey in selected countries (led by SWR/DLO)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.5 Report on the results of international survey on industrial buyers' attitudes and perceptions regarding cultures fish***.

Task 27.3 Opportunities and barriers for growth (led by DLO)

Task 27.3.1 Success-failure study

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.6 List of critical success factors for market acceptance***.

Task 27.3.2 Using the business model Canvas

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 27.7 Report on the analysis of the business models and supply chains of the participating SMEs***.

Deviations from Annex I and their impact:
There were no deviations from the planning in this work package.

Manuscripts that resulted from this Task

There were no publication that resulted from this WP, and none is expected due to the nature of the work for this WP.

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), José A. Pérez (P15), Rocio Robles (P18)			

Objectives

1. To develop new product concepts from selected species, by incorporating consumer and expert input,
2. To select product ideas and develop physical new products from the selected species,
3. To monitor the quality of new products in terms of organoleptic characteristics and nutrition-rearing history,
4. To make a technical assessment of the products.

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

Two subtasks were started and continued during the 2nd Reporting period within WP28: Sub-task 28.1.1 (led by P11. AU) and Sub-task 28.2.1 (led by P1. HCMR). The main outcomes of these two activities were:

- Design of a series of focus group discussions with consumers and experts in the selected countries of the project (UK, D, ES, F, I). The main objective of this task was to generate a set of ideas to be screened out and further developed into product concepts for testing in subsequent tasks in the new product development process.

- Estimation of optimum fish sizes for developing the selected new products. In this case the activities performed included somatometric measurements for the five species of interest (meagre, greater amberjack, pikeperch, wreckfish and grey mullet) as well as their chemical and sensory characterization.

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

In WP28 several product ideas have been identified per species on basis of focus group discussion in all selected countries. Some of these product ideas have been worked out to prototypes that have been sensory tested in the five selected countries. The results will be presented in this (3rd Period) report. During the second period (months 13-30) the following four different activities were completed and/or finished.

In the first activity (Sub-task 28.1.1) new ideas were explored and reported through focus groups with consumers and experts regarding the fish products resulting from the species under study in five focal markets: UK, Germany, Spain, France and Italy. Experts from different countries agreed that the created

products were attractive and feasible ideas that have potential in the market. They consider that in overall these ideas could increase profits of fish industry due to the higher diversity of choice. Generally, they stated that these ideas have a possible prospective if they are developed with good coordination between the fish farmers and consumers. In terms of general recommendations for new product development of selected fish species, the most important drivers and barriers for the choice of the new product ideas most relevant for consumers were analysed and discussed.

The second activity performed (Sub-task 28.1.2) focussed on generation and screening of ideas for new product development based on the market data of WP 27, the results obtained in the focus groups (Sub-task 28.1.1) and the evaluation of the different ideas by the scientists from different scientific areas. Technical limitations and the economic prospects efficiencies (i.e., within a socio-techno-economic study) were used to generate a pool of ideas about potential products. The selected ideas were assessed by means of technical, economic and market assessment criteria, among others.

In the third activity carried out (Sub-task 28.2.1) the optimum fish sizes for developing the new products identified in Sub-task 28.1.2 were described based on basic somatometric measurements and evaluation of losses. In addition, chemical-mechanical and sensory properties of fish species during cutting and minimal processing were obtained, which provided a definition of process solutions for each species based on technological, physical and sensory characteristics.

Finally, the fourth activity completed (Sub-task 28.2.2) focussed on the development of physical prototypes of new products from meagre, greater amberjack, pikeperch and grey mullet. The physical prototypes were developed based on the information provided by WP 27 (market potential of the new species), Sub-tasks 28.1.1 and 28.1.2 (products concept development: technical and consumer driven), Task 29.1 (consumer value perceptions and segmentation), physicochemical characteristics of each raw material (Sub-task 28.2.1), technical properties of the products and the process, and similar product availability in the market. Twelve different prototypes were elaborated based on ten selected ideas (idea numbers 1, 2, 4, 6, 9, 13, 21, 30, 33 and 34). Meagre fish was used for the development of the following ideas “frozen fish fillets with different recipes” (idea 1), “fish burgers shaped as fish” (idea 6) and “ready to eat meal: salad with fish” (idea 4). Pikeperch was used for the development of “fresh fish fillet with different ‘healthy’ seasoning and marinades” (idea 21), “ready-made fish tartar with additional soy sauce” (idea 30) and “fish spreads/pate” (idea 9). Grey mullet was used for the development of “thin smoked fillets” (idea 2), “ready-made fish fillets in olive oil” (idea 33) and “fresh fish fillet with different ‘healthy’ seasoning and marinades” (idea 21). Finally, greater amberjack was used for the development of “frozen fish fillet that is seasoned or marinated” (idea 13), “ready-made fish tartar with additional soy sauce” (idea 30) and “fresh fish steak for grilling in the pan” (idea 34). Information about how to elaborate these new products were provided as well as a number of guidelines, processing conditions, technical specifications and troubleshooting. In addition, basic information regarding the food products packaging, conservation conditions, preliminary product shelf life and consumer handling/cooking specifications were also reported. Since these prototypes have potential as fish product diversification, they will constitute the basis for further tasks in the project, including their consumer acceptability evaluation.

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

During this period all proximate composition as well as fatty acid analysis of the six developed products has been completed in duplicate. These products have also been characterized by means of sensory descriptive analysis, thus including the training of the panellist, the development of sensory references and the evaluation of their individual performance.

In order to correlate technical quality characteristics with previous nutritional - rearing history, different fish groups from varying farming histories have been identified for sampling. These animals will be analysed for the proximate composition and analytic fatty acid profiles (HCMR and ULL) and sensory characteristics (HCMR and/or IRTA). A detailed sampling protocol has been established and agreed between the partners involved in this sub-task.

Summary of progress towards objectives (49-60 Mo):

This period focused on monitoring the quality of the developed products and on relating the technical quality characteristics of the raw material with previous nutritional and rearing history. In addition, a technical assessment of the selected species has also been performed based on the results of all sub-tasks in WP28. Three different sub-tasks have been undertaken.

In the first subtask (***Deliverable 28.5 Report on results of quality evaluation study on basic quality characteristics of the developed products*** and deliverable ***D28.6 Report on results of sensory descriptive analysis of the developed products***) the main characteristics of the different developed products were assessed as well as the impact of the technological processes on them. Results indicated that processing had an effect on both the proximate composition and fatty quality of the products when compared to the raw fillet tissue. However, the effect depended on the processing method used as well as the inclusion of additional materials (such as olive oil) during the product formulation. Processing generally had a negative effect on nutritional quality reducing the proportion of essential fatty acids, i.e. EPA and DHA, of the majority of products when compared to the corresponding fish fillets. The proximate composition and fatty acid quality varied in a great extent across the processed products. This was expected, since the different post-mortem processing furthermore altered the initial variations of the species. The nutritional composition of the species was altered by the effect of processing, which was more intense for products that required additional materials and intense heat treatment. All processed products exhibited unique sensory profiles. The processed products showed more complicated sensory profiles with more attributes than the unprocessed cooked fillet of the species. The developed characteristics of the processed products in their majority could be connected to the added materials and/or the processing method. The products generated from grey mullet exhibited more similar profiles than those of meagre, which was attributed to the lack of added materials for the former, with the exception of olive oil. The fish steak was the product with the least altered sensory profile, when compared to the corresponding fish fillet of the species, which can be attributed to the low amount of processing it underwent.

The second subtask (28.3.2) (***Deliverable 28.7 Report on correlation of technical quality with nutritional - rearing history***) focused on exploring the relationship between the rearing history and the quality (main nutritional and sensory properties) of the raw material. In this case results indicated that filleting yields and protein contents did not seem to be influenced significantly by fish size or rearing and dietary histories at grow out stage. Greater amberjack displayed the highest filleting yields and final contents of protein, fat and especially EPA+DHA. However, further grow out trials are advisable in order to establish the best extruded diet to balance the final levels of saturated and 18:2n-6 fatty acids. Due to its vulnerability to fat oxidation, it is suggested that commercial sizes should be 1-2 kg with a relatively lower fillet fat content than bigger fish. Meagre filleting yield and protein content were quite attractive. Its total fat contents did not seem to be highly influenced by the dietary or growing history, displaying low contents of fat even in the wild, an attractive feature for low fat dietary regimes. However, the degree substitution of dietary marine origin ingredients by terrestrial vegetables components should be taken with more caution and highly controlled in this species, which displayed high contents of 18:2n-6 and lower comparable amounts of EPA+DHA. Grey mullet is confirmed as the best candidate for marine ingredients substitution either by terrestrial or marine origin vegetable sources. Independently of the rearing history, their fillets display a comparable high amount of EPA+DHA, low contents of 18:2n-6 and a good balance of saturated fatty acids, all of them nutritionally attractive and health promoting qualities. Regarding the sensory properties, diet had only an important effect on the sensory characteristics of the grey mullet, especially in aspects related to the fillet fat content and its oxidative stability.

Finally the third subtask (***Deliverable 28.8 Technical assessment of selected species***) focused on the technical assessment of the selected species to have a full image of the principles applying, the criteria for ensuring quality and safety of the product. The production in industrial scales of the developed products from the DIVERSIFY fish species can be a feasible task, subject to the application of certain principles and conditions. The technical yields that can be achieved are very satisfying for all products, thus providing high profit margins. The duration of high quality life ranges widely depending on the product nature but also on the optimization of processing and preservation procedures. Frozen products and sterilized (oil-preserved fish fillet and fish pate) have long high quality shelf life, expanded in months, while fresh

products have a high quality shelf life of few days, varying with the ingredients included (most sensitive ingredient is the limiting factor), the manufacturing process and the packaging type. The principles for proper production include three aspects: 1. Raw materials of good quality. 2. Good manufacturing (processing) practices. 3. Proper traceability. Freshness of the raw materials should always be ensured. The ISO and HACCP principles should apply throughout the whole processing chain and commercialization for ensuring safety and maximum quality. Food traceability systems should be implemented in all products but also in raw materials that are incorporated during processing. These rules are necessary and sufficient condition for high quality and economic sufficient products.

Altogether, these three subtask provide a broad picture of the main quality characteristics of the raw material and the developed products, thus given a valuable input for those interested in the commercialization of any of the studied products and/or species.

Details for each Task

Task 28.1 Product concept development: technical and consumer-driven (led by AU, Athanasios Krystallis)

Sub-task 28.1.1 (led by AU, Athanasios Krystallis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.1 Report with results of focus groups with consumers and experts regarding ideas for new fish products.***

Sub-task 28.1.2 (led by HCMR, Kriton Grigorakis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.2 List of ideas for new product development***.

Task 28.2 New Product Development (led by IRTA, Lluis Guerrero)

Sub-task 28.2.1 (led by HCMR, Kriton Grigorakis)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.3 Report on product and process solutions for each species based on technological, physical and sensory characteristics.***

Sub-task 28.2.2 (led by IRTA, Ricard Bou)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 28.4 Physical prototypes of new products from the selected species meagre, greater amberjack, wreckfish, pikeperch and grey mullet***.

Task 28.3 Monitoring technical quality of the products (led by HCMR)

The objective of this task was to monitor the quality of new products in terms of organoleptic characteristics and nutrition-rearing history. Next to that a technical assessment was made.

Sub-task 28.3.1 (led by HCMR, Kriton Grigorakis)

In this task a quality evaluation study is performed on basic of quality characteristics of the product developed, which will include somatometric measurement, fat deposition evaluation, proximate fillet composition and fatty acid analysis. Next to that a sensory descriptive analysis is done to describe the sesory characteristics of the developed products.

Methodologies

The proximate composition (protein, fat, moisture and ash) of physical prototypes was determined as described in the AOAC Official Methods. The fatty acids were extracted and esterified by a direct trans-esterification process in methanol-benzene. Esters were then analysed in a gas chromatograph – flame ionization detector (GC-FID). Carbohydrates were calculated by difference whereas the sugar content was determined after samples clarification with Carrez reagents. The salt content (Na content x 2.5) was determined by means of an Atomic Absorption Spectroscopy in sample extracts obtained after mineralization with nitric acid and hydrogen peroxide in a microwave digester. The gross energy content was determined in the freeze-dried samples, by an adiabatic calorimeter. Cholesterol, phytosterols, and squalene were determined by GC/FID after hot saponification.

Descriptive analysis (Report on results of sensory descriptive analysis of the developed products): all sensory analysis methodologies developed and used for this task have been described in 3rd Periodic report and analytically presented in Deliverable D28.6.

Results

The results indicated that greater amberjack exhibited the most distinctive proximate composition profile amongst the other species, which was connected to the significantly higher fat content, when compared to those (Table **28.3.1.1**). The respective proximate composition and energy contents of the generated products appear in Table **28.3.1.2**

Table 28.3.1.1: Mean values and coefficient of variation (CV) of fillet proximate composition parameters of wreckfish, greater amberjack, grey mullet, meagre and pikeperch (n=5). Different letters in the same row indicate statistically significant differences (P <0.05) between the mean values of each species.

Proximate composition (%)	Greater amberjack		Grey mullet		Meagre		Pikeperch	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Moisture	69.46b	0.04	76.53a	0.01	77.17a	0.04	76.58a	0.01
Protein	22.21a	0.07	21.37ab	0.03	20.65ab	0.09	21.80a	0.03
Fat	6.28a	0.64	0.58b	0.44	0.52b	0.36	0.06b	0.30
Ash	1.44a	0.01	1.27ab	0.01	1.35ab	0.07	1.30ab	0.01

Table 28.3.1.2. Proximate composition and energy contents. Mean (±CV) of the 6 generated products (n=5). Different letters in the same row indicate statistically significant differences (P <0.05) between the mean values of each species.

Proximate composition (/100g)	Steak[1]		Pate[2]		Salad[3]		Burger[3]		Smoked fillets[4]		Fillets in Olive oil[4]	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Moisture	72.0b[5]	2	66.0c	1	79.8a	1	71.8b	<1	63.3c	4	57.3d	11
Fat	3.67c	38	14.9b	3	2.93c	8	4.82c	3	2.87c	24	21.85a	16
Protein	22.4a	4	17.6cd	3	13.0d	8	18.8bcd	1	27.4a	8	19.4bc	23
inorganic c	1.48d	9	1.58cd	2	0.8e	5	2.47b	2	4.82a	8	1.99bc	20
total CHS	0	0	0.02b	0	8.4	0	0.09	0	0	0	0	0
sugars	0	0	0	0	6.0	0	0	0	0	0	0	0
fibers	0	0	0	0	1.9	0	0	0	0	0	0	0
salt (mg)	0.29	0	824	8	295	0	503.8	12	-*	-	0.16	0
Energy (kcal)	116b	7.6	66.0d	1.1	83.3cd	5.8	119.3b	1	184.2ab	4.3	287.8a	11

[1]Greater amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet, * missing value

The fatty acid profile of the products was altered mainly due to the addition of specific exogenous fat sources, which were added to the initial fillets and were presented in Deliverable 28.5. The changes undergone during the formation of products from initial fillet raw materials in composition and fatty acids appear in **Table 28.3.1.3**. Besides fatty acids, the lipid quality of the products was evaluated through their sterol contents (**Table 28.3.1.4**).

Table 28.3.1.3: Statistical changes of composition and main fatty acid groups in the processed products (and respective level of significance) when compared to respective raw fillets and the respective processed products in the proximate compositions.

	G. amberjack	pikeperch	meagre		grey mullet	
	steak	pate	salad	Burger	smoked fillets	fillets in olive oil
moisture	increase*	decrease*	increase**	decrease**	decrease**	decrease***
protein	-	decrease*	decrease**	decrease*	increase*	-
fat	decrease*	increase***	increase*	increase**	increase**	increase***
ash	-	increase*	decrease**	increase**	increase**	-
SFA	-	decrease**	-	increase***	decrease*	decrease*
MUFA	-	increase**	-	-	increase**	increase***
PUFA	decrease	-	-	decrease**	-	decrease***
n-9	decrease*	increase**	-	increase*	increase*	increase***
n-6	decrease*	increase***	increase*	decrease**	decrease*	decrease*
n-3	increase*	decrease***	decrease*	decrease*	decrease***	decrease***
18:2n-6	decrease	increase***	decrease**	decrease**	-	increase**
ARA	-	decrease**	decrease**	decrease*	decrease**	decrease***
EPA	increase*	decrease***	decrease**	decrease*	decrease*	decrease***
DHA	increase*	decrease***	decrease*	decrease*	increase**	decrease***
n-3/n-6	increase*	decrease***	-	increase*	-	decrease**

*P<0.05, **P<0.01, ***P<0.001

Table 28.3.1.4. Sterol contents of the 6 generated products (n=5)

	Steak[1]		Pate[2]		Salad[3]		Burger[3]		Smoked fillets[4]		Fillets in Olive oil[4]	
(mg/100g)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
squalene	0.04c	0.25	0.74	0.34	3.09b	0.29	0.09c	0.7	0.10c	0.8	72.0a	0.24
cholesterol	71.6b	0.15	67.5b	0.15	64.7b	0.04	104.8a	0.1	77.6b	0.16	49.0c	0.15
β-sitosterol	0	0	25.4a	0.19	2.31c	0.13	0	0	0	0	15.4b	0.10
5 Δ - avenasterol	0	0	0.68	0.82	0	0	0	0	0	0	0.96	0.14

[1]Greater amberjack, [2] Pikeperch, [3] Meagre, [4] Grey mullet,

All six processed fish product exhibited unique and discriminant sensory profiles as indicated in the PCA sensory maps of Figure 28.3.1.1. Furthermore the products exhibited significant ($P<0.05$) variations in all sensory aroma, taste, flavour and texture attributes examined. The main intense and discriminant characteristics of each product per modality were:

Pikeperch pate: garlic aroma; earthy and secondary potato flavour; pasty and secondary teeth adherence texture.

Meagre salad: lemon and secondary spicy and green aroma; sour taste; seafood flavour; crunchy and juicy texture.

Meagre burger: butter and toasted aroma; sweet and umami taste; cheese and secondary fatty flavour; rubbery and secondary greasy, juicy and chewy texture.

Smoked grey mullet fillets: smoked aroma; salty taste; sardine and secondary rancid flavour; chewy texture.

Grey mullet fillets in olive oil: sardine and oxidized aroma; canned tuna and secondary fatty, rancid and sardine flavour; fibrous and secondary greasy and chewy texture.

Greater amberjack steak: this product did not exhibit any specific discriminant characteristics, but was perceived as having equally high intensities with other processed products in several attributes. Specifically, the steak was perceived as having amongst the highest green aroma, potato and sardine flavour, teeth adherence and secondary fibrous and chewy texture.

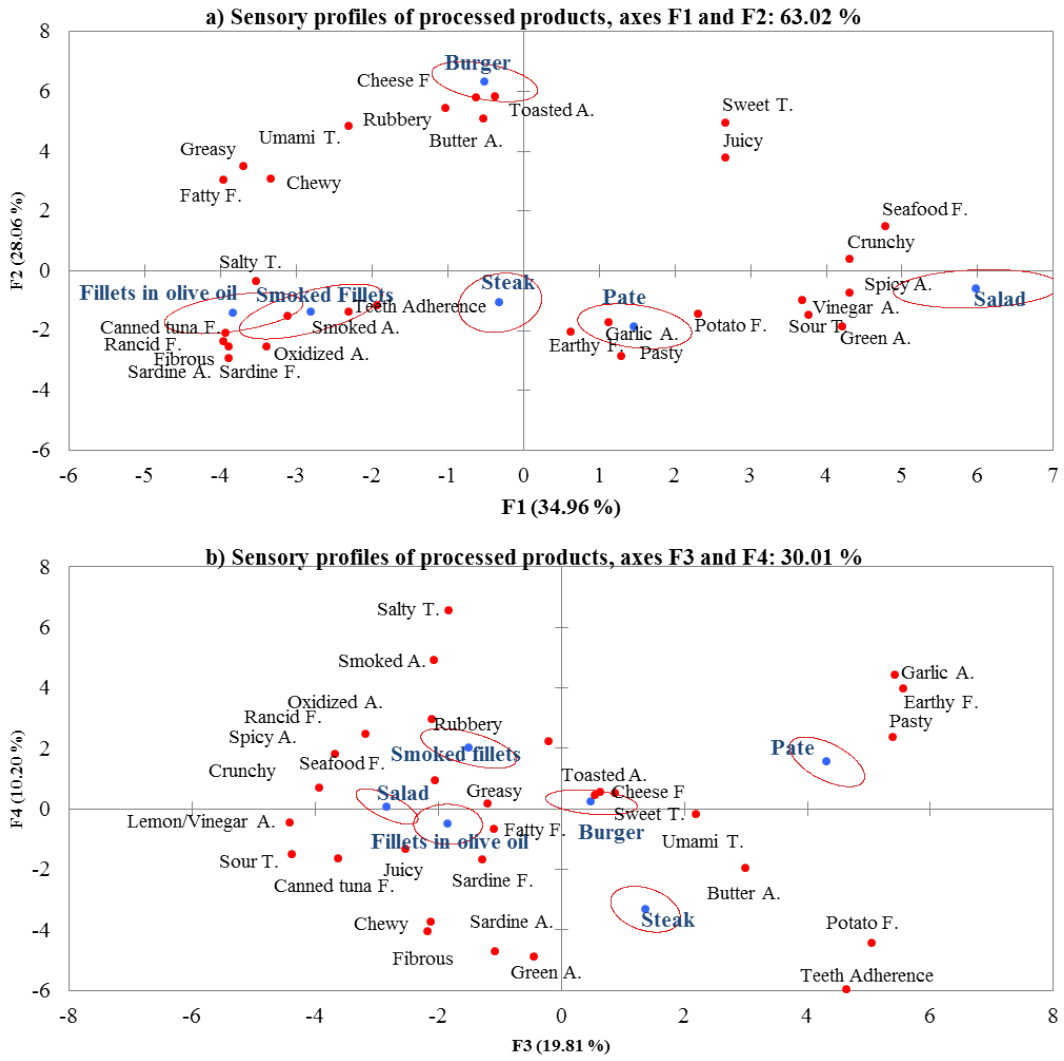

Figure 28.3.1.1: PCA sensory maps of processed products including confidence ellipses around products points indicating the uncertainty of sensory evaluations; the confidence ellipses are built via bootstrapping. Abbreviations used: A. aroma, T. taste, F. favour.

All products developed in D.28.4 were evaluated for their physicochemical properties and their chemical quality. This task has been resulted in ***Deliverable 28.5 Report on results of quality evaluation study on basic quality characteristics of the developed products.***

Next to that, a sensory descriptive analysis was done in order to analyse and describe the sensory characteristics of the developed products. This resulted in ***D28.6 Report on results of sensory descriptive analysis of the developed products.***

Sub-task 28.3.2 (led by ULL, Covadonga Rodriguez)

Task 28.3.2 evaluated the correlation between the fish dietary history (e.g. dietary fat and protein levels, fat sources, etc.) or other rearing parameters (e.g. rearing system, temperature, or density…) and the end product quality. A specific dossier per fillet product, including both, nutritional value in terms of protein,

fat and w-3 contents and sensory characteristics, was presented taking into account the fish origin, the rearing conditions and the feeding regimes. Task 28.3.2 attempts to correlate some technical quality features including nutritional value and sensory characteristics of greater amberjack, grey mullet, pikeperch and meagre with their previous nutritional - rearing history, in order to provide fish farmers and other potential chain partners a strong input for value positioning statement and communication claims of the four selected species. By relating new determinations made in the framework of WP21 (greater amberjack), WP22 (pikeperch), and WP23, (grey mullet), with the previous analyses (see D28.3, D28.5 and D28.6), it is intended to show how diet and other fish rearing conditions may influence the nutritional quality of the final product. Since many controversial issues in aquaculture regarding food safety, nutrition, and sustainability are directly related to the nutrition and feeds for farmed fish, the proximate and fatty acid composition of the extruded diets together with the main rearing history factors are also given and correlated with the technical and sensory quality of the fillets as well as their nutritional value expressed in absolute terms per 100 g serving portions. Furthermore, it is also shown that DIVERSIFY´s partners possess the knowledge and technology necessary to produce a fish product capable of meeting the nutritional, safety and environmental sustainability expectations of the international market, without losing the differential sensory characteristics of each of the 4 species selected in this work package. All new batches of fish evaluated were from reared origin (**Table 28.3.2.1**, in blue). **Tables 28.3.2.2** and **28.3.2.3** show the main characteristics of the samples analysed.

Table 28.3.2.1. Description of the batches used for each species (new batches in blue).

Species	Batch	Season	N	Origin-farming	Feed	Fish size	WP
Greater amberjack (*Seriola dumerili*)	1	Feb 2015	10	Farm (Corfu S.A.), NW Greece. Floating sea cages	Commercial extruded feed	1-1.5 kg	
	2	Apr 2015	8	Farm (Argosaronikos S.A.), Attiki, Greece. Floating sea	Commercial extruded feed	15-20 kg	
	3	Jan 2016	49	IEO Canarias, Tenerife, Spain (P15). Concrete ponds, FT rearing system	Commercial extruded feed (P20)	400-650 g	21
	4	Jul 2017	24	IEO Canarias, Tenerife, Spain (P15). Concrete ponds, FT rearing system	Commercial extruded feed (P20)	300-600 g	21
Grey mullet (*Mugil cephalus)*	1	Feb 2015	10	Wild fish. Bay of Cadiz, Spain. Earthen ponds	Natural feeding	500 g-1 kg	
	2	Dec 2016	6	P18/PISTRESA Cadiz, Spain. Earthen ponds	Natural feeding & experimental	150-300 g	23
	3	Dec 2017	4	PIMSA, Cadiz, Spain. Earthen ponds	Natural feeding & commercial	600-800 g	23
Pikeperch (*Sander lucioperca*)	1	Jul 2014	10	France. Fresh water intensive farm	Commercial extruded feed	1-2 kg	
	2	Jan 2018	4	Farm (P9/ P29 ASIALOR), NW France. Land based ponds, RAS	Commercial extruded feed (Le Gouessant)	300-500 g	22
Meagre (*Argyrosomus regius*)	1	Nov 2014	10	Farm (Andromeda Group), Burriana, Spain. Floating sea cages	Commercial extruded feed	1.5-2 kg	

Table 28.3.2.2 Average somatic indexes and technical yields from greater amberjack (*Seriola dumerili*), grey mullet (*Mugil cephalus*), pikeperch (*Sander lucioperca*) and meagre (*Argyrosomus regius*) produced within Diversify´s Grow out GWP

Greater amberjack			Grey mullet		Pikeperch		Meagre	
Batch	**3 & 4**		**2**		**1**		**1**	
Body weight (g)	452.8 ±104.9	b	200.3 ± 69.9	a	1096.6 ±217.2	c	1834.6 ±140.4	d
CI	2.02 ± 0.19	c	0.96 ± 0.08	a	0.71 ± 0.13	b	0.87 ± 0.05	a
Dressing yield (%)	94.0 ± 0.9	bc	95.1 ± 0.5	c	93.5 ± 1.3	b	91.1 ± 0.6	a
Filleting yield (%)	50.0 ± 5.0	b	46.8 ± 1.2	b	36.2 ± 4.4	a	38.4 ± 2.4	a
HSI	0.90 ± 0.18		0.84 ± 0.17		0.81 ± 0.18		0.87 ± 0.05	
VSI	4.80 ± 0.90	a	4.12 ± 0.51	a	5.73 ± 1.30	ab	5.75 ± 0.44	b

Data are means ± SD (n=38 for greater amberjack; n=6 for grey mullet; n=10 for pikeperch and meagre). CI: condition index; HSI: hepatosomatic index; VSI: viscerosomatic index.

Table 28.3.2.3 Comparison between proximal composition (% fresh weight), main fatty acid content (mg 100 g fillet^{-1}) and health lipid indexes of fillets from greater amberjack (*Seriola dumerili*), grey mullet (*Mugil cephalus*), pikeperch (*Sander lucioperca*) and meagre (*Argyrosomus regius*).

Greater amberjack			Grey mullet		Pikeperch		Meagre	
Batch	**3 & 4**		**2**		**2**		**1**	
Moisture	75.62 ± 1.40	a	77.98 ± 0.70	b	79.00 ± 0.75	b	77.18 ± 0.29	ab
Fat	3.07 ± 1.02	b	0.86 ± 0.17	a	0.93 ± 0.23	a	0.53 ± 0.36	a
Protein	20.75 ± 1.06	b	19.33 ± 0.80	ab	18.80 ± 1.01	a	20.66 ± 0.20	b
Ash	1.56 ± 0.22	b	1.21 ± 0.08	a	1.21 ± 0.19	a	1.35 ± 0.01	a
Total SFA	545.4 ±114.3	b	180.8 ± 43.4	a	165.3 ± 38.7	a	137.9 ± 11.3	a
Total MUFA	978.5 ±282.4	c	79.9 ± 23.4	a	156.0 ± 65.3	ab	145.9 ± 8.3	b
18:2n-6	**337.0 ± 76.9**	**d**	**10.8 ± 3.2**	**a**	**44.2 ± 19.9**	**b**	**105.2 ± 5.0**	**c**
20:4n-6	16.3 ± 1.8	b	21.7 ± 3.5	b	8.7 ± 1.4	a	7.0 ± 1.5	a
Total n-6 PUFA	372.8 ± 78.8	c	45.0 ± 8.6	a	56.4 ± 21.7	a	135.5 ± 15.9	b
18:3n-3	79.0 ± 21.8	b	18.4 ± 10.9	a	7.6 ± 3.8	a	6.2 ± 0.1	a
20:5n-3	115.0 ± 24.6	c	46.1 ± 10.3	b	47.2 ± 12.3	b	15.4 ± 1.4	a
22:6n-3	240.7 ± 47.8	c	116.1 ± 21.4	b	134.6 ± 20.4	b	65.5 ± 15.3	a
Total n-3 PUFA	528.3 ±114.2	c	215.9 ± 45.5	b	208.1 ± 40.8	b	92.8 ± 17.0	a
EPA+DHA	**335.7 ± 70.2**	**c**	**162.2 ± 65.9**	**b**	**178.8 ± 43.3**	**b**	**80.9 ± 16.7**	**a**
n-3/n-6	1.45 ± 0.22	b	4.90 ± 1.22	c	4.02 ± 0.99	c	0.68 ± 0.04	a
IA	0.38 ± 0.01	a	0.55 ± 0.05	c	0.47 ± 0.01	c	0.42 ± 0.01	b
IT	0.22 ± 0.01	b	0.21 ± 0.01	ab	0.21 ± 0.01	a	0.30 ± 0.01	c

Data are means ± SD (greater amberjack, n=30; pikeperch, n=8; grey mullet, n=6; meagre, n=3). IA, Index of atherogenicity; IT, Index of thrombogenicity

With certain uncertainties due to the limited number of specimens from some of the selected species, or limited number of different rearing histories, some interesting aspects have been confirmed and some new suggestions can be also drawn upon the results presented here.

The four fish species were studied for their fillet composition, technical yields and fillet sensory properties. The main results obtained were:

1) The dressing yields for all species are quite similar, slightly exceeding 90%.
2) Greater amberjack showed high fillet fat reaching 3-4% in 0.5-1.5 kg fish, whereas all other fish exhibited very low fillet fat, not exceeding 1%.
3) Filleting yields and protein contents did not seem to be influenced significantly by fish size or rearing and dietary histories at grow out stage. All species showed similar and typical fillet protein content close to 20%.
4) Greater amberjack displayed the highest filleting yields and final contents of protein, fat and especially EPA+DHA. However, further grow out trials are advisable in order to establish the best extruded diet to balance the final levels of saturated and 18:2n-6 fatty acids. Due to its vulnerability to fat oxidation, it is suggested that commercial sizes should be 1-2 kg with a relatively lower fillet fat content than bigger fish.
5) Meagre filleting yield and protein content were quite attractive. Its total fat contents did not seem to be highly influenced by the dietary or growing history, displaying low contents of fat even in the

wild, an attractive feature for low fat dietary regimes. However, the degree substitution of dietary marine origin ingredients by terrestrial vegetables components should be taken with more caution and highly controlled in this species, which displayed high contents of 18:2n-6 and lower comparable amounts of EPA+DHA.

6) Non exclusively marine fish species, *i.e.* the grey mullet and the pikeperch, display good filleting yields as well as nutritious high protein and healthy low-fat contents.
7) Grey mullet is confirmed as the best candidate for marine ingredients substitution either by terrestrial or marine origin vegetable sources. Independently of the rearing history, their fillets display a comparable high amount of EPA+DHA, low contents of 18:2n-6 and a good balance of saturated fatty acids, all of them nutritionally attractive and health promoting qualities.
8) Pikeperch fillet was also attractive in terms of protein and fatty acid profiles. The carnivorous nature of the species might not allow such a high degree of dietary inclusion of vegetable components, but still its high dynamic to retain DHA in the flesh and its low fat content makes it a highly attractive and health-promoting fish.
9) In the case of greater amberjack, the different tested culture densities seem to have a negligible effect on the sensory quality of the final product.
10) The diet had an important effect on the sensory characteristics of the grey mullet, especially in aspects related to the fillet fat content and its oxidative stability.
11) Pikeperch had neutral sensory characteristics, which makes it an ideal fish for those people looking for mild flavours.
12) In the case of the pikeperch it is important to control the ante-mortem treatment in order to guarantee the absence of sensory defects (earthy flavour).

This task has been completed during this reporting period and the full description of the work and results have been provided in Deliverable 28.7 ***"Report on correlation of technical quality with nutritional - rearing history".***

Sub-task 28.3.3 (led by IRTA, Lluis Guerrero)

The objective of this task was to provide a technical assessment on basis of the former sub-tasks of WP28 for all selected species (meagre, greater amberjack, pikeperch., Atlantic halibut wreckfish and grey mullet). This technical assessment is essential for the product development and especially in cases of advanced processing to have a full image of the principles applying, the criteria for ensuring quality and safety of the product.

Methodologies

This technical assessment covered:

• a general description of the products

• the list of essential characteristics relevant for high quality consumption and intended use of the products;
• the methods and criteria for assessing the performance of the product in relation to those essential characteristics
• principles and conditions for the production, control and packaging of the new products to guarantee quality of consumption

Results

The products created from the species included three different processing levels (minimum, medium, high). The technical yields appear in Table 28.3.3.1

Table 28.3.3.1 Products technical yield. Indicative fish commercial size and indicative average filleting yield are presented for each species. Based on the fish fillet quantity required for each product, the number of products per fish is calculated.

	fish commercial size (g)	filleting yield (%)	fish quantity per product (g)	nr of products per fish
Greater amberjack	10000	60		
Idea 13: Frozen fish filet that is seasoned or marinated			400	15
Idea 30: Ready-made fish tartar with additional soy sauce			100	60
Idea 34: Fresh fish steak for grilling in the pan	2000g (smaller fish)	50	450-500	2
Grey mullet	500	35		
Idea 2: Thin smoked fillets			60-100	2-3
Idea 21: Fresh fish fillet with different 'healthy' seasoning and marinades			200	approx. 1
Idea 33: Ready-made fish fillets in olive oil			220	approx. 1
	fish commercial size (g)	filleting yield (%)	fish quantity per product (g)	nr of products per fish
Meagre	2000	40		
Idea 1: Frozen fish fillets with different recipes			1200 (3 fillets) or 800 (2 fillets)	2/3 or 1
Idea 4: Ready to eat meal: salad with fish			45	17

Idea 6: Fish burgers shaped as fish		scenario 1 (from fish fillet)	85	10
		scenario 2 (from filleting discards 2%)		1/2
Pikeperch	2000	40		
Idea 9: Fish spreads / pate			100	8
Idea 21: Fresh fish fillet with different 'healthy' seasoning and marinades			150	5
Idea 30: Ready-made fish tartar with additional soy sauce			100	8

The essential characteristics of the products are related to their nature and processing degree (Table 28.3.3.2). The shelf life assessment has taken place including both microbiological and sensory criteria. High-quality shelf life can be termed as the duration of storage for which product retains mostly its freshness characteristics, is definitely shorter than the total shelf life.

Table 28.3.3.2 Products' nature, high-quality shelf life in days (d), months (mo) or years (y), safety issues and essential characteristics for retaining high quality during storage and commercialization (HPP: hydrostatic pressure processing, MAP: modified atmosphere packaging, VP: vacuum packed, VSP: vacuum skin packaging)

	product nature	high quality shelf life	safety measures*	essential characteristics
Greater amberjack				
Idea 13: Frozen fish filet that is seasoned or marinated	marinated, frozen, VP	6 mo		deep freeze cold chain retaining at -20 °C ± 2 throughout storage
Idea 30: Ready-made fish tartar with additional soy sauce	fresh, VP (or preferably VSP)	approx. 3d	pH<5, allergen labeling (soya sauce, sesame seeds, mustard and sherry vinegar (may contain sulphites)	retaining cold chain throughout commercialization (<3 °C), retaining packaging
Idea 34: Fresh fish steak for grilling in the pan	fresh, VP	6d	hygiene during filleting	retaining cold chain throughout commercialization (<3 °C)
Grey mullet				
Idea 2: Thin smoked fillets	thermally processed, dried, VP or MAP, cold storage	21d		retaining cold chain throughout commercialization (<3 °C)
Idea 21: Fresh fish fillet with different 'healthy' seasoning and marinades	fresh, MAP	7d	allergen labeling (depending on sauce)	retaining cold chain throughout commercialization (<3 °C), retaining MAP
Idea 33: Ready-made fish fillets in olive oil	oil-preserved, sterilized, sealed in airtight glass container	<1y	sterilization after filling into vessel/glass container	avoid oxidation (retain packaging)

	product nature	high quality shelf life	safety measures*	essential characteristics
Meagre				
Idea 1: Frozen fish fillets with different recipes	frozen, VP	9mo	hygiene during filleting and freezing	deep freeze cold chain retaining at -20 °C ± 2 throughout storage, properly sealed vacuum package (avoid oxidation)
Idea 4: Ready to eat meal: salad with fish	vinegar-marinated fish, fresh, MAP	5d (vegetable salad is the limiting factor)	24 h freeze fish before processing, proper acidi-fication of fish, aller-gen labeling (gluten, mustard)	retaining cold chain throughout commercialization (<3 °C), retaining MAP
Idea 6: Fish burgers shaped as fish	thermally processed, frozen	6mo	allergen labeling (milk protein)	deep freeze cold chain retaining at -20 °C ± 2 throughout storage, properly sealed vacuum package (avoid oxidation)
Pikeperch				
Idea 9: Fish spreads / pate	thermally processed (pasteurized or sterilized) tube-sealed	30d in sealed tube (3d after opening)	proper heat treatment (pas-teurizing and aseptic tube filling), aller-gen labeling (milk protein)	retaining cold chain throughout commercialization (<3 °C), retaining integrity of tube
Idea 21: Fresh fish fillet with different 'healthy' seasoning and marinades	fresh, MAP	6d	allergen labeling (depending on sauce)	retaining cold chain throughout commercialization (<3 °C), retaining MAP
Idea 30: Ready-made fish tartar with additional soy sauce	fresh, pressurized (HPP), VSP	approx 6d for the non-pressurize d and ≤15 d for press.	pH<5, allergen labelling (soya sauce, sesame seeds, mustard and sherry vinegar	retaining cold chain throughout commercialization (<3 °C), retaining packaging

*safety measures for all products irrespectively include the application of HACCP principles.

The principles for proper production include three aspects: 1. raw materials of good quality. 2. good manufacturing (processing) practices. 3. proper traceability. These three aspects were analyzed in detail and explained in ***Deliverable D28.8 Technical assessment of selected species***.

Deviations from Annex I and their impact:

In the DOW, it was mistakenly (by copy paste) written the description of Deliverable D28.5 as being exactly the same as D28.6. The correct description of is as following: "Report on results of quality evaluation study on basic quality characteristics of the developed products. The report will refer: to the total proximate composition of the products (protein, lipid moisture, inorganic content and carbohydrates), the energy contents of the selected products the quantitative nutritional value in aspects of fatty acids".

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Alexi, N., Byrne, D.V., Nanou, E., Grigorakis, K., 2018a. Investigation of sensory profiles and hedonic drivers of emerging aquaculture fish species. Journal of the Science of Food and Agriculture 98, 10.1002/jsfa.8571.

Alexi, N., Nanou, E., Lazo, O., Guerrero, L., Grigorakis, K., Byrne, D.V., 2018b. Check-All-That-Apply (CATA) with semi-trained assessors: Sensory profiles closer to descriptive analysis or consumer elicited data? Food Quality and Preference 64, 11-20.

Grigorakis, K., 2017. Fillet proximate composition, lipid quality, yields, and organoleptic quality of Mediterranean-farmed marine fish: A review with emphasis on new species. Crit Rev Food Sci Nutr 57, 2956-2969.

Lazo, O., Claret, A. and Guerrero, L. (2016). A comparison of two methods for generating descriptive attributes with trained assessors: Check-All-That-Apply (CATA) vs. free choice profiling (FCP). J. Sensory Studies, 31, (2), 163-176.

Lazo, O., Guerrero, L., Alexi, N., Grigorakis, K., Claret, A., Pérez, J. and Bou, R. 2017. Sensory characterization, physico-chemical properties and somatic yields of five emerging fish species. Food Research International, 100, 396-406.

Lazo, O., Claret, A., Bou, R., Robles, R and Guerrero L. Consumer's performance compared to trained assessors when using Check All That Apply (CATA) over clear different samples having different degree of sensory complexity (In preparation).

WP 29 Socioeconomics – Consumer value perceptions and behavioral 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):	Marija Banovic (P11) and Athanasios Krystallis (P38)			
Other Scientists participating:	Machiel Reinders (P6), GemmaTacken (P6), Luis Guerrero, (P3), Kriton Grigorakis (P1), Rocio Robles (P18), Hellas-Maria Saltavarea (P38)			

Objectives

1. To analyze 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 behavior 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 (13-30 Mo):

The 2nd Periodic Report covered Task 29.2 (Consumer sensory perceptions) and the first part of Task 29.3 (Optimization of intrinsic-extrinsic attribute combinations), namely its Sub-task 29.3.1.

The objective of task 29.2 was to develop the actual product samples from the selected fish species for the sensory testing with consumers in the five countries investigated (i.e. France, Germany, Italy, Spain and the UK) (Deliverable 29.3). In this task, the different physical product prototypes developed and tested in Tasks 28.1 - 28.3 related to new product development and the monitoring of technical quality of the products were manufactured according to the amount needed and following strict hygienic conditions.

These product samples were the basis for the acceptability test done in task 29.2 (resulting in Deliverable 29.4). This task provided all the information needed to handle, store and prepare the different samples, the statistical design followed in each location (order of presentation, sample distribution among participants, etc.) as well as some practical recommendations that were necessary to carry out the test and recruit the participants properly. More specifically, participants were recruited in each of the five selected countries (France, Germany, Italy, Spain and UK) based on the consumer segments identified in Task 29.1 (see Deliverable D29.2 report on the segmentation analysis for more information). Further, all the sensory tests were performed under controlled conditions in a central location per country. All the product samples were shipped in advance to each location in the right conditions and guaranteeing the cold chain. Samples were sent with detailed instructions about the right procedure to store them until analysis. Finally, ten tasting sessions were held in each location in two consecutive days. In each tasting session, consumer assessed overall expectations with the different physical product prototypes developed and tested in Tasks 28.1 - 28.3, followed by blind tasting and overall expectation in informed condition (i.e. upon provision of pictures with full description of the product from deliverable 28.2).

In terms of results, products with a higher degree of processing were those who generated lower expected acceptance, although all of them were perceived positively. The most important parameter affecting liking expectations was the expected taste of the product. Health, nutritional and well-being related issues were relevant as well in order to increase individuals' expectations, but to a lower extent. These findings seem to indicate that, in general, consumers are unwilling to sacrifice taste by an improvement in health or functional properties. In a general sense, the perception of these products was similar across countries. Once products were blind tasted, the acceptability results obtained confirmed those previously reported regarding consumers' expectations, and also seems to indicate a tendency to prefer the low processed fish products. Even though the different products were perceived similarly in the different locations regarding acceptability ratings, they were described in a clearly different way when dealing with the main intangible dimensions that might define them (taste, convenience, environmental impact, etc.).

The objective of sub-task 29.3.1 was to incorporate a number of extrinsic quality attributes (i.e. product labelling elements) into the physical product prototypes developed in WP 28 (see Deliverable 28.2 and Deliverable 28.4) and based on the results from Task 29.2 (and Deliverable 29.4). The main goal was to

develop experimental product mock-ups with optimal intrinsic-extrinsic attribute combinations for use in the experimentation with consumers.

Based on a review of secondary data and a detailed literature review of studies dealing with consumer behaviour towards fish product following similar methodologies (i.e. experimentation with product mock ups in simulated choice tasks), sub-task 29.3.1. ended up with the selection of the most appropriate extrinsic quality attributes to be incorporated onto the label of the experimental product mock-ups for further testing in sub-task 29.3.2. The extrinsic attributes selected were: a) product's country of origin (i.e. EU or domestic), b) a quality guarantee of ethical nature (i.e. ASC logo), c) health claims (i.e. improves cardiovascular function and improves brain function), d) nutrition claims (i.e. rich in Omega 3 and high in proteins), and finally e) three price levels (*i.e.* average, +10% premium and +15 premium).

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

The WP29 objectives relevant for the 3rd Periodic Report are Objectives 3 and 4, namely:

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

In terms of significant results in relation to Objective 3, the most relevant attributes for all three investigated products were 'Country of Origin (COO)' and 'Price', followed by 'Existence of an ASC logo', 'Existence of a nutrition claim' and 'Existence of a health claim'. Consumer preferred the product lower the higher prices were. Higher price sensitivity across the investigated countries has been observed for the case of fish fillets in olive oil compared to the other two products. Results further suggested an increasing probability of choosing a fish product that has been 'produced in own (domestic) country'. Furthermore, fish product alternatives possessing an 'ASC logo' also increased the probability of choice. Nevertheless, consumer preferences for nutrition and health claims varied across products and countries.

Finally, in with respect to Objective 4, the effect of communication on attitude towards the product was significantly higher when the goal message was associated with the lower level of product processing across all three goal messages (i.e. about products' healthiness, tastiness, and traceability). This was evident in the case of low processed product - fresh fish steak and the health goal message. Besides health, the traceability goal message worked well across all three products (i.e. primes). The results further showed that the highest effect on product's purchase probability had the positively and negatively evoked emotions. This finding was evident especially for the experimental conditions with the traceability and taste goal messages primed with medium (i.e. smoked fillet) - and high (i.e. fish burger) - processed products.

Summary of progress towards objectives (49-60 Mo):

No work was undertaken in this WP, as all was completed during previous reporting periods

Details for each Task

Task 29.1 Consumer value perceptions and segmentation (led by AU, Athanasios Krystallis).

Sub-task 29.1.1 (lead by DLO, Gemma Tacken, prepared by Machiel Reinders)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.1 Dataset of consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the selected species in the five countries investigated***.

Sub-task 29.1.2 (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU))

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.2 Report on the segmentation analysis based on consumer value perceptions about the selected species in the five countries investigated (value-based segmentation task)***.

Task 29.2 Consumer sensory perceptions (led by IRTA, Lluis Guerrero).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.3 Development of the actual product samples from the selected species for the sensory testing with consumers in the five countries investigated*** *and* ***Deliverable 29.4 Report on the actual product's sensory profiling in the five countries.***

Task 29.3 Optimization of intrinsic-extrinsic attribute combinations (led by AU, Athanasios Krystallis).

Sub-task 29.3.1 (led by AU, Athanasios Krystallis, prepared by Marija Banovic)

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.5 Development of the product mock-ups for use in the experimentation with consumers in the five countries investigated***

Sub-task 29.3.2 (led by AU, Athanasios Krystallis, prepared by Marija Banovic (AU))

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.6 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.***

Task 29.4 Communication effectiveness in behavioural change (led by AU, Athanasios Krystallis).

This task has been completed during the previous reporting periods and the full description of the work and results have been provided in ***Deliverable 29.7 Development of the stimulus (i.e. written and broadcasted information material) that will be used in the communication experiments in the five countries investigated*** and ***Deliverable 29.8 Report on the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers attitudes and behaviour towards the products coming from the selected fish species.***

Deviations from Annex I and their impact:

There were no deviations from the calendar of activities established.

Manuscripts that resulted from this Task (if not published, indicate Submitted, Accepted or In Preparation**)**

Banović, M., Krystallis, A., Guerrero, L., Reinders, M.J., 2016. Consumers as co-creators of new product ideas: An application of projective and creative research techniques. Food Research International 87, 211-223.

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.

Lazo, O., Guerrero, L., Alexi, N., Grigorakis, K., Claret, A., Perez, J.A., Bou, R., 2017. Sensory characterization, physico-chemical properties and somatic yields of five emerging fish species. Food Res Int 100, 396-406.

Banovic, M. Reinders, M.J. Claret, A. Guerrero, L. and Krystallis, A. ""One Fish, Two Fish, Red Fish, Blue Fish" or How Ethical Beliefs Impact "Blue" Products Purchase Intention?", J. Bus. Eth. [SUBMITTED]

Banovic, M. Reinders, M.J. Claret, A. Guerrero, L. and Krystallis, A. "Take it or leave it: Impact of eco-label, health and nutrition claims, and country-of-origin on consumer choice of aquaculture products", Food Policy [SUBMITTED]

WP 30 Socioeconomics – Business model and marketing strategy development

WP No:	30	**WP Lead beneficiary:**		P10. TU/e
WP Title (from DOW):	Socioeconomics – Business model and marketing strategy development			
Other beneficiaries (from DOW):	P3. IRTA	P6. DLO	P11. AU	P12. APROMAR
P18. CTAQUA	P23. ARGO	P25. DOR	P28. CANEXMAR	P39. F2B
Lead Scientist preparing the Report (WP leader):	Edwin Nijssen			
Other Scientists participating:	Michel van der Borgh (P10), Lluis Guerrero (P3), Gemma Tacken, Machiel Reinders and Mariët van Haaster – de Winter (P6), Athanasios Krystallis (P11), Javier Ojeda (P12), Rocio Robles (P18)			

Objectives

1. To identify business models for sustainable profitability and improved competitiveness of the sector for all the DIVERSIFY species,
2. To devise marketing strategies for the newly developed products from the DIVERSIFY species, aiming to develop a market that is as large and profitable as possible,
3. To come up with policy/strategy recommendations for further development and market expansion.

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

In the DOW, this WP is not planned to start until project month 43. However some activities were already initiated, because the work for this work package is highly dependent on work done in other work packages (*e.g.*, WP 27, WP 28 and WP 29). A PhD candidate was selected (Maren Vos) who will execute a large part of the work for WP30. Next to that, we consulted with P6. DLO in order to make sure that work executed in Sub-task 27.2.3 and Sub-task 27.3.2 is aligned with work to be executed in WP 30.

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

In WP 30 no activities were performed in this reporting period.

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

The results of Task 30.1 show that for several of the species business models are still difficult (greater amberjack and grey mullet) or even problematic (wreckfish and Atlantic halibut). Production problems make process outcomes uncertain and a constant supply difficult. Selling to large retail chains thus will be hard because it requires a controlled and continuous stream of products. Therefore, for the suppliers of experimental species, selling to smaller retailers/parties and local restaurants makes more sense. It generates cash flow, but without the risk of not living up to expectations of being a reliable partner who

creates and delivers high quality products on promise. For the producers of the new species collaborating with innovative channel partners who are willing to co-create and co-invest is their best bet (compare Coviello and Joseph 2012).

The most promising business opportunities and thus models identified concern pikeperch and meagre. For these species, most bottlenecks in production have been subsidised. The challenge now is to grow customer demand and market acceptance. The newly developed products can help give an impulse to these efforts. The products developed for meagre included (i) a fish-burger aimed at children and (ii) a fish salad for consumers who like convenience. By targeting the segment of involved innovative customers (Deliverable 29.2) and in particular those consumers interested in convenience, progress can be made. Unfortunately, while these two species are most production ready they and their products had not been selected for additional consumer research to establish the best value specification and communication message (Deliverable 29.6). Still, suggestions were made towards building of business models for these species and the products developed for them.

Overall, our business model development showed a coherent business story for all four focal species, which is the first litmus test for any business model (Margretta 2002). Although farmers will benefit from continuing to work with their business partners to enhance their production processes and increase product quality/growth and decrease production cost, it is clear that serious investments in marketing and sales/channel management, i.e. market development are important and needed. Only with a buy in from distribution partners and adequate marketing efforts can consumers be reached and convinced to adopt and continue purchasing these new products. It benefits from using country/region of origin branding and health claims (e.g. Omega3), among others. Building a reputation or brand can help create differentiation necessary to prevent or resist price erosion when production begins to increase significantly.

The results of task 30.2 show that most firms are indeed focused on R&D for the species and thus have a partner or alliance portfolio consisting of equipment providers, hatcheries, feed manufacturers, and research institutes. On the one hand, this would appear logical because of the experimental stage of development of most species. However, on the other hand, farmers' (particularly meagre and greater amberjack) limited involvement in marketing and key customer alliances is troublesome. It suggests that the farmers are not very actively cultivating these relationships. Consequently, they may fail to achieve an early buy in, co-development, and other possible roles that customers can play in this process (Coviello and Joseph 2012). Although farmers do recognize the need for creating more market awareness of customers for the new species limited marketing investments and attention could result in involving downstream partners too little and too late.

Firms particularly need to pay more attention to their marketing efforts and relationship building with channel partners in order to succeed. Part of these efforts and channel partner involvement should be the creation of or compliance with a quality/sustainability certificate. Lack of such a certificate has been shown to prevent firms from gaining access to the retail sector and thus the consumer market.

Summary of progress towards objectives (49-60 Mo):

The results of Task 30.2 presented the development of the marketing strategies of the new species in the five target countries. The aim was to draw on previous findings of particularly deliverables D30.1, D29.8 and D29. The unique aspect of this research was studying acceptance of the new species in competitive setting. The focus was on the most prototypical species of the project, i.e. greater amberjack. The design included three parts: (1) an experiment to test the importance of communicating the new, unfamiliar species' nearest neighbour species as referent, (2) an experiment testing the new species in an online

supermarket context, and finally (3) a model for simulating product acceptance using a different launch strategies together with production capacity effects. Based on this research design an online supermarket store was build and data collected by HRH from Greece. Data were collected and results reported as part of task 30.2.2. The systems dynamics model focusing on narrow and full launch and different degrees of advertising investments was developed by TU/e.

The results of task 30.2.2 i.e. of the preliminary categorization experiment confirm the importance of offering category information for unfamiliar food products, i.e. in our case for greater amberjack fillets. It supports results from prior food research. Extra information about structure and taste of greater amberjack is less or not important. Communicating new and offering category information is very useful and thus recommended. Particularly if the information comes from a credible source, e.g., fish monger, it will be trusted and thus accepted. It will also help overcome perceived risk of not having tasted the fish before buying.

The results of the online test showed that the new product was well accepted, i.e. performed rather well in this supermarket context. The new species' fillets were approximately 12.5% of consumers' first choice and another 10-12.5%'s second choice (willingness to switch when offered the opportunity). However differences in acceptance levels between countries could be noted. Southern countries were more inclined to try and buy the new species than Northerners. Particularly Italian consumers seemed open and interested in adopting the new greater amberjack fillets. It could be targeted first, i.e. used as lead country. These observed differences noted between national markets match those reported in Deliverable 29.4 regarding consumers' variations in their initiative to try new fish species.

Finally, the systems dynamics modelling effort drew attention to other aspects of the market launch. A general model of new product launch was adapted for the marketing of fresh fish. Several extra strategic launch decisions, such as channel and advertising strategies, specifically narrow versus full-fledged or fat launch, push versus pull advertising and limited versus large marketing budget were explored. The results suggested that channel strategy is the most important variable driving adoption and market diffusion of the new product. A full launch reaches the whole market. It benefits from heavy advertising. This investment in advertising is also necessary to compensate for the lower effectiveness of the type of outlet (i.e., retailers). A narrow launch, i.e. niche approach works well too. However, this does not benefit from high advertising expenditures. High advertising expenditure in combination with narrow launch may even work counterproductively; demand surpasses (the initially lower) supply and may deplete stocks causing product shortage. If mongers and consumers respond negatively to such out of stock situation it could seriously hurt the new species' market development. Providers thus should be aware of this potential problem.

Details for each Task

Task 30.1 Business models (led by TU/e, Edwin Nijssen)

Sub-task 30.1.1 Value proposition

The objective of this deliverable was to develop business models for the SMEs participating in DIVERSIFY for the focal species and their products. The full description of the work and results is provided in ***Deliverable 30.1+30.2 titled Report on value propositions for the producers and Partners***

Sub-task 30.1.2 Resources (TU/e)

This task objected to define the resources necessary to create value for the customer. The full description of the work and results is provided in ***Deliverable 30.1 and 30.2 Report on value propositions for the producers and partners.***

Subtask 30.1.3 (TU/e)

In this task cost structures and possibilities to further drive down costs will be analysed together with the SME Partners. The full description of the work is provided in ***Deliverable 30.4 Revenue (pricing & costs structures) model per species.***

Deliverables 30.1 to ***30.4*** resulted in business models for the selected species, and more in detail for the ones for which new products are developed. Similarly, current relationships in the supply chain and opportunities to cultivate buyer-supplier relationships to develop the business will be identified and reported. Also bottlenecks will be studied and identified and potential solutions suggested. The full description of the work is provided in ***Deliverable 30.3 Guidelines to cultivate buyer-supplier relationships per species***

Task 30.2 New strategy development (led by TU/e, E. Nijssen)

Sub-task 30.2.1 (TU/e)

The objective of this report was to develop new product marketing strategies per species and product. The study and its report discusses the plan for developing and detailing marketing strategies for the new species and their products of the DIVERSIFY project. In the absence of actual products of the farmed species, the real-life market test was substituted by a virtual one. Focusing on the most prototypical species, i.e. greater amberjack, and the product of fillets, two experiments were designed.

Three parts were planned.

Part 1: In a first experiment the importance of offering consumers extra information about a new fish species was studied. While some consumers might be triggered by the label 'new', others may require more information in order to be convincing to try a new alternative on the market. Drawing on prior research on adoption of new products and extension of product categories we explored the need for customers for a referent product to accept a new unfamiliar species (*i.e.* information about its closest neighbour or referent product). We chose the setting of the fish monger store and the fish monger offering advice and information about the species. The data for the experiment were collected using a professional market research agency focusing on UK consumers. The focus was on greater amberjack as most representative species using tuna as closest neighbour.

Part 2: The second experiment that was designed involved an online store of a virtual national retailer. In this second experiment the effects of price discount; traceability label and communication of referent product were studied simultaneously. The experiment was performed in all five target countries, *i.e.* UK, France, Italy, Spain and Germany. By focusing on online context, we begin to explore the potential of using the innovative new channel. It complements the setting of the first experiment that focused on fish mongers.

Customers got the task to select a fish for a meal that they had to prepare for their family. In the experiment their first saw the online store landing page (fish counter) and then 5 fillets to choose from.

Part 3: Drawing on previous results a simulation model was used to explore the effect of different market entry strategies. The model explored effects of narrow versus full-fledged channel strategy and use of different levels of advertising budget to gain market acceptance. In the stimulation of diffusion of the species/product in the market place required production volumes were accounted for too.

The aim of the simulations was showing the effectiveness of the market strategy while accounting for the interplay of variables and mechanisms launching in each country, i.e. market. The full results are published in the ***Deliverable 30.5 Project plan for market test and development of marketing strategy.***

Sub-task 30.2.2 Testing of the proposed market strategy (led by TU/e, E. Nijssen)

The objective of this Deliverable is to report results of experiments and simulations regarding the effectiveness of marketing strategies devised for the newly developed products from the DIVERSIFY species, aiming to develop a market that is as large and profitable as possible.

While prior research studied consumer reactions to the new species without offering consumers alternatives to choose from, the current study focused on *market launch* in a retailer setting. Exposed to multiple alternatives we wanted to see the percentage of consumers that selected the new alternative in the market.

Consistent with the set up/design of ***Deliverable 30.5*** consumer data was collected. The results pertain to greater amberjack as species because this species was considered most prototypical for the project. Fillets were selected as its products.

Part 1: preliminary categorization experiment. The scenarios we developed used the consumer's local fishmongers as informant about the new species to the customers. Fishmongers are experts and therefore a credible source of information. Respondents were given **a task** to buy fish fillets for a meal for their family at the monger. The monger suggested three fillets for their dish: cod, tuna, and greater amberjack. The monger continued to (i) (just) promote new greater amberjack, or (ii and iii) promote it as new but with different levels of extra information about its nearest neighbor (its category). The scenarios were supported with pictures of each species and their fillets on ice as to mimic their display in the monger's fish counter. In between the monger's comments and before respondents gave their evaluation of the fish fillets, we asked respondents about their thoughts regarding the monger's advice (open question), and to list their purchase criteria for this decision in this setting.

Data from a sample of 445 UK consumers was collected. Only consumers that consumed fish products at least once a month and were able to cook, i.e. to prepare a meal at home, were selected to participate.

The results showed that our model explained a reasonable level of variance (adj. R^2) in the dependent variables, varying from 14.9% of attitude towards monger's advice to 20.4% of variance in attitude towards greater amberjack and 39.0% of willingness to buy greater amberjack is explained. More importantly, we found a significant effect of our different experimental conditions (level of information of the new species, i.e. new, category and information about taste/structure) on attitude towards advice of the fish monger and willingness to buy the new fish. The impact of the experimental condition i.e. manipulation on attitude towards our new species was only marginally significant (p=.092).

The detailed results confirmed that information about category provided by the monger had positive effects on attitude and acceptance of the new species. However, extra info beyond category e.g., on taste let to reduced evaluations. Both results are in line with prior findings by Tuorila et al (1998).

Part 2. Online experiment. In response to online grocery sales as a new, emerging channel of the future the market test was performed in an online setting. Customers were provided with the **scenario** to shop online for a meal for their family, and had to decide to use fish as its main ingredient. Manipulations included in the experiment were: price discount, traceability label, and environmental cue/claim.

The objective of the experiment was to uncover which innovation paths and marketing cues are best used in the launch process in an attempt to optimize the final launch strategy for the new species and its product.

The unique aspect of the experiment was the *competitive retail setting*. The experiment preferred validity over control. Finally, customers who did not choose the new species were offered extra information about the product after which they were asked whether they wanted to reconsider their choice and switch or at least try the new species.

Building on results of ***Deliverable 29.6*** (see p.25 Table 9) we used the Diversify brand sticker, and a health claim (Omega3) on the package of our new fish product. Further we used the EU as country of origin. The use of the Diversify brand helped with comparability of settings and thus findings across the experiments. The health claim had been found to have a positive effect on attitude in all target countries, and thus most likely also on adoption. In accordance with the findings from the pre-test (part 1 –see above) we communicated the closest neighbour of the new fish species to facilitate the new fish's acceptance/trial.

Results showed that respondents had a positive attitude towards the website (see **Figure 30.2.1** for screenshot) that had been developed and was presented to them. In all countries the respondents evaluated the website in the 4.5 – 4.9 range (on a seven points scale). It suggests a generally positive and realistic setting (5=agree).

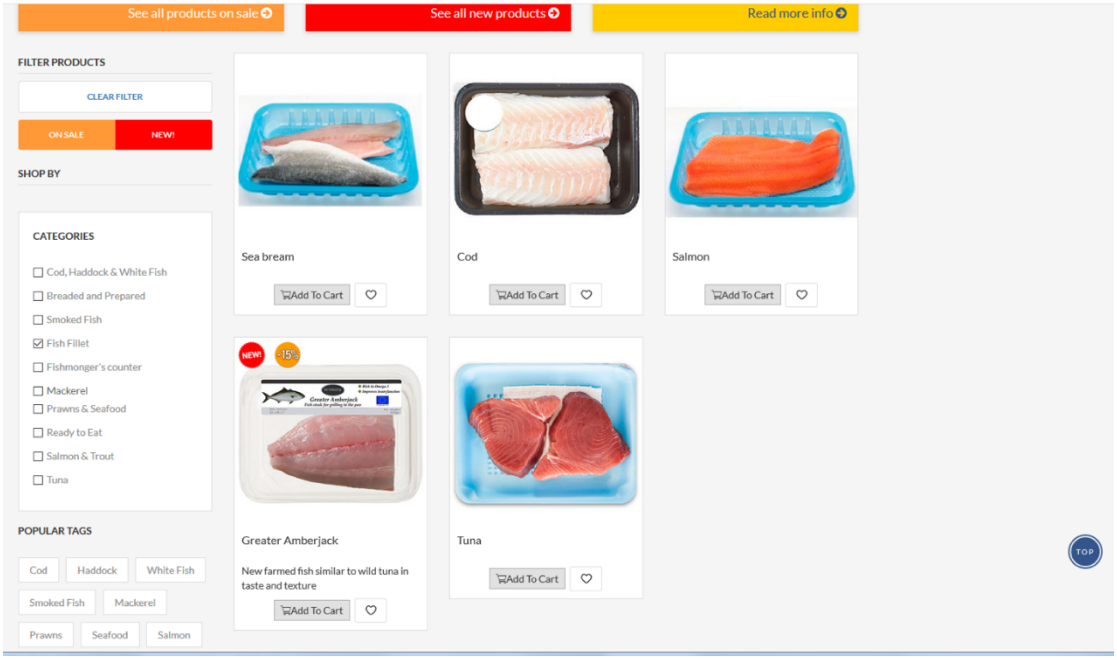

Figure 30.2.1 Screen shot of page 2 of the online- shop

Table 1.1 shows the percentage of consumers that purchased the new fish in the virtual market test experiment. The percentage of first time buyers is rather similar across all five countries; approximately 12.5% chose to purchase greater amberjack in each country, except for France where percentage of consumers that bought greater amberjack was slightly (10.0%) and Italy where it is significantly higher (16.2%)!

Those that did not select greater amberjack in the virtual market test received additional information and then were asked if they wanted to reconsider their decision. A healthy number of consumers switched

towards the new species; overall an 11.0% switched (see **Table 30.2.1**, last column second line). The highest levels of switching occurred in France and Spain, i.e. 14.7 and 13.8%, respectively.

Combining both those that directly purchased greater amberjack and those that bought it after receiving extra information (something that might also happen when offered an in store trail) we note a total of 23.5% overall 'adopters'. Further exploring this percentage that directly and indirectly bought greater amberjack fillets (see bottom of Table 1.1 for sum of options 1+2) we note a *split between Southern versus Northern countries*: The former have significant higher levels of joint acceptance than the latter, i.e. $\pm$ *25% versus 20%.*

Table 30.2.1. Acceptance of greater amberjack by consumers in the 5 countries.

Acceptance of greater amberjack	Country					
	UK	Germany	France	Italy	Spain	Total
1.Great amberjack chosen	38	39	32	52	40	201
	12.0%	12.0%	10.0%	16.2%	12.5%	12.5%
2.Not selected. but willing to switch	27	30	47	28	44	176
	8.5%	9.2%	14.7%	8.7%	13.8%	11.0%
3.Not selected, but willing to consider/try	130	142	98	160	158	688
	41.0%	43.7%	30.6%	49.8%	49.4%	42.9%
4.Not selected, and indifferent to extra info	122	114	143	81	78	538
	38.5%	35.1%	44.7%	25.2%	24.4%	33.6%
Aggregate percentage chose & switched to greater amberjack, i.e. 1+2	*(20.5%)*	*(21.2%)* ↔	*(24.7%)*	*(24.9%)*	*(26.3%)*	*(23.5%)*
Total	317	325	320	321	320	1603
	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

$\chi^2(12)= 62.890$. $p < 0.001$. Note in italics dominant switching pattern of segment.

Looking at the detailed results of **Table 30.2.1** we note a dichotomy in the French market. The French are positive towards greater amberjack on the one hand but also have a major share of people uninterested and even rejecting the species (44.3%)! In contrast consumers uninterested in the Spanish and Italian market is rather low, only some 25% making for a maximum acceptance potential of 75% of consumers in these markets.

Based on these results, the best market to first enter for a provider of greater amberjack would be Italy probably followed by Spain. In addition the drivers of acceptance of greater amberjack by respondents of the virtual market test of the 5 target markets (see results Hayes PROCESS analyses of Deliverable 30.6).

The original model that was estimated focused on the impact of consumer innovativeness (and a set of covariates) on (i) consumer attitude towards greater amberjack, and (ii) consumer acceptance of greater amberjack. The objective was to identify drivers across our five markets.

The results showed that consumer innovativeness, category knowledge, and a positive attitude towards tuna were significant antecedent of a positive attitude towards greater amberjack in most countries. This is consistent with the fixed communication cues that were provided in the experiment of 'new' and 'similar to tuna'.

Because environmentalism had a positive effect in some countries this could also be considered as extra unique selling point when designing the final marketing strategy. Traceability and safety played a minor, none significant role in our results. Remarkably, price promotion (proneness) played a negative role rather than positive role, except in Spain. It could be due to less price-concerned innovators being the major driving force behind great amberjack acceptance in our experiment.

Part 3. Simulations of marketing strategies/launch. Finally, the systems dynamics modelling effort using a general model of new product launch helped explore several extra strategic launch decisions such as channel and advertising strategies, specifically narrow versus full-fledged or fat launch, push versus pull advertising and limited versus large marketing budget.

The results suggested that channel strategy is the most important variable driving adoption and market diffusion of the new product. A full (fat) launch reaches the whole market. Consequently, it benefits from heavy advertising. However, this investment in advertising is also necessary to compensate for the lower effectiveness of the type of outlet, i.e., retailers compared to mongers/specialty stores. A narrow launch, i.e. niche approach works well too. This does not require high advertising to make it work. High advertising expenditure in combination with narrow launch even works counterproductive; demand surpasses (the initially lower) supply and can deplete stocks completely. If mongers and consumers respond negatively to such out of stock situation it may hurt the market development for the new species.

Additional inventory gap analyses (see **Figure 30.2.2**) confirmed the supply issue. Results stress the challenge, in particular in the case of narrow launch and high advertising, to ensure a continuous supply. It should help prevent outlets from deciding to leave the channel/sales relationship once the provider is unable to deliver the product on time. It is important for mongers but even more so for supermarkets that demand secure and constant deliveries to keep carrying an item, i.e. keep the species in their assortment. A large period of shortage (supply shortage) could result in consumers and particularly retailers abandoning the product, i.e. dis-adopting the new species.

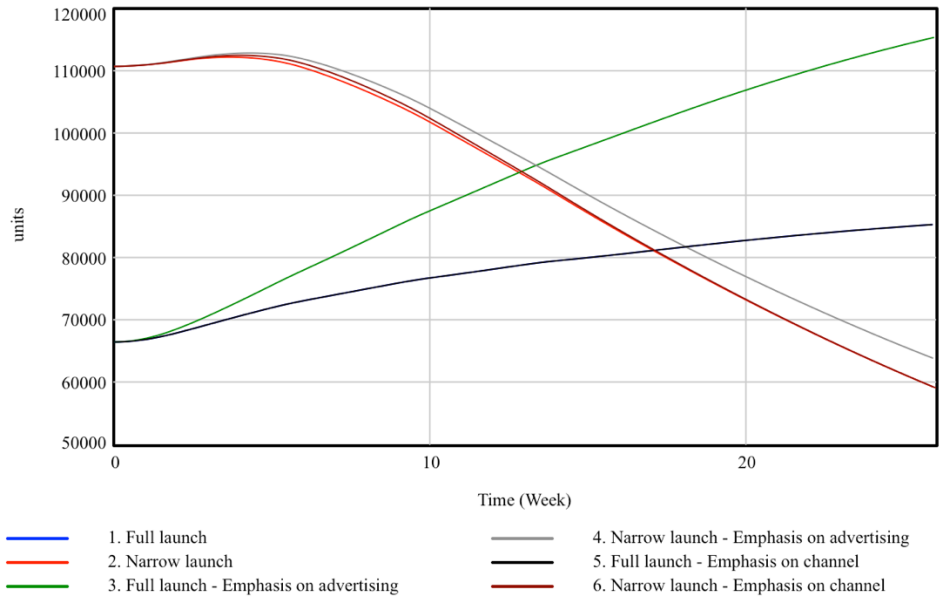

Figure 30.2.2. The inventory gap over time.

The system dynamics modelling results point out that the choice for a fat or narrow launch approach will mainly be determined by the production capacity issue and the ability of the provider to ramp up production levels. As long as production remains limited a narrow approach (in a single country) is to be favoured.

The current test results clearly show that market demand for the new species is present and provide information for providers and their partners to develop a solid market launch strategy.

The full results are published in ***Deliverable 30.6 Report on results of test markets per species.***

Task 30.3 Recommendation for industry development and international expansion (led by SWR, led by G. Tacken)

Sub-task 30.3.1 Feasibility study (led by SWR, R. Stokkers)

The objective of the feasibility study was to make an analysis on basis of the technical assessment (WP28), market information (WP 29, resource and cost analysis (Task 30.1) and the result of the tested strategies (Task 30.2). 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 social and market impact assessment and a stakeholder identification to introduce the products in the market.

The study learned that Greater Amberjack and Atlantic Halibut currently have the highest margins and therefore the best financial feasibility. The margins for pikeperch can be improved significantly when the cannibalism problems are solved and a specific feed with good conversion is developed. For meagre and grey mullet no financial information was available at the end of the project.

The environmental impact of the species is not researched in DIVERSIFY, but in general the impact of the different species on environment is not better or worse than any other production in ponds, RAS or tank system or cages in the sea.

The social impact of introduction of the species is most likely to be highest in Norway and the Mediterranean. Greater Amberjack and Meagre are species that have potential in the south of Europe, while Atlantic Halibut can be the diversification option next to salmon. Pikeperch is the middle European option for aquaculture, but the margins are not high enough yet.

How large the market impact is of the species is mainly dependent of the acceptance by retail buyers and consumers. This can't be quantified yet, but a good positioning can help a lot. All species need further research and effort on market positioning, marketing, and sensory research. On basis of this project all products have potential.

In Europe, **greater amberjack** shows the most promising market opportunities, given its large size, processing abilities and superior sensory characteristics. **Grey mullet** is a very interesting species due to the higher sustainability of its production methods. No specific preference region has been identified for this species. **Atlantic halibut** is especially interesting as alternative in the flatfish market since quota are decreasing and demand is still high. **Pikeperch** is especially interesting as the controlled and fresh alternative for Pangasius in mid-EU market and **meagre** has physical characteristics and is of interest in Southern Europe as start market. Wreckfish was not included in this study since it has no grow out yet.

The full results are published in ***Deliverable 30.7 Feasibility study.***

Sub-task 30.3.2 Global market approach (led by SWR, M. Reinders)

The objective of this deliverable was is to identify opportunities for the new species based on a synthesis of the expertise developed in the socio-economic Work Packages of the DIVERSIFY project resulting in providing policy and strategy recommendations with the potential to make the European aquaculture sector more competitive, and to create more of a level playing field in relation to aquaculture production in developing countries. Apart from this, system dynamics simulation models that help predict international diffusion of the EU produced fish species of this study are developed. The models factor in the SME's international relations and other (e.g. cultural) linkages between geographical markets.

So this Deliverable was a final wrap up of all work done in socio-economic GWP7 and based on system dynamics modelling a diffusion model is made for greater amberjack.

Part I of Deliverable 30.8 builds on the virtual market test reported in Deliverable 30.6. Based on consumer's willingness to buy and observed need for extra information assumptions regarding innovation and social contagion are made. It allows for modelling speed of adoption per country. Based on this and adoption levels /volume suggestions regarding which launch strategy to use in the EU are developed.

In **Part II** of this Deliverable, based on above-mentioned results of the work done in the GWP, overall conclusions will be drawn. More specifically, an overview of the relevant trends that set the stage for the DIVERSIFY-species is provided as well as specific recommendations per species on how their market can be developed. This part ends with strategy and policy recommendations for further market development and expansion of the DIVERSIFY species.

In part I the diffusion curves are calculated for greater amberjack in each of the five target countries of the DIVERSIFY project.

Figure 30.3.1 shows the results for the exercise using the *standard p, q* values. All curves follow the standard pattern of evolving from zero to 100% adoption/diffusion (vertical axis: 0 and 1.0 respectively). Consistent with the results of Deliverable 30.6, Italy is found to be the market with most speedy adoption and fastest diffusion of the new species. Spain is the second most innovative market, i.e. second quickest take off and steepest curve. These two countries should be targeted first. The German, UK and French markets could be addressed next, although we do note that the size of the German market is limited in volume. Interesting is that the French market lags behind. This is caused by its low *p* and *q* values and ratio. The low set of initial adopters gives it a slow start while the ration explains it less pronounced curvature.

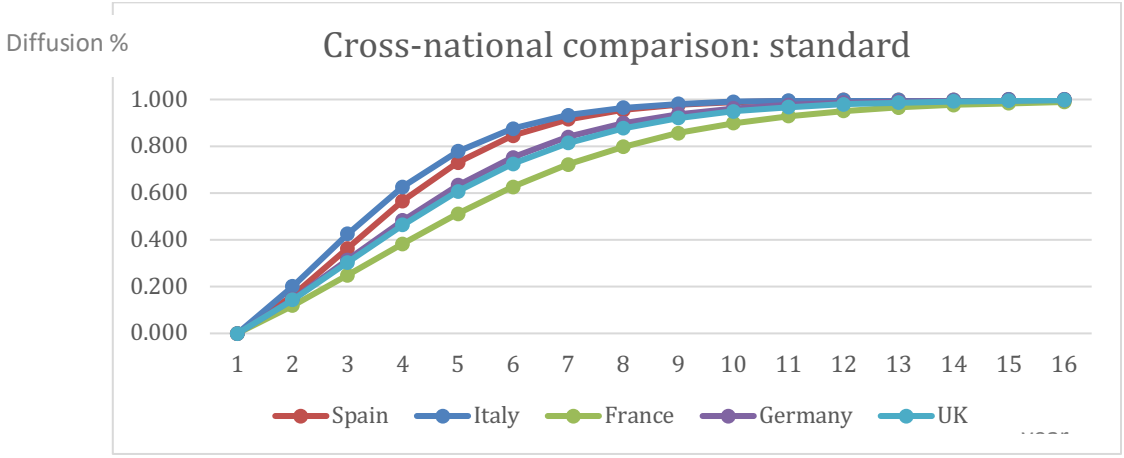

Figure 30.3.1 Diffusion curves for the five target countries using standard settings for *p,q*

Figure 30.3.2 shows the results using the more *innovator based/focused settings* for *p, q*. Here too, the Italian market is the most innovative and fastest diffusing one. However, accounting better for the innovators in the market place, now Spain and Germany ex aequo end up in second place. Like in **Figure 2.1**, it can be observed in **Figure 2.2** particularly that the French market lags behind. This is a new insight since in Deliverable 30.6, France seemed at least moderately attractive. As mentioned, the difference can be traced back to the market's low initial adoption rate and a much lower set of people willing to consider the new fish species after receiving extra information (i.e. low *p* and *q*).

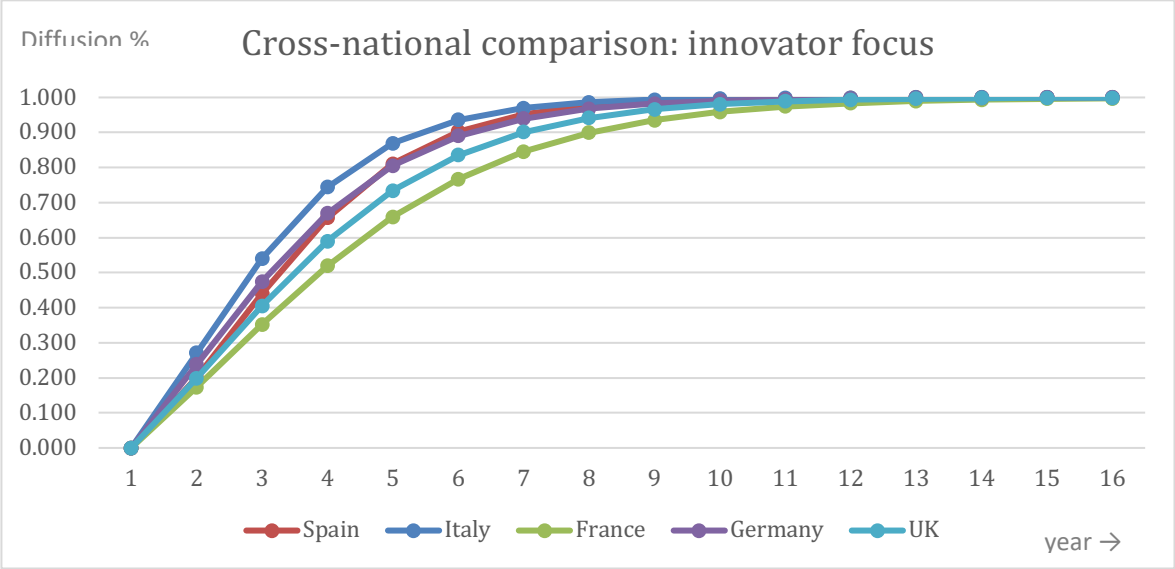

Figure 30.3.2 Diffusion curves for the five target countries using innovator-oriented settings for *p*,*q*

The input for the models was the market research data from the online store test reported on in Deliverable 30.6. Consistent with results of this prior deliverable, current results show that Italy is the most innovative and attractive market with the fastest adoption and diffusion of the new species. Spain is the second most innovative and thus interesting market to consider. Italy and Spain are also the most interesting based on market volume; expected market potential is 84 and 177 tons annually, respectively. However, we should note that this conclusion may be limited to greater amberjack fillets and not processed products. Italian consumers had lower acceptability values for the different processed products developed (see ***Deliverable 29.4***).

Interestingly, the current results show the French market to be less attractive than we expected based on the static results of Deliverable 30.6. The dichotomy in the market between adopters and consumers who reject the new product, but also the lower percentage of initial adopter as well as low percentage of people willing to consider the new species played a much more significant role in the dynamic perspective, suggesting that diffusion in France may be more difficult and slower. This result should be compared and weighted against the large size of the French market. The German market is, in fact, more interesting – except that the latter country's market size (volume) is rather small. Still, Germany may be a wild card for launching the new species, particularly if production volume for farmed greater amberjack is and remains limited.

The results of the exploration of the two internationalization strategies, i.e. waterfall and sprinkler, provided important new insights regarding production volumes required for each alternative internationalization approach. Since current production levels of farmed greater amberjack are extremely limited this is important to consider. The results suggested that a sprinkler approach requires the ability to ramp up production quickly. It requires a tremendous production capacity, which is currently unavailable. Hence, the waterfall approach focusing on Italy and then (in year 2 or later) Spain, makes more sense. A benefit of this approach also is that the launch can be tailored to local conditions and offers the provider and its partner i.e. retailers the opportunity to learn from the launch in the (previous) lead country.

Still, in the short run production capacity may even be too low for the waterfall option involving both Italy and Spain. If so, the provider(s) may choose either a launch on a limited scale, e.g. in Italy only and, for

example, limiting itself to a particular region before scaling up. Alternatively, the firm may decide to first experiment in a small volume country like Germany. Production location and market access may, of course, play a role in these decisions too.

In part II strategic recommendations and policy recommendations are made for introduction of the five species.

Build market position

The European fish market has many opportunities that are underexploited by the European aquaculture sector yet. In the DIVERSIFY project, research has been done aiming at the future market development and expansion opportunities for a number of new, emerging finfish species with a great potential for the expansion of the European aquaculture sector. One topic that needs to be taken under consideration is how to build a market position for the new species by paying attention to marketing segmentation, communication and the rising of the level of consumer awareness. Marketing communication plays an essential role in building brand and/or product reputation. The introduction of new aquaculture species (and products) should therefore be accompanied by a well thought out communication strategy. There is a clear need for aquaculture industry to promote new products and persuade/engage consumers that they can find an excellent source of sustainable and quality health promoting food. Having said that:

- Use of a country-of-origin (COO) indication in general, and 'Produced in own (domestic) country' in particular makes EU consumers to think more positively about the product besides increasing the probability of its purchase. This study shows that locally produced products have added value for fresh fish products. Locally produced products also provide good branding opportunities, as locality could help to establish consumer identification with the brand.
- Although not considered by all consumers as important as COO and eco-friendly labels, the use of nutrition and health claims could nevertheless provide market opportunities. As shown in this project, some segments, "nutritious conscious", would be more likely to choose products that contain nutrition and health claims. Moreover, for the general public, the use of nutrition and health claims would actually help consumers making more informed choices, stimulating health-related behaviour.
- Overall, being certified becomes more important. Certification is a requirement for business-to-business buyers. Certification requirements are buyer dependent not country dependent. We would like to highlight certification with respect to sustainability too. This is becoming increasingly important and is expected to become a market access requirement throughout Europe and has importance for consumers too. For instance, the ASC logo currently does play an important role in consumers' fish product choices and may even become more important in the future. Having the required certificate(s) is a licence to supply.

Another key element in building market position is to address the right consumer segments for the new species. Involved fish consumers are most interested in new species. Involved traditionals generally prefer the traditional fish products, which imply fresh products in the south of Europe and convenience, more processed, fish products in northern Europe. This segment is most open for aquaculture. Additionally, the segment of involved innovators is not only open for new species but also for new products. The fact that the two main groups of potential consumers of the new fish products, i.e., the involved traditionals and the involved innovators, are identified in substantial size in all research countries (France, Germany, Italy, Spain and UK), shows that a marketing approach for the species could be pan-European. In addition to this, the convergence of supermarkets makes that eating patterns change as well and that traditional buyers of fresh whole fish tend to buy fresh fillets too for convenience reasons. For aquaculture producers, prices are determined by their production costs and the decision on the selection of a market segment will depend on the development of production and processing as well as efficiency in organizing its supply chains.

Concentrating exclusively on price competition against competitors is a risky decision and means addressing a single market segment. A better option is to DIVERSIFY in market segments and products by processing these into added value products. This strategy spreads the risks and is a better defence against substitute products.

Apart from this, still much has to be done on awareness and recognition. Looking at the products that are currently in the European market, a preference for white fish, large fillets and convenience products (especially in the Northern Europe) exists in consumers where fish species from aquaculture such as salmon dominate the market. However, in the Southern Europe, adopting species and fish consumption from aquaculture requires marketing and awareness raising campaigns, as consumers still prefer wild over farmed fish. In line with this, most DIVERSIFY species are still not (well) known to consumers and professional buyers of supermarkets. The new species will face competition from established aquaculture species, when penetrating the market. Prices will be a crucial factor, besides quality, service and availability. It is suggested to put energy to overcome the bottlenecks and do not let lack of availability interfere with growth ambitions.

Build together

"If you want to go fast, go alone. If you want to go far, go together" (African proverb). European fish farmers are in favourable position thanks to direct sourcing, transparency, sustainability and locality. Consumers give added value to a locally produced aquaculture product. An encouraging trend is that at the supply side parties are looking increasingly to source directly from farmers.

Points of attention for farmers or producer organisations are than that most buyers do not have the logistics to maintain the quality of fresh fish, so support till store can give a lot of added value to supermarket buyers. Furthermore, buyers need to be able to position the product and see where the product would fit in their assortment. So, in approaching buyers, farmers should be open to provide full information on their entire production process, feed, and logistics (knowing that buyers prefer suppliers that decrease risks).

Suggestions for improvement refer to the second part of the African proverb ("…If you want to go far, go together") and encompass cooperation. After all, mainly engaged in resolving biological issues i.e. production bottlenecks and driving down costs, farmers and other consortium partners focused first and foremost on the parties directly before and behind them in the supply chain. They showed little awareness for and thus paid little attention to more distant, strategic parties and issues. They were focused on the next level in the supply chain and not (yet) on the end-customer. However, what ultimately determines the success of establishing markets for new fish species is the *cooperation* between farmers mutually (e.g., producer organisations) and other stakeholders, and market their product together. Mutual cooperation between fish farmers also provides opportunities for better alignment with feed suppliers.

Another level of cooperation refers to working buyer-driven. Marketers should consider involving consumers, retailers and mongers more in the co-creation of new aquaculture product ideas. The work done in DIVERSIFY shows for example that projective and particularly creative techniques should be considered ideal in the first stages of new product development, since these techniques provide a valuable pool of new ideas where the 'voice of the consumer' is loudly heard. Moreover, the chosen approach in this project, a customer-centric view was fully adopted all along, might be usable or inspiring for market introduction or development of other species. A few ideas for co-creation per species are delivered in this project. More ideas could be generated with local partners.

Species overview

We conclude this section with some remarks concerning the business opportunities of the DIVERSIFY species. Atlantic halibut and greater amberjack seem to have the best opportunities to succeed. Both are good quality fillets/meat, a favourable cost structure, and already benefitting from a market position, which allow for a positive outlook and profit margin. Still, below we summarize the main recommendations for each species (in alphabetical order):

- **Atlantic halibut** is a very good aquaculture substitute for sole and turbot, and has therefore a good market potential in a large existing market for flatfish. The product can be sold as a high-end product since substitutes are wanted and very good priced too.
- **Greater amberjack** is attractive since it has good flesh characteristics. From a competitive viewpoint it has better qualifications than tuna, but the same product quality. For both species, growth in aquaculture production is needed to keep up with the growth in market demand.
- **Grey mullet** from European waters is unique and could serve consumers all over Europe. Grey mullet is very well known for people with an Arabic background and is regarded as an important segment to start distribution of these fish throughout Europe. It provides fish roe and is a suitable candidate to use plant based alternatives to fish meal an oil raw materials.
- **Meagre** is comparative to European sea bass and gilthead sea bream, but demand for meagre is still low as it is relatively unknown to the consumer. Problematic is the fact that meagre is known by consumers under different, local names. Hence, the main challenge is in marketing communication to stimulate demand for meagre all over Europe.
- **Pikeperch** is a fresh water fish with multi possibilities for preparations including the fact that the skin of pikeperch could be of value too. So, a marketing strategy for both the fish and the skin, could give an interesting business model. A niche positioning to justify high prices might remedy the unfavourable cost level.
- **Wreckfish** is relatively far in technical development to give relevant business model projections. In the market research studies this species was not considered.

Policy recommendations

Supporting further growth and market expansion of European aquaculture needs **removing barriers and increase promotion**.

Research in D27.2 and D27.5 learns that there is not a on common certification program for aquaculture products all over Europe for all clients that is most accepted. Although there is a common ground, each business-to-business buyer has their own requirements for the products they buy and are not controlled whether they use the same certification grounds for all products they buy. **It is company policy that determines what certification qualifications are asked.** From a policy viewpoint, it is therefore very difficult to **create a level playing field** for all species sold in the EU. As such, more uniformity in sustainability and product certification could be valuable to give local production a chance in the European market. Since sustainability is getting more important for food products throughout Europe, the aquaculture sector could be helped with uniform requirements. Consumers' demand for sustainability will continue to grow and the responsible sourcing of fish will become even more important when selling fish in Europe. There are however still relatively few options for simple certification. The use of some type of official EU Ecolabel for responsibly farmed fish could be of high interest, also for the DIVERSIFY species. Furthermore, certification demands a high administrative burden. Providing clear guidelines for sustainable aquaculture and facilitate the certification process can help.

This study learns that there is a **market potential for increased fish consumption within the EU**. The PESTEL analysis in this project shows that a lot of countries have stimulation policies for fish consumption both for fresh fish and aquaculture (D27.1). Consumers in both the segmentation study and the focus group discussions (D28.1 and 29.2) confirm to be open to consume more and new fish products, different than and on top of what they consume now.

Especially **for new species of the DIVERSIFY project, positioning and reputation is a major point of attention** since farmed fish has to deal with an image, based on bad media reports about farming conditions at fish farms in Asian and African countries. Instead, **European aquaculture should start building a good positioning and reputation**, also given the fact that EU farmed fish is in a good position to be positioned as sustainable: they relieve pressure from the wild stocks and they provide more reliable and controlled supplies. Explicit positioning of local production could give added value to consumers, since consumers already give added value to aquaculture products from their own country. General communication for EU aquaculture has no added value for consumers.

All species have chances in their own target market. However**, positioning of the species in relation to the main competitor in wild catch or aquaculture products produced inside and outside the EU is necessary and needs support**. Support to do this is necessary, since most of the firms producing aquaculture don't have the means to compete with species like Pangasius (on price) and salmon (in marketing). Overproduction of other species like sea bass and sea bream makes that alternatives, although far away in product characteristics might be preferred. The online market test found out that positioning the added value of the new species in relation to well-known species is essential. However, if this alternative is cheaper, price competition has to be conquered by quality and other added value. This added value needs communication and support.

The full results are published in deliverable ***D30.8 Report on EU and international market development plans and recommendations.***

Deviations from Annex I and their impact:

No deviations

Dissemination (WP 31)

WP No:	31	**WP Lead beneficiary:**		P18. CTAQUA
WP Title (from DOW):	Dissemination			
Other beneficiaries (from DOW):	P1. HCMR	P3. IRTA	P6. DLO	P7. IMR
P8. IEO	P9. UL	P10. TU/e	P.11 AU	P12. APROMAR
P13. UNIBA	P15. ULL	P19. CMRM	P33. FGM	P34. BVFi
P35. MASZ	P36. ANF	P37. EUFIC		
Lead Scientist preparing the Report (WP leader):	Rocio Robles			
Other Scientists participating:	Constantinos C. Mylonas, Maria Papadaki and Ioannis Fakriadis (P.1), Alicia Estévez (P.3), Neil Duncan (P.3), Luis Guerrero (P.3), Mathias Keller (P.34), Maria Banovic (P.11), Javier Ojeda (P.12), Blanca Álvarez (P.8), Covadonga Rodriguez (P.15), Gemma Tacken (P.6), Aldo Corriero (P.13), Fátima Linares (P.19), Laslo Varadi (P.35), Martiña Ferreira (P.36), Carlos Abundancia (P.37).			

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 audio-visual material
- D31.5 Collaboration agreement with food industry and consumer organization; linkage of websites.
- D31.6 Annual presentation of DIVERSIFY (Y1) at a relevant conference (Aqua Europe 2014).

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

During the 2nd Reporting Period and according to Task 31.1 (Project website and brochure), Task 31.2 (Annual Coordination Meetings), Task 31.3 (Presentation of DIVERSIFY at the AQUA EUROPE meetings), and Task 31.7 (Dissemination to the food industry and consumers), the following Deliverables were reported in the previous Reporting Periods (13-30 Mo):

- D31.1 Establishment of the Project website (www.diversifyfish.eu) including information on the objectives and main tasks of the project and the adaptions of the web structure.
- D31.4, D31.7, D31.8, D31.12 and D31.13 Production and release of audiovisual material
- D31.9 Annual presentation of DIVERSIFY (Y2) at a relevant conference (Aqua Europe 2015)
- Presentations of DIVERSIFY at the aqua Europe meetings (Diversification Sessions by the Species leaders (Y2).

Summary of progress towards objectives and details for each Task (31-48 Mo)

In the 3rd Reporting Period further work has been performed on the Tasks described in the DoW, 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 Deliverables that have been reported in the previous Reporting Periods (30-48 Mo) were the following:

- D31.1 Establishment of the Project website (www.diversifyfish.eu): the website has been re-structured and specific pages related to the production of scientific articles and the organization of the Promotional Workshops and Species Seminars have been incorporated.
- D31.15, D31.17 and D31.22 Production and release of audiovisual material
- D31.14 and D31.19 Annual presentation of DIVERSIFY (Y3, Y4) at a relevant conference (Aqua Europe 2016 and 2017)
- D31.16 and D31.18 Promotional Workshops (1st and 2nd) for specialized audience in fish market sector (Germany and Spain).
- D31.21 presentation of DIVERSIFY at the European Seafood Expo.
- Presentations of DIVERSIFY at the aqua Europe meetings (Diversification Sessions by the Species leaders (Y4).

Summary of progress towards objectives and details for each Task (49-60 Mo)

- Task 31.1 Project website and brochure (led by CTAQUA): project web has been updated with the events and important news related to the achievements of the different Research Areas, popular and scientific publications, information and content of the annual coordination meetings, update of the intranet, etc.
- Task 31.2 Annual Coordination Meetings (led by HCMR). Two annual coordination meetings have been organized (Mo50 and the final meeting in Mo60).
- Task 31.3 Presentation of DIVERSIFY at the AQUA EUROPE meetings (led by HCMR): DIVERSIFY had a full-day Special Session at the recent AQUACULTURE EUROPE 2017 conference in Dubrovnik (Croatia) in October 2017
- Task 31.4 Scientific presentations and submission of manuscripts (led by HCMR). A total of 51 pre reviewed scientific articles have been published as a result of the research done in the project.
- Task 31.5 Full-day seminars on “Know-how Transfer” of the aquaculture of each of the DIVERSIFY species (led by CTAQUA and the Species Leader Partner). Six Species Seminars have been organized in six different European countries.
- Task 31.6 Promotional workshops (led by CTAQUA). Specialized 1-day workshops have been organized in 4 strategic countries (Spain, Greece, Germany and Italy) for the promotion of the project activities and results (CTAQUA).

- Task 31.7 Dissemination to the food industry and consumers (led by APROMAR and EUFIC). ANFACO, APROMAR and BVFi have translated to Spanish and German the Species Technical Manuals and promoted the documents among their associates in the corresponding country.

Details of the Tasks.

Task 31.1 Project website and brochure (led by CTAQUA, Rocio Robles).
Website development

Although the Deliverables corresponding to this Task have been already reported in the previous reporting period and 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 been updated continually, providing essential information on project activities. The webpages have been re-structured to incorporate new pages such as:

The Dissemination tab includes now:

- Fish Health Manuals: includes a Diagnostic- Recommendation technical manual for meagre health management and a Diagnostic- Recommendation Technical manual for greater amberjack health (both from HCMR). Both manuals are available in the project web: https://www.diversifyfish.eu/fish-health-manuals.html **(Fig. 31.1.1).**
- Aquaculture Europe publications, which contains all the DIVERSIFY related publications in the Aquaculture Europe magazine, along the five year project duration. A total of 9 articles have been published and a final compilation issue has been edited by the Aquaculture Europe editors. All the publications are freely available in the web. https://www.diversifyfish.eu/aquaculture-europe-magazine.html (**Fig. 31.1.2).** The compilation issue has been distributed during the last Coordination and Dissemination meeting held in Brussels, November, 2018.
- Promotional Workshops: a summary of each of the Promotional Workshops is available in the web, including all relevant information of the event (**Fig. 31.1.2).** All the presentations given during the events are available in the web.
- Species Workshops: this page includes a summary with all the relevant information on each of the workshops and a Technical Manual per species (scroll down menu), incorporating all the significant information on the culture of the species and providing the results of the experimental work carried out with the species in the different Research Areas of the project (Fig. 31.1.3). All the presentations given during the Species Workshops have been uploaded in the web and they are available for public consultation. Spanish and German translated Technical Manual are also available in the page. *A complete and detailed description of the Technical Manuals (originally mentioned as Technical Leaflets in the DoW) have been submitted as Deliverable 31.24 "Technical Leaflets"*. For each of the Seminars a technical leaflet (manual) have been elaborated with the input of the species leaders and the scientists involved in the species and research areas. The design of each manual includes a front page, table of contents, presentation of the results on each research area and bibliography. The seminars have had a great acceptance in the industry. Farmers and fish producing companies have attended the meetings with great interest and have requested further technical information on the culture of the species during the meetings. The technical manuals are available for free download in the project web:

  https://www.diversifyfish.eu/grey-mullet-workshop.html

  https://www.diversifyfish.eu/pikeperch-workshop.html

  https://www.diversifyfish.eu/wreckfish-workshop.html

  https://www.diversifyfish.eu/halibut-workshop.html

  https://www.diversifyfish.eu/amberjack-workshop.html

  https://www.diversifyfish.eu/meagre-workshop.html

- Scientific articles tab: includes all the published pre-reviewed articles resulted from the project Research Areas (**Fig. 31.1.4).**

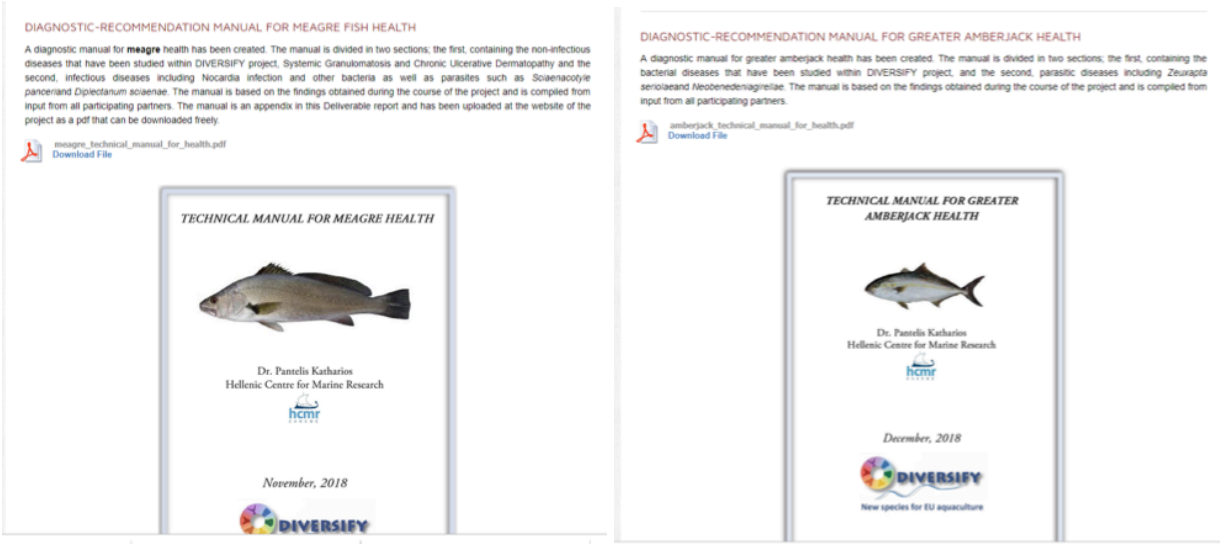

Figure 31.1.9. Screen capture with the front page of the Technical Manual for meagre health (left) and Technical Manual for greater amberjack health (right).

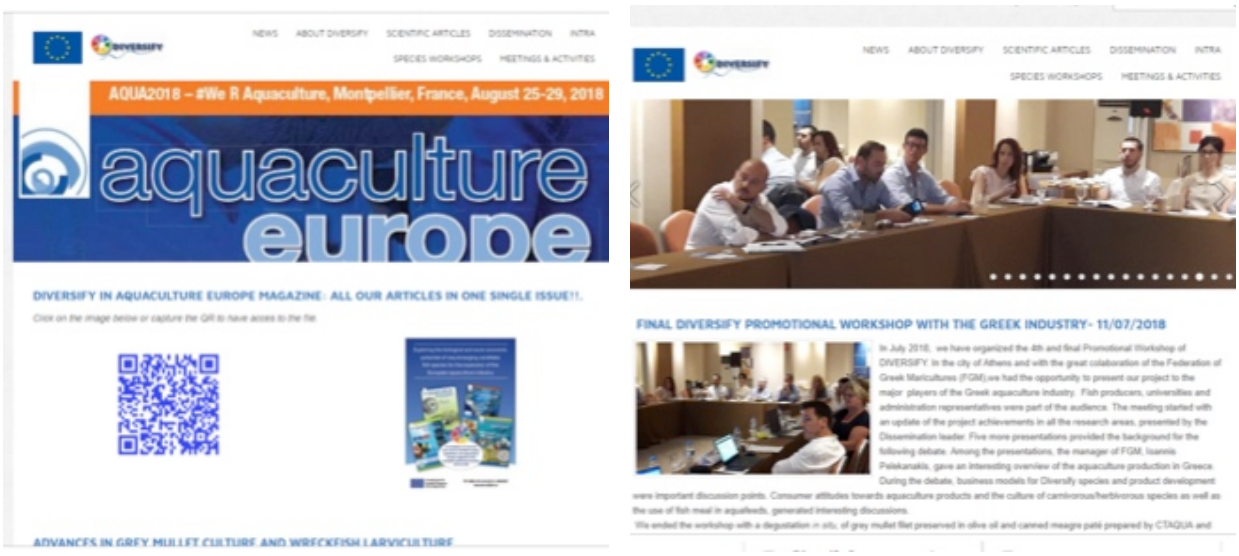

Figure 31.1.10. Screen capture of the Aquaculture Europe publications page; in the left image, the front page of the compilation issue. On the right, the summary of the last Promotional Workshop held in Athens, July, 2018.

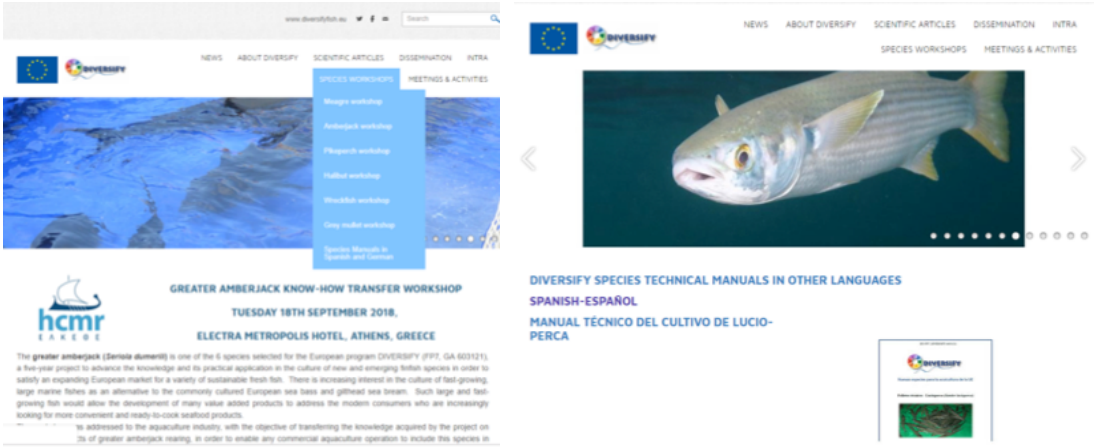

Figure 31.1.11. Image of the Species Workshop page (left), including the Spanish and German translation of the Technical Manual per species (right).

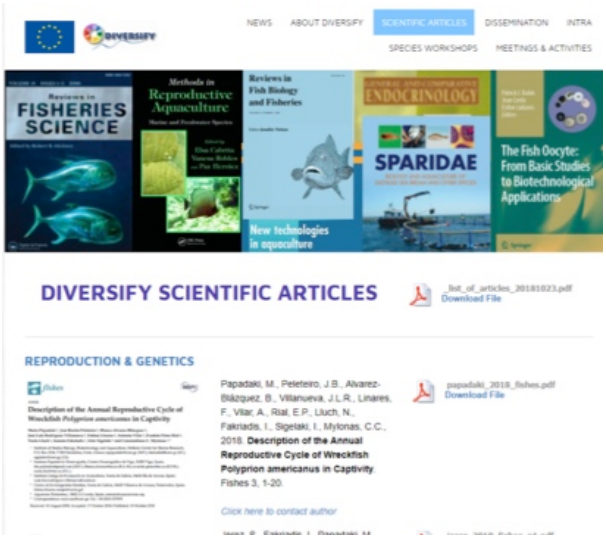

Figure 31.1.12. Screen capture of the Scientific articles page. Authors can be contacted directly from the page.

The rest of the webpage sections remain as it was reported in the 3rd reporting period. The information on these sections has been regularly updated with the new information when available.

DIVERSIFY LOGO

As it has been reported previously, the updated logo design keeps being used in all the pages and subpages of the website, and it is included in all the dissemination material produced within the project, in the profiles of the social networks, Facebook, twitter and as part of the signature of the partners. With the occasion of the last Coordination and Dissemination meeting, a key keeper (floater to be used in aqua production environments) with the logo and the message of "New species for EU aquaculture" have been produced and distributed among the stakeholders, producers associations and partners (**Fig. 31.1.5).**

Figure 31.1. 13. Key keeper floater designed with the logo of DIVERSIFY and distributed among stakeholders and partners.

New brochure

During this reporting period, a final brochure of the project has been designed, printed and distributed with the occasion of the final Coordination and Dissemination meeting in Brussels. The final folder presents the aim and impact of the project, a short summary with the most relevant results obtained with each species, an extended summary of the work developed within the Socioeconomic Area and the list of partners in the back page (**Fig. 31.1.6)**.

Figure 31.1.14. Final folder of the project. Top: front and backside page; bottom: internal part with major achievements per species.

PRODUCTION AND RELEASE OF DOWNLOADABLE AUDIOVISUAL MATERIAL

All the videos of the project are included following a chronological order in the "News" page of the project website (http://www.diversifyfish.eu/). A summary of the videos from previous reporting period has been included in the 3rd Dissemination Report. During the period December 2017 to November 2018, a podcast and several videos have been produced and uploaded in the web.

- Podcast with an interview to the Species leaders of meagre, Dr. Alicia Estévez, wreckfish, Dr. Blanca Álvarez and greater amberjack Dr. Nikos Papandroulakis. A short interview from each of the species leader summarized the main results of the experimental and trial done with each of the species. *The full description of the work and results is provided in Deliverable 31.22 Production and release of audio-visual material.*
- Two short videos documenting the first wreckfish (*Polyprion americanus*) juveniles produced in Galicia, Spain, after years of research in the reproduction and larval rearing of the species in the framework of the DIVERSIFY project.
- A short video with an interview to the PC and the Dissemination leader has been recorded during the final Coordination and Dissemination meeting. They make a closing up summary of the project and invite all the interested aquaculture stakeholders to contact for further information on the project results. The video is available at https://goo.gl/5AUU9w. (**Fig. 31.1.7)**. *The full description of the work and results is provided in Deliverable 31.25.Production and release of audiovisual material.*
- A final video as compilation of the project videos and about the articles on DIVERSIFY work published in Aquaculture Europe magazine; the video is available in youtube https://youtu.be/dYzp_Wgthko (**Fig. 31.1.7)**. Detailed description of this video has been submitted *as Deliverable 31.15. Production and release of audiovisual material*.

Figure 31.1.15 . Screen capture of the short video interview to the PC and Dissemination leader during the final Coordination and Dissemination meeting (left) and an image of the final video compilation.

ADDITIONAL DISSEMINATION ACTIVITIES

Multiple dissemination activities have been implemented during this reporting period to broadcast project activities to the scientific community and the aquaculture industry, as well as the general public (**Table 31.1.1**). During this period, dissemination of the major achievements in the research areas through the Promotional Workshops (more focused on the Socioeconomic research) and the Species Workshops (focused on the biological-technical research) have been the centre of the knowledge transmission of the project. These activities will be further documented within this report in the corresponding section.

Table 31.1. 5. Representative **d**issemination activities of DIVERSIFY during 2018, uploaded in the "Dissemination activities" site of the SESAM application of the Participant Portal (only the first page is reproduced here. See Final report for the full list).

284	Press releases	HELLENIC CENTRE FOR MARINE RESEARCH	Workshop on greater amberjack (Seriola dumerili) aquaculture: results from the DIVERSIFY project	23/04/2018	Fishing News Magazine, Vol 431, April 2018	Scientific community (higher education, Research) - Industry - Policy makers	500	Greece	VALIDATED	
285	Articles published in the popular press	ASOCIACION NACIONAL DE FABRICANTES DE CONSERVAS DE PESCADOS Y MARISCOS-CENTRO TECNICO NACIONAL DE CONSERVACION DE PRODUCTOS DE LA PESCA	"Nuevas especies para la acuicultura de la UE", special article translated into Spanish from Aquaculture Europe 42(2), september 2017	15/05/2018	https://www.diversifyfish.eu/publicaciones-en-espantildeol.html	Industry		España	VALIDATED	
286	Posters	FUNDACION CANARIA PARQUE CIENTIFICO TECNOLOGICO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	Effects of different dietary levels EPA + DHA on egg quality of greater amberjack (Seriola dumerili).	05/06/2018	International Symposium Fish Nutrition and Feeding 2018. Las Palmas de Gran Canaria	Scientific community (higher education, Research)	500	International	VALIDATED	
287	Posters	FUNDACION CANARIA PARQUE CIENTIFICO TECNOLOGICO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	Effect of different dietary n-3 long-chain polyunsaturated fatty acids levels on stress response of meagre (Argyrosomus regius, asso 1801) juveniles	05/06/2018	International Symposium Fish Nutrition and Feeding 2018. Las Palmas de Gran Canaria	Scientific community (higher education, Research)	500	International	VALIDATED	
288	Posters	FUNDACION CANARIA PARQUE CIENTIFICO TECNOLOGICO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	The effect of fish density and dietary supplementation of vitamin C, manganese, zinc and selenium on the development of systemic granulomatosis in juvenile meagre (Argyrosomus regius)	05/06/2018	International Symposium Fish Nutrition and Feeding 2018. Las Palmas de Gran Canaria	Scientific community (higher education, Research)	500	International	VALIDATED	
289	Oral presentation to a scientific event	FUNDACION CANARIA PARQUE CIENTIFICO TECNOLOGICO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	Effect of increasing dietary levels of n-3 long-chain polyunsaturated fatty acids on liver composition and histopathology of meagre (Argyrosomus regius, Asso 1801) fingerlings	05/06/2018	International Symposium Fish Nutrition and Feeding 2018. Las Palmas de Gran Canaria	Scientific community (higher education, Research)	500	International	VALIDATED	
290	Oral presentation to a scientific event	FUNDACION CANARIA PARQUE CIENTIFICO TECNOLOGICO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA	Dietary use of mannan oligosaccharides in greater amberjack juveniles: effects on growth performance, immune gene expression and disease resistance against Neobenedenia girellae	05/06/2018	International Symposium Fish Nutrition and Feeding 2018. Las Palmas de Gran Canaria	Scientific community (higher education, Research)	500	International	VALIDATED	
291	Oral presentation to a scientific event	HELLENIC CENTRE FOR MARINE RESEARCH	BROODSTOCK MANAGEMENT AND SPAWNING INDUCTION OF GREATER AMBERJACK Seriola dumerili REARED IN SEA CAGES IN GREECE	04/06/2018	11th International Symposium on Fish Reproductive Physiology, 4-8 June 2018, Manaus, Brazil	Scientific community (higher education, Research)	230	International	VALIDATED	
292	Posters	INSTITUT DE RECERCA I TECNOLOGIA AGROALIMENTARIES.	REPRODUCTIVE CONTROL OF MEAGRE (Argyrosomus regius) TO OBTAIN FAMILIES FOR GENETIC BREEDING PROGRAMS	06/06/2018	11th International Symposium on Fish Reproductive Physiology, 4-8 June 2018, Manaus, Brazil	Scientific community (higher education, Research)	230	International	VALIDATED	
293	Posters	INSTITUT DE RECERCA I TECNOLOGIA AGROALIMENTARIES.	RECOMBINANT FOLLICLE-STIMULATING HORMONE INDUCES VITELLOGENESIS AND SPERMATOGENESIS IN FLATHEAD GREY MULLET (Mugil cephalus)	07/06/2018	11th International Symposium on Fish Reproductive Physiology, 4-8 June 2018, Manaus, Brazil	Scientific community (higher education, Research)	230	International	VALIDATED	
294	Posters	ISRAEL OCEANOGRAPHIC AND LIMNOLOGICAL RESEARCH LIMITED	OVERCOMING REPRODUCTIVE DYSFUNCTIONS IN CAPTIVE GREY MULLET (MUGIL CEPHALUS): AN EXPANDED TOOL BOX FOR SUCCESSFUL BREEDING	07/06/2018	11th International Symposium on Fish Reproductive Physiology, 4-8 June 2018, Manaus, Brazil	Scientific community (higher education, Research)	230	International	VALIDATED	

357	Press releases	FUNDACION CENTRO TECNOLOGICO ACUICULTURA DE ANDALUCIA	El potencial de la producción y comercialización de la lisa	26/09/2018	http://www.ctaqua.es/180626-potencial-lisa-diversify-acuicultura-ctaqua.aspx#.XABmsOhKhPY	Scientific community (higher education, Research) - Industry - Civil society - Policy makers	5000	EU, world	PENDING	
358	Press releases	FUNDACION CENTRO TECNOLOGICO ACUICULTURA DE ANDALUCIA	Italia acogerá un completo taller sobre el cultivo de la lisa Mugil cephalus	11/04/2018	http://www.ctaqua.es/180411-taller-workshop-lisa-diversify-bari-italia-acuicultura-ctaqua.aspx?id=1	Scientific community (higher education, Research) - Industry - Civil society - Policy makers	5000	EU, world	PENDING	
359	Press releases	FUNDACION CENTRO TECNOLOGICO ACUICULTURA DE ANDALUCIA	DIVERSIFY consigue establecer protocolos de producción para la seriola	27/02/2018	http://www.ctaqua.es/180227-seriola-diversify-acuicultura-ctaqua.aspx#.XABIluhKhPY	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias	5000	EU, world	PENDING	
360	Press releases	FUNDACION CENTRO TECNOLOGICO ACUICULTURA DE ANDALUCIA	DIVERSIFY celebrará su tercer taller promocional en Verona	08/02/2018	http://www.ctaqua.es/180207-diversify-taller-promocional-verona-acuicultura-ctaqua.aspx#.XABn--hKhPZ	Scientific community (higher education, Research) - Industry - Civil society - Policy makers - Medias	5000	EU, world	VALIDATED	
361	Articles published in the popular press	FUNDACION CENTRO TECNOLOGICO ACUICULTURA DE ANDALUCIA	Fishing for new ways to expand the EU's aquaculture industry	01/09/2018	Research EU- Results Magazine Nº 75	Scientific community (higher education, Research) - Industry - Civil society - Medias	1000	EU	VALIDATED	
362	Oral presentation to a wider public	INSTITUTO ESPANOL DE OCEANOGRAFIA	INVESTIGACION EN ACUARIOS PUBLICOS PROYECTO DIVERSIFY	09/03/2018	Centro de educación Ambiental de Cordoba	Scientific community (higher education, Research)	100	Spain	VALIDATED	
363	Oral presentation to a wider public	UNIVERSIDAD DE LA LAGUNA	LA IMPORTANCIA DEL CONSUMO DE PESCADO EN LA SALUD HUMANA PROBLEMÁTICA ACTUAL EN LA PRODUCCIÓN DE OMEGA-3, DÍA MUNDIAL DE LA PESCA	28/11/2018	ESCUELA DE PESCA, CABILDO DE LANZAROTE, SPAIN	Civil society	50	SPAIN	PENDING	
364	Exhibitions	UNIVERSIDAD DE LA LAGUNA	FLYERS, PPT, LEAFLETS, VIDEO AND BOOK MARKERS OF DIVERSIFY PROJECT	28/11/2019	INNOVAZUL, FIRST INTERNATIONAL MEETING ON KNOWLEDGE AND BLUE GROWTHCÁDIZ	Scientific community (higher education, Research) - Industry - Civil society - Policy	1200	EU, WORLD	PENDING	

PRESS RELEASES

During this reporting period, several press releases have been produced:

- P .15, ULL:
- Organization of the ACM held in Tenerife, Spain. https://www.ull.es/portal/noticias/2018/la-ull-acoge-encuentro-coordinacion-anual-del-proyecto-europeo-acuicultura-diversify/
- Update on the DIVERSIFY research work, https://www.ull.es/portal/noticias/2018/comienza-reunion-diversify-ull-2018/
- New species for aquaculture https://www.ull.es/portal/noticias/2018/responsables-diversify/
- Information on the ACM (twitter) https://twitter.com/CIBICANarias/status/958283885393084416
- Information on the ACM (facebook) https://www.facebook.com/cibicanarias/
- New species for aquaculture (twitter) https://mobile.twitter.com/diversifyfish?s=08
- P.1 HCMR, Greater amberjack Workshop, Fishing News Magazine, vol. 431, April 2018.
- P.19 CMRM, Closing the wreckfish culture cycle, www.lavozdegalicis.es/noticia/maritima/2018/07/09/galicia-cierra-ciclo-cultivo-mero/0003_201807G9P22
- **P.18 CTAQUA:**
- Halibut and greater amberjack as potential aquaculture species http://www.ctaqua.es/181009-diversify-resultados-seriola-fletan-diversificacion-acuicultura.aspx#.W-
- DIVERSIFY achievement: production protocols available for greater amberjack production: http://www.ctaqua.es/180227-seriola-diversify-acuicultura-ctaqua.aspx#.XC-AZFxKg2x
- 3rd Promotional Workshop of DIVERSIFY in Verona, Italy. http://www.ctaqua.es/180207-diversify-taller-promocional-verona-acuicultura-ctaqua.aspx#.XC-CSVxKg2y
- Species workshop on grey mullet, http://www.ctaqua.es/180411-taller-workshop-lisa-diversify-bari-italia-acuicultura-ctaqua.aspx?id=1#.XC-B6lxKg2w
- Species workshop on pikeperch, http://www.ctaqua.es/180906-diversify-seminarios-ponencias-acuicultura.aspx#.XC-CnVxKg2x
- Grey mullet: potential of the species in aquaculture http://www.ctaqua.es/180626-potencial-lisa-diversify-acuicultura-ctaqua.aspx#.XC-Dp1xKg2x
- Species workshop on meagre, http://www.ctaqua.es/181128-diversify-resultados-corvina-diversificacion-acuicultura.aspx#.W_6PH-hKh

ARTICLES

DIVERSIFY has continued the close collaboration with the European Aquaculture Society editors and during the present reporting period, two more dedicated articles have been published in the magazine Aquaculture Europe, issue September 2018, Vol. 43(2) of the (**Fig. 31.1.8).**: the first article article covers the "Advances in larval and juvenile grey mullet (*Mugil cephalus*) culture: The DIVERSIFY project" and the second one is "Breakthrough in the reproduction and larval rearing of wreckfish in the DIVERSIFY project" (**Fig. 31.1.8).**

Moreover, thanks to the good collaboration established between DIVERSIFY and the European Aquaculture Society, a compilation issue of the Aquaculture Europe magazine including the 9 DIVERSIFY articles, has been published in October 2018. This compilation issue has been distributed (hard copies) during the Final Coordination and Dissemination meeting in Brussels and the online version is available at the DIVERSIFY web (https://www.diversifyfish.eu/) and at the European Aquaculture Society web (https://www.aquaeas.eu/) (**Fig. 31.1.9).**

WORKSHOPS

The workshops organized during the present reporting period have been dedicated to the Promotional Workshops of the project and to the Species Workshops ("Know-how transfer seminars for the aquaculture

industry presenting the progress achieved in DIVERSIFY in the technology for the six species of the project). These workshops will be described in detail in Task 31.5 and Task 31.6.

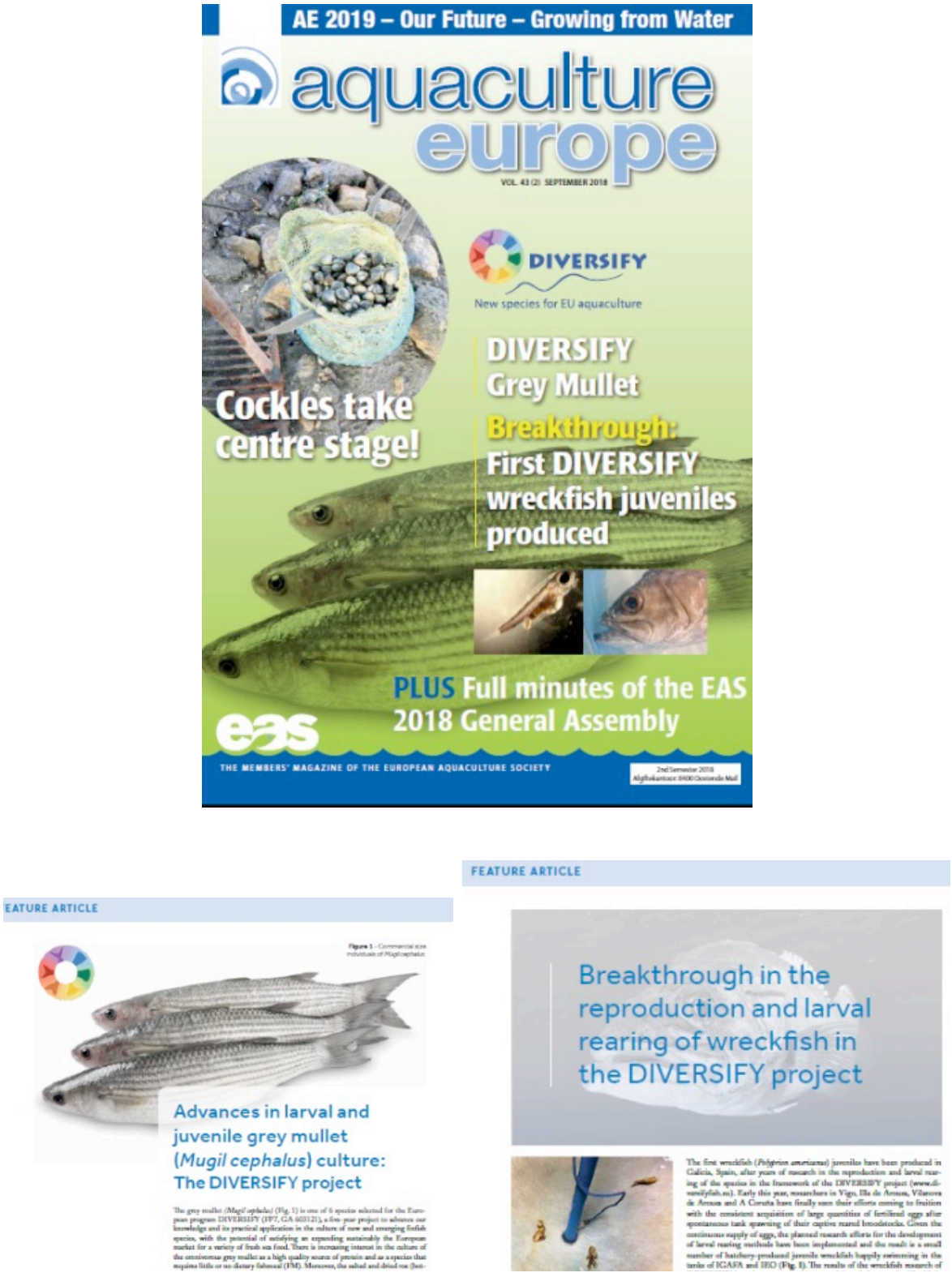

Figure 31.1. 16. Front page of the Aquaculture Europe magazine September 2018 issue, Vol. 43(2) (top) including the two articles on grey mullet (bottom left) and wreckfish larviculture (bottom right).

Figure 31.1. 17. Desktop capture with the front page of the DIVERSIFY compilation issue of the European Aquaculture magazine and the corresponding QR.

Task 31.2 Annual Coordination Meetings (led by HCMR, Constantinos Mylonas).

The full description of the work and results of the ACM 2018 (January 2018) has been submitted as ***Deliverable D1.10 Annual Coordination Meeting for Y5***. Also, the full description of the work and results of the Final ACM 2018 (November 2018) has been submitted as ***Deliverable D1.12 Annual Coordination Meeting (Nov 1028 Final)***. A brief presentation is also provided here.

The ACM 2018 was hosted by Drs. José Peréz and Covadonga Rodríguez (ULL), and Salvador Jerez and Virginia Martín (IEO). It was held at two venues between 23-25 January 2018 (**Fig. 31.2.1**). The task-specific presentations during Days 1 and 2 took place at the Faculty of Sciences, ULL. The Group Work Package (GWP) workshops took place at the IEO facilities in Santa Cruz. The 3-day meeting was attended by 79 persons: 78 coming from the DIVERSIFY consortium and only 1 invited guest from outside the consortium. No representative attended from Beneficiaries P25. DOR, P26. GEI, P27. FORKYS, P34. BVFi and 35. MASZ, the latter two having previous commitments that they could not modify, while the PI from P2. FCPCT was again missing (having missed four ACMs so far, out of a total of five).

As for all previous ACMs, information regarding the meeting was uploaded continually on the project's web site (**http://www.diversifyfish.eu/2018-annual-coordination-meeting-jan.html**) to ensure that all participants had access to the most updated information. The Agenda (**Tables 31.2.1 and 31.2.2**) was developed with assistance from GWP leaders and consisted of:

(a) DAY 1 and 2: A common session for all participants presenting Task-specific presentations from various WPs,
(b) DAY 2: A presentation of the WP 31 Dissemination presenting the dissemination activities of the consortium, and organizing the preparation of Deliverables as well as of manuscripts for scientific articles,
(c) DAY 2: Presentations by the Species leaders of the organization of the Species-specific Knowledge-transfer Workshops that will be held during 2018,
(d) DAY 2: Presentation by the Project Coordinator (PC) dealing with the status of the 3rd Periodic Report, financial issues, the preparation of Amendment 4, various management issues and the preparation of two (2) books from DIVERSIFY research.
(e) DAY 3: The Group Work Package (GWP) workshops for each scientific discipline, for the coordination of the work during Y5 of the project.


Figure 31.2.1. The two venues of the Annual Coordination Meeting 2018, held in Tenerife, Spain. The Faculty of Sciences, University of La Laguna (left) and the IEO facilities in Santa Cruz (right).

The morning session started with a welcoming by the following dignitaries, who honoured our meeting with their presences and expressed their great satisfaction for their organizations' participation in DIVERSIFY and in hosting the meeting:

1. The Vice Chancellor for Research of ULL, Dr. Francisco Almeida
2. The Dean of the Faculty of Sciences of ULL, Dr. Nestor Torres
3. The director of IEO Canary Islands, Dr. Luis Lopez

Then followed a presentation by the Project Coordinator (PC), Dr. C.C. Mylonas, presenting the Agenda for the meeting and covering some logistics. The format of the task-specific presentations for DAY 1 & 2 (**Table 31.2.1** and **31.2.2**), as it was adopted also for the ACM 2017 meeting in Barcelona, Spain, allowed a large number of the RTD partners to present their work (some for the first time) –which in many cases was done in collaboration with the SMEs and Large companies participating in the project, as well as work to be presented from all Scientific Disciplines. In total, 21 presentations from 16 RTD partners were presented, representing collaboration with the two large companies and six SMEs from the DIVERSIFY consortium.

At the end of the specific task presentations of Day 2, there was a presentation by the WP 31 Dissemination leader, Dr. Rocio Robles. As always, and to remind our partners of the great significance of this WP for our project, the presentation begun with a brief reiteration of the WP's many objectives, emphasizing the need for all Partners to participate actively in the preparation of dissemination materials and activities. Then there was a presentation of the various dissemination activities carried out during the 3rd Reporting Period (2016-2017), which included the publication of Newsletters that are uploaded at the website of the project and two more species-focused articles published at the quarterly magazine of the European Aquaculture Society (for Atlantic halibut and wreckfish), as well as a special 21-page featured article presenting work from DIVERSIFY in all species published in September 2017, just before the annual EAS meeting in Dubrovnik.

The Dissemination leader also discussed about the scientific articles that have been published so far and the number of manuscripts in preparation. The partners were encouraged to submit their work for publication as soon as the Deliverables are submitted, in order to disseminate the work carried out in the project.

Table 31.2.1. Agenda of DAY 1 of the Annual Coordination Meeting 2018, which took place on the 23-25 January 2018, at the Faculty of Sciences, University of La Laguna, Spain.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda **2018 Annual Coordination Meeting** **Tenerife 23-25 January 2018** **Uni La Laguna**

DAY 1		23-Iαν	Tuesday (Open Day presentations)		
Start	**End**		**Title**	**Presenter**	**Details**
8,00	**9,00**		**Registration**		**Pick up badges**
9,00	**9,30**		Welcome-Logistics	Perez, Jose Antonio & Mylonas, Constantinos	HCMR/ULL
9,30	**9,50**	**1**	Induced gametogenesis in flat-head grey mullet using recombinant gonadotropins	Ramos, Sandra	IRTA
9,50	**10,10**	**2**	Annual cycles of gonadotropins and sex steroids in plasma of farmed and wild-caught female Atlantic halibut	Norberg, Birgitta	IMR
10,10	**10,30**	**3**	Spawning induction of F1 greater amberjack in eastern Atlantic	Jerez, Salvador	IEO
10,30	**10,50**	**4**	Broodstock management and spawning induction in greater amberjack in tanks and sea cages in Greece	Mylonas, Constantinos	HCMR
10,50	**11,30**	**Coffee**			
11,30	**11,50**	**5**	Comparison of progammed and auto-demand type feeding of meagre in tanks	Duncan, Neil	IRTA
11,50	**12,10**	**6**	Effect of dietary fatty acids on spawn quality in greater amberjack broodstock	Djellata, Adnane	FCPCT
12,10	**12,30**	**7**	Some insights in lipid metabolism of larvae from novel aquaculture candidate species	Rodriguez, Covadonga	ULL
12,30	**12,50**	**8**	How to achieve predictable and stable juvenile production in marine fish- an industrial approach	Erstad, Borre	SWH
12,50	**13,10**	**9**	Effect of of phospholipids on lipid metabolism in Atlantic halibut	Sæle, Øystein	NIFES
13,10	**15,00**	**Lunch on site (Faculty of Sciences), 10 euro**			
15,00	**15,20**	**10**	Designing weaning diets based on the ontogeny of digestive tract enzyme activity during the carnivorous-omnivorous transition in grey mullet juveniles	Koven, Bill	IOLR
15,20	**15,40**	**11**	Greater amberjack larval rearing in IEO: effect of live prey enrichments and feeding regime	Martin, Virginia	IEO
15,40	**16,00**	**12**	Requirements for n-3 HUFA of meagre fingerlings	Carvalho, Marta	FCPCT
16,00	**16,20**	**13**	Test of different feeding methods on growth performance and feeding behavior of meagre	Papadakis, Ioannis	HCMR
16,20	**17,00**	**Coffee**			
17,00	**17,20**	**14**	Identification and expression of type I interferons in meagre	Secombes, Chris	UNIABDN
17,20	**17,40**	**15**	Dietary use of prebiotics in greater amberjack juveniles: effects on growth performance, immune gene expression and disease resistance against *Neobenedenia girellae*	Fernandez Montero, Alvaro	FCPCT
17,40	**18,00**	**16**	Epitheliocystis disease; results and progress	Katharios, Pantelis	HCMR
20,30		**Dinner at REAL CASINO de Tenerife, Plaza de la Candelaria 12 (consortium dinner)**			

Table 31.2.2. Agenda of DAY 2 of the Annual Coordination Meeting 2018, which took place on the 23-25 January 2018, at the Faculty of Sciences, University of La Laguna, Spain.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda **2018 Annual Coordination Meeting** **Tenerife 23-25 January 2018** **Uni La Laguna**

DAY 2 Start	End	24-Ιαν	Wednesday (Open Day presentations & Consortium Management) Title	Presenter	Details
8,00	9,00		Registration		Pick up badges
9,00	9,20	1	Overview of consumer behavior-related affairs in the frame of DIVERSIFY and their key findings	Krystallis, Thanassis	HRH
9,20	9,40	2	The effect of message framing on consumers' attitudes and purchase intentions towards new DIVERSIFY products	Banovic, Marija	AU
9,40	10,00	3	Feasibility study, the contributions of partners needed	Stokkers, Robert / Tacken,Gemma	SWR/DLO
10,00	10,20	4	Business model and marketing strategy development	Nijssen, Ed / van der Borgh, Michel	TU/e
10,20	10,40	5	EU funded projects: why communication matters?	Abundancia, Carlos	EUFIC
10,40	11,30		**Coffee**		
11,30	12,30		Dissemination activities, articles and species leaflets	Robles, Rocio	CT-AQUA
12,30	13,00		Meagre one-day workshop	Estevez, Alicia	IRTA
13,00	13,30		Greater amberjack one-day workshop	Papandroulakis, Nikos	HCMR
13,30	15,00		**Lunch on site (Faculty of Sciences), 10 euro**		
15,00	15,30		Pikeperch one-day workshop	Fontaine, Pascal	UL
15,30	16,00		Atlantic halibut one-day workshop	Norberg, Birgita	IMR
16,00	16,30		Wreckfish one-day workshop	Alvarez, Blanca	IEO
16,30	17,00		Grey mullet one-day workshop	Koven, Bill & Corriero, Aldo	IOLR/UNIBA
17,00	17,30		**Coffee**		
17,30	18,15		Preparation of books on meagre and amberjack biology and culture	Mylonas, Constantinos	HCMR
18,15	19,00		3rd Periodic Report, Amendment 4, Budget and Deliverables - General Asssembly	Mylonas, Constantinos	HCMR
Dinner on your own, explore the city!					

The **ACM 2018 Final** was hosted by Matthias Keller (P34. BVFi) and Carlos Abundancia (P38. EUFIC). It was held in the Thon Hotel Brussels Center on the 22-23 November 2018 (**Fig. 31.2.2**). As proposed during the previous ACM 2018 held in Tenerife, (Spain) in January 2018, and according to the suggestion of our EU Scientific Officer Dr. Marta Iglesias, this final ACM was held in Brussels (Belgium). This central location was decided in order to be able to invite a large number of relevant officers from the European Commission, the Parliament and relevant stakeholders that have their headquarters nearby. As we had already completed the six "Species-specific Knowledge Transfer workshops", which were organized between May and October 2018 with the objective of disseminating the acquired knowledge to

the aquaculture industry (farmers, feed companies, veterinarians, etc.), we considered important to broadcast the results of the project to this different audience.

Figure 31.2.2. The venue of the Final Annual Coordination Meeting (ACM) 2018, held in Brussels, Belgium.

Invitations went out to specific persons suggested by our EU Scientific Officer, and also to other people and organizations decided by the Project Coordinator and the Dissemination leader. These included officers from the EC such as DG RTD, DG MARE and DG ENVIRONMENT, REA, members of the European Parliament Fisheries Committee, the European Aquaculture Technology and Innovation Platform (EATIP), the Federation of European Aquaculture Producers (FEAP), the European Aquaculture Society (EAS), the Aquaculture Advisory Council (AAC), and the European Market Observatory for fisheries and aquaculture (EUMOFA) and the International Union for Conservation of Nature (IUCN). A total of 16 people from the above organizations accepted our invitation (**Fig. 31.2.2**), while a number of people excused their absence.

The 1-day meeting was attended by 58 persons: 43 coming from the DIVERSIFY consortium and 15 invited guests from the list. No representative attended from Beneficiaries P2. FCPCT, P5. UNIABD (PI was retired), P14. IFREMER (PI was retired), P23. ARGO (death in the family) P25. DOR, P26. GEI, P27. FORKYS, P28. CANEXMAR, P31. IRIDA (prior commitment). The PI from P2. FCPCT did not attend the meeting (as in the previous five occasions) and the GWP for Nutrition belonging to the same institution could not attend due to economical limitations of his institution. Nevertheless, once the Project Coordinator was aware of the problem, he offered financing for the travel; however, nobody from the institution attended the final project meeting.

As it has been done for all previous ACMs, the information regarding the meeting has been uploaded on a continuous basis on the project's web site (https://www.diversifyfish.eu/2018-final-coordination-meeting-nov.html) to ensure that all participants had access to the most updated information. The Agenda (**Tables 31.2.3 and 4**) was developed with assistance from GWP leaders and consisted of:

(a) DAY 1: A dissemination session open to outside guests, where summary presentations were made for the work achieved in each of the six species, in Fish health, Socioeconomics and New Product Development, and in Dissemination,
(b) DAY 2: Presentation by the Project Coordinator (PC) dealing with the preparation of the 4th Periodic and Financial Report, and the preparation of the Final Report; and presentation by the WP31 Dissemination leader Dr. Rocio Robles discussing issues related to preparation of Scientific papers and to the continuation of dissemination activities beyond the end of the project.

The morning session started with a welcoming by the Project Coordinator Dr. Constantinos C. Mylonas who gave a brief presentation regarding the project and the consortium, some highlights of major achievements and a presentation of the Agenda for the meeting (**Fig. 31.2.3 and Table 31.2.3**). Then the Species Leaders continued with the presentations on the objectives and achievements in all six species included in the project.

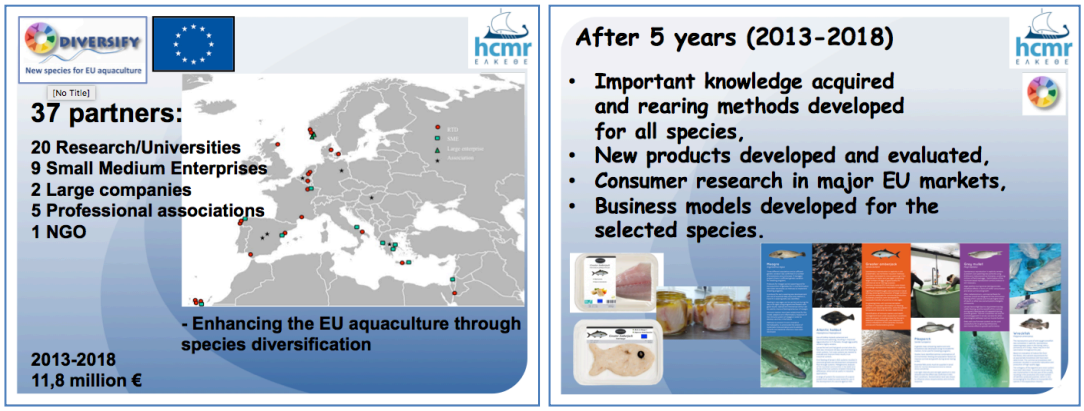

Figure 31.2.3. Representative slides from the presentation of the PC at the Final ACM 2018, in Brussels.

Table 31.2.3. Agenda of DAY 1 of the Final ACM (Nov 2018), which took place on the 22-23 November 2018, in Brussels, Belgium.

DIVERSIFY					
7FP-KBBE-2013-603121					
Meeting Agenda		2018 Final Coordination Meeting		Brussels 22-23 November 2018	Thon Hotel
DAY 1	22-Nov		Thursday (Open Day presentations)		
Start	End		Title	Presenter	Details
8,30	9,30		Registration		Pick up badges
9,30	10,00	1	The DIVERSIFY project	Dr Constantinos Mylonas (HCMR, Greece), Project Coordinator	
10,00	10,30	2	The meagre (*Argyrosomus regius*): objectives and progress	Dr Alicia Estevez (IRTA, Spain), meagre leader	
10,30	11,00	3	The greater amberjack (*Seriola dumerili*): objectives and progress	Dr Nikos Papandroulakis (HCMR, Greece), greater amberjack leader	
11,00	11,30		Coffee		
11,30	12,00	4	The pikeperch (*Sander luciperca*): objectives and progress	Dr Pascal Fontaine (UL, France), pikeperch leader	
12,00	12,30	5	The Atlantic halibut (*Hippoglossus hippoglossus*): objectives and progress	Dr Birgitta Norberg (IMR, Norway), Atlantic halibut leader	
12,30	13,00	6	The wreckfish (*Polyprion americanus*): objectives and progress	Dr Blanca Alvarez (IEO, Spain), wreckfish leader	
13,00	14,30		Lunch on site		
14,30	15,00	7	The grey mullet (*Mugil cephalus*): objectives and progress	Dr Bill Koven (IOLR, Israel), grey mullet leader	
15,00	15,30	8	Perspectives of the DIVERSIFY species and the European aquaculture market	Dr Gemma Tacken (SWR/DLO, Netherlands) Socioeconomics leader	
15,30	16,00	9	Technical assessment of the DIVERSIFY species and new product development	Dr Lluis Guerrero (IRTA, Spain)	
16,00	16,30	10	Fish health	Dr Pantelis Katharios (HCMR, Greece)	
16,30	17,00		Coffee		
17,00	17,30	11	Dissemination	Dr Rocio Robles (CTAQUA, Spain), dissemination leader	
17,30	18,00	12	Future directions-discussion	Dr Constantinos Mylonas (HCMR, Greece), Project Coordinator	
20,00			Dinner at local restaurants		

Following the species-specific presentation, the leader of the Socioeconomics WPs Dr. Gemma Tacken (SWR/DLO, Netherlands) gave a presentation on the "Perspectives of the DIVERSIFY species and the European aquaculture market", and Dr Lluis Guerrero (IRTA, Spain) presented the "Technical assessment of the DIVERSIFY species and new product development". Then, Dr Pantelis Katharios (HCMR, Greece) gave a presentation on the Fish health work carried out in the project.

The presentations closed with the Dissemination leader Dr Rocio Robles' who presented a summary of the dissemination activities carried out during the project. Special emphasis was given to the Promotional Workshops and the Species Workshops (Know-how transfer for the project species).

The Promotional Workshops were one-day workshops organized in specific countries where fish production and processing are relevant economic activities. The workshops were designed for specific audience, such as fish producers, processors and retailers, consumer organizations, and fisheries and aquaculture authorities. Four Promotional Workshops have been successfully organized in Germany, Spain, Italy and Greece with the collaboration of the fish processors' associations and fish producers' associations of the project. Project achievements with special emphasis on the Socioeconomical research area were presented to the audience.

The Species Workshops were "Know-how Transfer" seminars for the aquaculture industry, presenting the progress achieved through DIVERSIFY in the production technology of each of the six project species. The seminars included presentations on selected aspects of the culture given by DIVERSIFY Partners, but also from some authorities in the species, whose work was considered relevant. Fish farmers, feed companies, European aquaculture support companies (feed, pharmaceutical, equipment, engineering, etc.), researchers and educators and government organizations have been part of these meetings. Six Species Workshops have been organized in Italy (for grey mullet), France (pikeperch), Spain (wreckfish and meagre), Norway (Atlantic halibut) and Greece (greater amberjack). A Technical Manual for each species has been elaborated and uploaded in the website of DIVERSIFY. In collaboration with the producers' and processor associations, the Technical Manuals have been translated to other EU languages: Spanish (APROMAR and ANFACO) and German (BVFi). Translated Manuals are also available in the project web.

All the presentations from the Promotional workshops (https://www.diversifyfish.eu/promotional-workshops.html) and the Species Workshops (https://www.diversifyfish.eu/species-workshops.html) are available in the project website.

The preparation of the 4th Periodic Report and the Final Report was the focus of the presentation by the PC during the Day of the meeting (**Table 31.2.4**), which was only for consortium members. As before, the partners were given instructions as to the process that was going to be followed for the preparation of the 4th Periodic Report, which covered the last 12 months of the project. The PC would prepare the format documents for all WPs, which would include summaries of the worked done in previous reporting periods, so the partners would only have to include the data obtained in the last reporting period. The time schedule for the conclusion of the various steps was agreed.

Table 31.2.4. Agenda of DAY 2 of the Final ACM (Nov 2018), which took place on the 22-23 November 2018, in Brussels, Belgium.

DIVERSIFY 7FP-KBBE-2013-603121					
Meeting Agenda		2018 Final Coordination Meeting		Brussels 22-23 November 2018	Thon Hotel
DAY 2	23-Nov	Friday (Consortium Management)			
Start	End		Title	Presenter	Details
8,00	9,00		Registration		Pick up badges
9,00	10.00		Scientific and Financial Reporting	Constantinos Mylonas, PC (HCMR)	
10.00	11.00		Dissemination activities after the end of the project	Rocio Robles, (CTAQUA)	
11.00	11.30		Coffee		
11.30	13.00		Future actions for the promotion of DIVERSIFY II	Constantinos Mylonas, PC (HCMR)	
13.00	15,00		Lunch at a local restaurant for the ones staying the day in Brussels		

Task 31.3 Presentation of DIVERSIFY at the AQUA EUROPE meetings (led by CTAQUA, Rocio Robles).

During the present reporting period, DIVERSIFY work has been presented at the 2018 Aquaculture Europe conferences, organized by the European Aquaculture Society and organized in Montpellier, France. The AQUACULTURE EUROPE 2018 conference was held between 25-29 August 2018 (**Fig. 31.3.1**). This year the WP 31 Dissemination leader Dr Rocio Robles gave a presentation in the Special Session "Socio-economics".

Figure 31.3.1. The announcement poster of AQUACULTURE EUROPE 2018 (left) organized every year by the European Aquaculture Society, and a representative slide of the summary presentation of DIVERSIFY during the Socio-economic session presented by the Dissemination leader**.**

Major results of the project in the different research areas were presented according to the species: meagre, greater amberjack, halibut, pikeperch, grey mullet and wreckfish. The socioeconomic work was described more in detail in accordance with the topic of the session where DIVERSIFY was presented. Main objectives of the work package were introduced**.** Consumer segmentation, new product development (meagre, amberjack, grey mullet and pikeperch), consumer evaluation of these products and business model for each DIVERISFY species were addressed during the talk. The developed products were: fresh frozen greater amberjack filet, ready-to-eat meagre salad, grey mullet in olive oil, smoked grey mullet filet, pikeperch hamburger with the shape of a fish and fish pate (made with pikeperch)**.**. The products were selected to cover an ample range of processing, following an increasing degree of processing (from the fresh filet to the pate). The degree of processing was an important part of the consumer's evaluation. Moreover, an o-line market has been also performed and the results are under evaluation at this moment.

The full description of this task is provided in ***Deliverable 31.28. Annual presentation of DIVERSIFY (Y4) 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).

Presentations of DIVERSIFY work have also been given in other International conferences held in Europe and around the world as it is shown in **Table 31.1.1.** Some of these conferences are: Advances in Fish Reproduction and their Application to Broodstock Management, organized by CIHEAM at Torre de la Sal, Castellón, Spain; the 11th International Symposium on fish reproductive Physiology, 4-8 June 2018, Manaus, Brazil; the International Symposium of fish nutrition and feeding, 3-7 June 2018, Las Palmas de Gran Canaria and the International Congress on Applied Ichthyology and Aquatic Environment, 8-11 November 2018, Volos, Greece.

Submission of manuscripts to scientific journals

The following list contains the scientific articles already published in pre-reviewed scientific journals:

Alexi, N., Byrne, D.V., Nanou, E., Grigorakis, K., 2018a. Investigation of sensory profiles and hedonic drivers of emerging aquaculture fish species. Journal of the Science of Food and Agriculture 98, 10.1002/jsfa.8571.

Alexi, N., Nanou, E., Lazo, O., Guerrero, L., Grigorakis, K., Byrne, D.V., 2018b. Check-All-That-Apply (CATA) with semi-trained assessors: Sensory profiles closer to descriptive analysis or consumer elicited data? Food Quality and Preference 64, 11-20.

Andree, K.B., Roque, A., Duncan, N., Gisbert, E., Estevez, A., Tsertou, M.I., Katharios, P., 2015. Diplectanum sciaenae (Van Beneden & Hesse, 1863) (Monogenea) infecting meagre, Argyrosomus regius (Asso, 1801) broodstock in Catalonia, Spain. A case report. Veterinary Parasitology: Regional Studies and Reports 1–2, 75-79.

Baekelandt, S., Redivo, B., Mandiki, S.N.M., Bournonville, T., Houndji, A., Bernard, B., El Kertaoui, N., Schmitz, M., Fontaine, P., Gardeur, J.N., Ledore, Y., Kestemont, P., 2018. Multifactorial analyses revealed optimal aquaculture modalities improving husbandry fitness without clear effect on stress and immune status of pikeperch *Sander lucioperca*. Gen Comp Endocrinol 258, 194-204.

Baekelandt, S., Mandiki, S.N.M., Schmitz, M., Kestemont, P., 2019. Influence of the light spectrum on the daily rhythms of stress and humoral innate immune markers in pikeperch Sander lucioperca. Aquaculture 499, 358-363.

Banović, M., Krystallis, A., Guerrero, L., Reinders, M.J., 2016. Consumers as co-creators of new product ideas: An application of projective and creative research techniques. Food Research International 87, 211-223.

Campoverde, C., Estevez, A., 2017. The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae. Aquaculture 478, 16-24.

Campoverde, C., Milne, D.J., Estévez, A., Duncan, N., Secombes, C.J., Andree, K.B., 2017a. Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*). Fish & Shellfish Immunology 69, 200-210.

Campoverde, C., Rodriguez, C., Perez, J., Gisbert, E., Estevez, A., 2017b. Early weaning in meagre Argyrosomus regius: Effects on growth, survival, digestion and skeletal deformities. Aquaculture Research 48, 5289-5299.

Campoverde, C., Andree, K.B., Milne, D.J., Estevez, A., Gisbert, E., Carella, F., 2018. Ontogeny of lymphoid organs and mucosal associated lymphoid tissues in meagre (Argyrosomus regius). Fish Shellfish Immunol.

Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M., 2018. Dietary requirement for n-3 long-chain polyunsaturated fatty acids for fast growth of meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture 488, 105-113.

Colchen, T., Faux, E., Teletchea, F., Pasquet, A., 2017. Is personality of young fish consistent through different behavioural tests? Applied Animal Behaviour Science 194, 127-134.

Colchen, T., Fontaine, P., Ledoré, Y., Teletchea, F., Pasquet, A., 2019. Intra-cohort cannibalism in early life stages of pikeperch. Aquaculture Research 0, 1-10.

Duncan, N.J., Mylonas, C.C., Milton Sullon, E., Karamanlidis, D., França Nogueira, M.C., Ibarra-Zatarain, Z., Chiumento, M., Aviles Carrillo, R.O., 2018. Paired spawning with male rotation of meagre *Argyrosomus regius* using GnRHa injections, as a method for producing multiple families for breeding selection programs. Aquaculture 495, 506-512.

El Kertaoui, N., Hernández-Cruz, C.M., Montero, D., Caballero, M.J., Saleh, R., Afonso, J.M., Izquierdo, M., 2017. The importance of dietary HUFA for meagre larvae (Argyrosomus regius; Asso, 1801) and its relation with antioxidant vitamins E and C. Aquaculture Research 48, 419-433.

Fakriadis, I., Lisi, F., Sigelaki, I., Papadaki, M., Mylonas, C.C., 2018. Spawning kinetics and egg/larval quality of greater amberjack (Seriola dumerili) in response to multiple GnRHa injections or implants. Gen Comp Endocrinol.

Fernández-Montero, A., Caballero, M.J., Torrecillas, S., Tuset, V.M., Lombarte, A., Ginés, R.R., Izquierdo, M., Robaina, L., Montero, D., 2017. Effect of temperature on growth performance of greater amberjack SERIOLA DUMERILI Risso 1810) Juveniles. Aquaculture Research 49, 908-918.

Fernández-Montero, Á., Torrecillas, S., Izquierdo, M., Caballero, M.J., Milne, D.J., Secombes, C.J., Sweetman, J., Da Silva, P., Acosta, F., Montero, D., 2019. Increased parasite resistance of greater amberjack (Seriola dumerili Risso 1810) juveniles fed a cMOS supplemented diet is associated with upregulation of a discrete set of immune genes in mucosal tissues. Fish & Shellfish Immunology 86, 35-45.

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.

Grigorakis, K., 2017. Fillet proximate composition, lipid quality, yields, and organoleptic quality of Mediterranean-farmed marine fish: A review with emphasis on new species. Crit Rev Food Sci Nutr 57, 2956-2969.

Jerez, S., Fakriadis, I., Papadaki, M., Martín, M., Cejas, J., Mylonas, C.C., 2018. Spawning induction of first-generation (F1) greater amberjack *Seriola dumerili* in the Canary Islands, Spain using GnRHa delivery systems. Fishes 3, 1-22.

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At the moment of writing this report, the following articles have been submitted for publication and are under revision by the referees:

- Banovic, M. Reinders, M.J. Claret, A. Guerrero, L. and Krystallis, A. "One Fish, Two Fish, Red Fish, Blue Fish" or How Ethical Beliefs Impact "Blue" Products Purchase Intention?", *Journal of Business Ethics* –
- Banovic, M. Reinders, M.J. Claret, A. Guerrero, L. and Krystallis, A. "Take it or leave it: Impact of eco-label, health and nutrition claims, and country-of-origin on consumer choice of aquaculture products", *Food Policy* .

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)

During the last year of the project, six full-day seminars have been organized in different European countries. The objective of this task is to organize a "Know-how Transfer" seminar for the aquaculture

industry, presenting the progress achieved through DIVERSIFY in the production technology of the DIVERSIFY finfish species. The seminars include 30 min presentations on selected aspects (e.g., reproduction and spawning induction, final product diversification and quality, socioeconomic issues and marketing, etc.), given by DIVERSIFY partners, but also from any authorities in the species, whose work was not part of the project. Aquaculturists (mainly), but also European aquaculture support companies (feed, pharmaceutical, equipment, engineering, etc.), researchers and educators, government organizations and other important institutions were invited to attend these meetings.

From each of the Species Seminars a Technical Manual have been elaborated and uploaded in the DIVERSIFY website for free downloading https://www.diversifyfish.eu/species-workshops.html . Likewise, all the presentations from all the Species Seminars are available in the project web. The Seminars started with the organization of the **Grey mullet Species Seminar.** The workshop was organized jointly by Dr Aldo Corriero (P13. UNIBA) and Dr Bill Koven (P4. IOLR), the latter being the Species Leader for grey mullet. It was held in Palace Hotel, Bari, Italy on the 14th of May 2018, and was advertised via an announcement sent to a number of potential stakeholders, such as National Producers associations, FEAP, EAS, DG-Mare, DG R&I, FAO, etc. as well as via the DIVERSIFY website, which includes a dedicated page (**Fig. 31.5.1**).

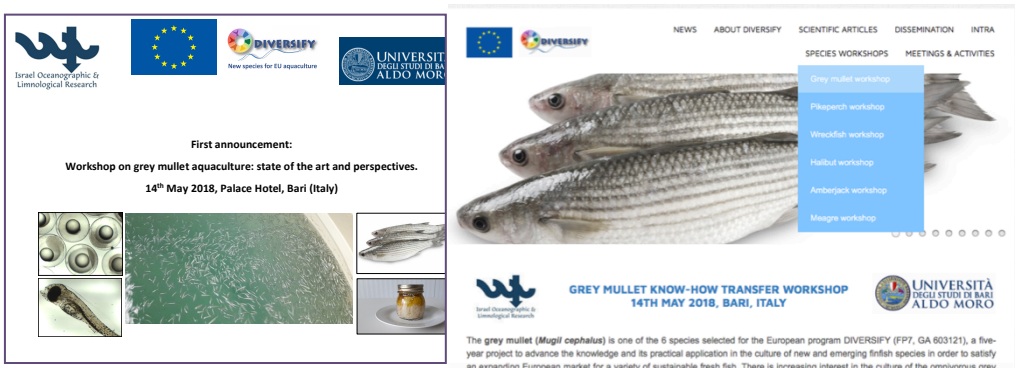


Figure 31.5.1. Desktop captures of the Grey mullet Species Seminar announcement (left) and the dedicated page for the workshop in the DIVERSIFY website**.**

The full description of the Grey mullet Species Seminars has been already fully described in the **Deliverable 31.34 "Know-how Transfer" seminar for the aquaculture industry (Italy), presenting the progress achieved in DIVERSIFY in the technology for grey mullet**.

The second seminar was dedicated to **pikeperch** and it was organized by the Species leader Dr. Pascal Fontaine from the University of Lorraine, Nancy (France). The seminar for pikeperch has been organized over one full day during Y5 (27th June, 2018). A total of 68 people attended the seminar (**Fig. 31.5.2**), including pikeperch producers, feed producers, aquaculture systems designers (RAS), consultants in aquaculture, scientists and representatives from the administration. The participants were coming 10 different European countries, mainly from Belgium, Denmark, France, Germany and Switzerland (at least 6 participants pre country). Seven presentations from Diversify partners and 4 from invited speakers were included in the agenda of the event.

The full description of the work and results is provided in ***Deliverable 31.31. Pikeperch "Know-how Transfer" seminar for the aquaculture industry (potential location: France, Belgium, Denmark), presenting the progress achieved through DIVERSIFY in the production technology***.

Figure 31.5.2. General view of the attendees to the pikeperch seminar at the aula of the Faculty of Sciences and Technologies (University of Lorraine, Nancy, France) (left) and a desk-capture of the website with the information on the Seminar.

The following seminar was the Wreckfish Species Seminar, organized in Vigo, Spain. The seminar has been organized during the19th and the 20th of July 2018 at the facilities of the Instituto Español de Oceanografía, IEO of Vigo (Pontevedra- Spain). Major achievements on the culture of the species were presented during the full-day seminar (day 19th). The seminar was included 8 presentations from DIVERSIFY partners and 3 presentations from wreckfish experts outside the consortium.

In 2018 wreckfish larvae of more than 25 day have been obtained and are grown at the facilities of Igafa, Galicia (Spain). This centre holds one of the wreckfish broodstock batches as well as the first fry of wreckfish obtained in 2018 **(Fig. 31.5.3).** As it has been done for all the Species Seminars, a Technical Manual of the species has been elaborated and make available at the project website.

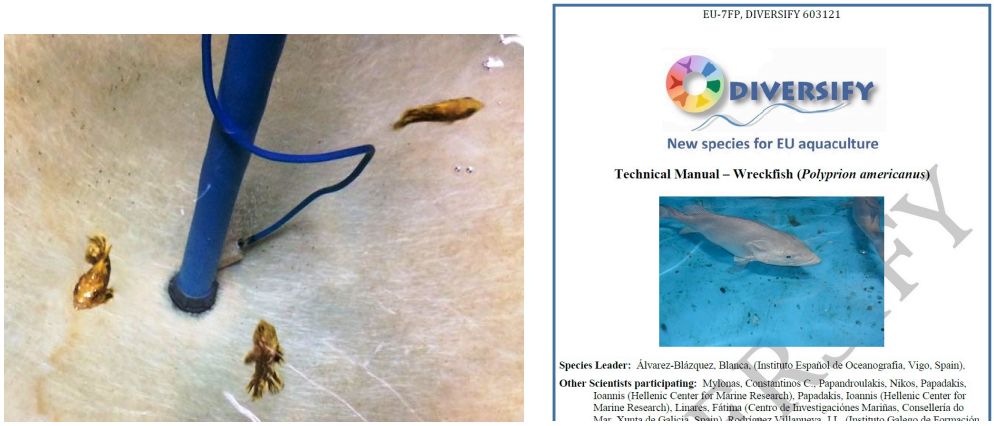


Figure 31.5.3. First wreckfish juveniles obtained in captivity at Igafa (left) and front page of the Technical Manual for wreckfish (right).

The full description of the work and results is provided in ***Deliverable 31.33. Wreckfish "Know-how Transfer' seminar for the aquaculture industry (Spain), presenting the progress achieved in DIVERSIFY in the production technology***.

The Atlantic halibut Species Seminar was held on September 11, 2018, at SPA Hotel Velvære, Hjelmeland, Norway. A specially prepared dinner, with five different halibut courses, was arranged at the main office of P.22, Sterling White Halibut. All active European Atlantic halibut producers were invited and were present at the workshop. A number of presentations were given and the most important results from the

DIVERSIFY project were presented by the project partners. The seminar was followed by an excursion to the production sites (**Fig. 31.5.4)** of Sterling White Halibut on September 12.

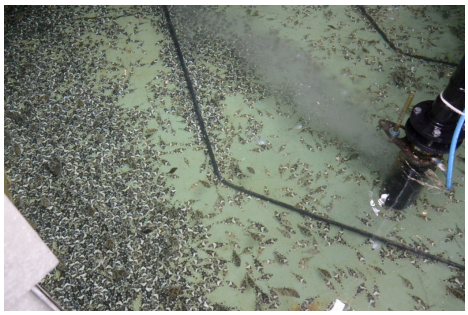


Fig. 31.5.4. Atlantic halibut juveniles in the culture tank.

The full description of the work and results is provided in ***Deliverable 31.32. Atlantic halibut "Know-how Transfer" seminar for the aquaculture industry presenting the progress achieved through DIVERSIFY in the production technology***.

For the **greater amberjack** Species Seminar, a full-day seminar was held on September 18, 2018, at Electra Metropolis Hotel, Athens, Greece (**Fig.35.5.5)**. The meeting was attended by 84 persons, coming mainly from the Aquaculture industry in Greece (Nireus SA., Selonda SA and Andromeda SA), but also Spain, France and Turkey, as well as from feed companies (INVE SA, BIOMAR) and national funding organizations. A number of presentations were given and the most important results from the DIVERSIFY project were presented by the project partners.

As in the case of the previously reported seminars, the full description of the work and results is provided in ***Deliverable 31.32. Greater amberjack "Know-how Transfer" seminar for the aquaculture industry presenting the progress achieved through DIVERSIFY in the production technology***.

Figure 185.5.5. Representative slides from the presentations of DIVERSIFY researchers**.**

Last Species Seminar was dedicated to **meagre.** The seminar was held on October 9, 2018, at Palau Macaya, Barcelona, Spain. The seminar included brief presentations on selected aspects given by DIVERSIFY partners, including reproduction and artificial fertilization, nutrition of larvae and juveniles, feeding behaviour, cage culture husbandry, final product diversification and quality, socioeconomic issues and marketing, etc. The workshop was attended by 38 people from various European countries representing commercial aquaculture companies, feed producers and veterinarians, but also researchers and University professors (**Fig. 35.5.6**).

Figure 35.5.6. DIVERSIFY partners with the banner of the workshop (left) and audience attending the workshop (right).

The full description of the work and results is provided in ***Deliverable 31.29. Meagre "Know-how Transfer" seminar for the aquaculture industry presenting the progress achieved through DIVERSIFY in the production technology***.

Task 31.6 Promotional workshops (led by CTAQUA, Rocio Robles).

During the last two years of the project, specialized one-day workshops are organized in specific countries where fish production and processing are relevant economic activities. The workshops are designed for specific audience, such as fish producers, processors and retailers, consumer organizations, and fisheries and aquaculture authorities

Two Promotional workshops were already organized and reported in the 3rd Reporting Period:

- 1st Promotional Workshop held in Bremen Germany
- 2nd Promotional Workshop held in El Puerto de Santa Maria, Cádiz, Spain.

The complete description of the work and results have been submitted as *Deliverable 31.16 Promotional Workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) 1st Workshop* and as *Deliverable 31.18 Promotional Workshops (2nd) for specialized audience in fish market sector (Spain, Greece, UK or Italy).*

In the present reporting period (Mo 49-60), the two remaining Promotional Workshops have been organized in Italy (3rd Promotional Workshop) and Greece (4th Promotional Workshop). Likewise the previous workshops, the complete description of the work and results have been submitted as the corresponding deliverable. *Deliverable 31.23 Promotional Workshops (3rd) for specialized audience in fish market sector (Spain, Greece, UK or Italy)* and as *Deliverable 31.27 Promotional Workshops (4th) for specialized audience in fish market sector (Spain, Greece, UK or Italy).* A summary of both events is provided in the following paragraphs.

The **3rd Promotional Workshop** has been organized in Verona, Italy, which is the location of the headquarters of the Italian fish Producer Association (API, **Associazione Piscicoltori Italiani** in Italian). The Association is a **non-profit corporation. I**t promotes any financial, scientific, technical, insurance, professional, union and legal interventions which may prove necessary to reach this target.

The agenda of the event was distributed to fish farmers, fish processing and fish industry stakeholders in Italy as well as to the members of API. The meeting was organized including five presentations from DIVERSIFY partners and a presentation from Andrea Fabris, Director of API, who gave a view on the aquaculture production in Italy (**Fig. 31.6.1)**. After the six presentations and the following debate, it was organized a degustation session with one of the products developed within the project: meagre pate (WP 28 Socioeconomics: New product development prepared by IRTA).

Figure 191.6.1. Left: Representative slides of the presentation of Andrea Fabris *"Aquaculture production in Italy "*; right: Vladimir (API secretary); Rocio Robles (DIVERSIFY Dissemination leader) and Andrea Fabris (API Director) (from left to right).

After the presentations, there was an interesting debate where the fish producers claimed more attention for the actual Mediterranean species to develop new products before starting with new species. Certain concerns on the carnivorous feeding habits of most DIVERSIFY species lead to interesting discussion with the audience. We provided information on the reasoning behind the species selection of the project and also on the actual moment of the fish feed industry where a substantial fish meal replacement is being applied to the fish feed formulations with alternative sources of protein.

The Species Seminars were also presented during the debate. The audience was very interested in this type of knowledge transfer. Frequent visit to our web site was indicated as the best way to be updated on the events. The complete description of the workshop has been submitted as ***Deliverable 31.23 Promotional Workshops (3rd) for specialized audience in fish market sector (Spain, Greece, UK or Italy).***

The **4th Promotional Workshop** was organized in close collaboration with the Federation of Greek Mariculture (P.30) in the meeting room Syntagma of the Hotel Central, 21 Apollonos str. Athens, Greece. The meeting was attended by 22 people, including sales responsible from the main aquafarms in Greece, fish farmers, universities and administration representatives. The meeting started with an update of the project achievements in all the research areas, presented by the Dissemination leader, Rocio Robles from Ctaqua (P.18), Spain. Five more presentations from the DIVERSIFY partners provided results and relevant information on the Socioeconomic Research area of the project.

The presentations were followed by the debate "Consumer attitude to diversification in aquaculture fish products: trust of consumer in aquaculture products, sustainability and health- related behaviour*"* which was moderated by Rocio Robles (CTAQUA) y Gemma Tacken (SWR). The debate counted with the active participation of the audience that raised interesting questions on marketing of aquaculture products. The companies in Greece see market opportunities mainly with greater amberjack, since it is unique, and also with meagre. Marketing results presented during the workshop were very appreciated by the companies present in the audience.

The Species Seminars were also presented during the debate. The audience was very interested in this type of knowledge transfer. Frequent visit to our web site was indicated as the best way to be updated on these events (https://www.diversifyfish.eu/species-workshops.html) . The workshop ended with a degustation *in situ*, of grey mullet filet preserved in olive oil and canned meagre pate prepared by CTAQUA and IRTA respectively (new fish products developed within the DIVERSIFY project) (**Fig.31.6.2)**.

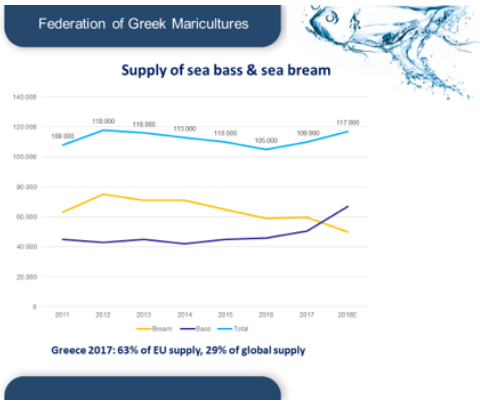


Figure 31.6.2. View of the Syntagma meeting room during the presentations (left) and representative slide from the talk given by Ioannis Pelekanakis (FGM, P.30) showing the market share of farmed fish from Greece in EU and in the world.

The complete description of the workshop has been submitted as ***Deliverable 31.27 Promotional Workshops (4th) for specialized audience in fish market sector (Spain, Greece, UK or Italy***).

Task 31.7 Dissemination to the food industry and consumers (led by APROMAR and EUFIC, Javier Ojeda and Laura Fernández).

The objective of this task is the promotion project results to processors, retailers, caterers, food scientists, etc. During the present reporting period, P.37 EUFIC has produced and disseminate a series of video interviews with the occasion of the final Coordination and Dissemination meeting in Brussels. PC, (CC Mylonas), Dissemination leader (Rocio Robles), Socioeconomic Work-package leader (Gemma Tacken), IRTA product development responsible (Luis Guerrero) and the Project Officer (Marta Iglesias) were interviewed during the meeting. These short interviews aimed to target the general public. They not only summarised the main results of the project but also tackled general misconceptions around the topic of aquaculture. The questions asked were selected with the help of the PC and the Dissemination leader (**Fig. 31.7.1)**. They can be found on YouTube .
Concerning the elaboration and publication of the second dedicated article about DIVERSIFY in Food Today to summarised the main findings of the project, as the final project leaflet already accomplished this objective, it was internally agreed (with the approval of the PC) to substitute the production of the dedicated article with the recording of the final Coordination and Dissemination meeting on video as well as several short interviews. The recording and editing of the videos was done externally by the company Focusbiz.

All 12 sessions from the final meeting were recorded, edited and made available on YouTube. In January, they will be published on both the EUFIC and DIVERSIFY websites, and disseminated through Twitter via @SciFoodHealth (a EUFIC-run account communicating on food & health EU-research projects with more than 12K followers) and @diversifyfish, the main account of the project. They will be available on both the EUFIC and DIVERSIFY websites. Plus, they will be promoted individually through the two social media channels.

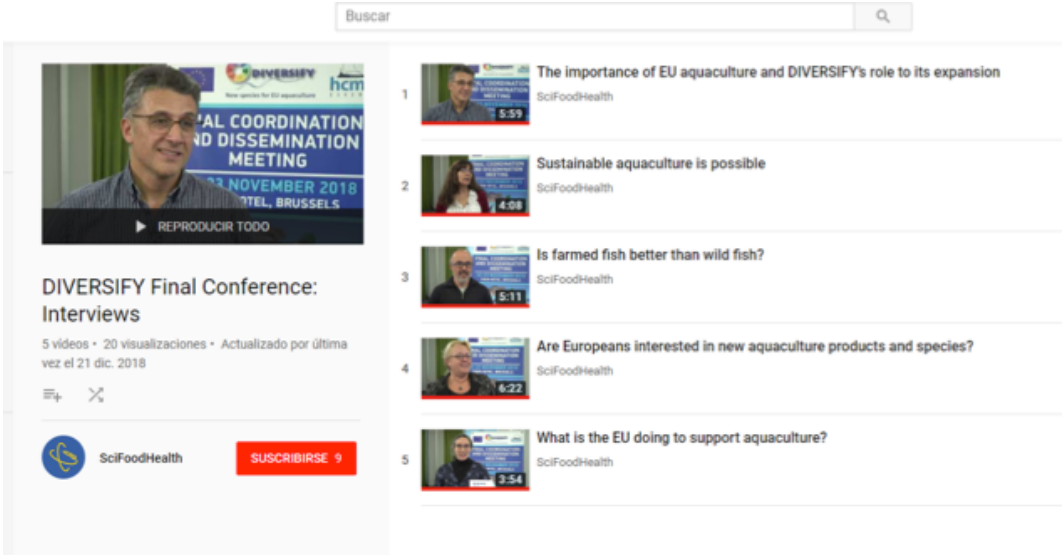


Figure 31.7.1. Screen capture of the series of video interviews with the occasion of the final Coordination and Dissemination meeting in Brussels in YouTube . .

The full description of this work has been submitted as ***Deliverable 31.26 Audio-visual popularization document and publication of the second article in Food Today, electronic journal of EUFIC***.

With regard to the Technical Manuals produced for each of the DIVERSIFY species, they have been translated to Spanish (by ANFACO and APROMAR) and German (BVFi). They are free for downloading at the project web site (**Fig. 31.7.2).** ANFACO-CECOPESCA has contributed to the dissemination of the project through the distribution of the project leaflet in the events attended by the staff involved in the project: AQUA 2018 joint conference & tradeshow of the World Aquaculture Society WAS and the European Aquaculture Society EAS (25th-28th August, Montpellier, France) and the Association of Scottish Shellfish Growers annual conference (4th-5th October, Oban, UK). Furthermore, project leaflets were handed out in business meetings and to some visitors as part of ANFACO-CECOPESCA's general communication activity. Martiña Ferreira participated as a speaker in a training workshop organized by ARIEMA S.L. (Spain) in the context of the project SEMILLA, funded by Fundación Biodiversidad (belonging to the Spanish Ministery for the Ecological Transition). SEMILLA aimed to train employees of the agriculture and aquaculture sectors in resource management, energy efficiency, preservation of the environment and eco-innovation. The training workshop addressed to employees of the aquaculture sector was held in Vigo and Martiña Ferreira gave a dissertation entitled "Market challenges and opportunities for aquaculture products: presentation, processing, preservation and marketing". In this dissertation, publicly available contents of DIVERSIFY were used to illustrate a practical case about the development of business models for aquaculture products. ANFACO has also translated into Spanish the species Technical Manuals (**Fig. 31.7.2)**.

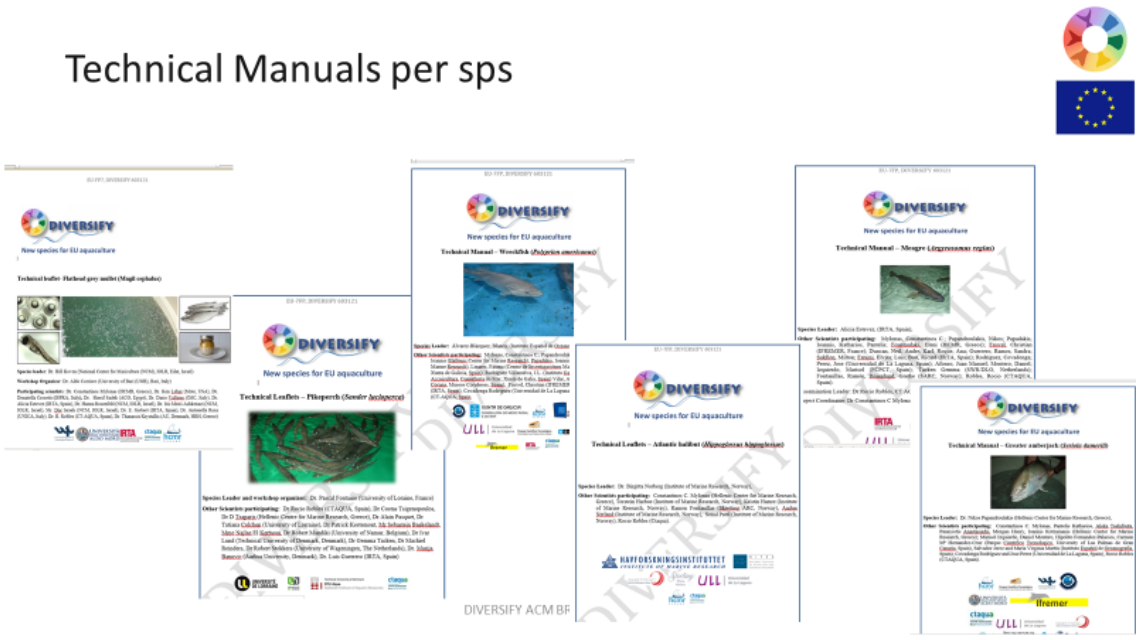


Figure 31.7.2. Representative slide of the Dissemination presentation at the final Coordination and Dissemination meeting including the 6 species Technical Manuals.

In the case of APROMAR, during the 4th Report Period, the association has participated in different Dissemination tasks, such as the participation with a presentation of *Traceability, labelling and certification of aquatic products* in the 3th Promotional Workshop in Verona (Italy); dissemination of project results to its associates companies and to the Experts Committee of REMA (Aquaculture Marine Experimentation Network); disseminating DIVERSIFY activities and results in APROMAR's Social Media Profiles (LinkedIn, Facebook, Twitter, Youtube and Instagram) and its Website (www.apromar.es) and with the translation of the Technical Manuals of Greater Amberjack, Grey Mullet, Halibut and Meagre into Spanish.

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.

For Grant Agreements related to infrastructures (Annex III to the Grant Agreement), the access provider shall include a section in the periodic reports on the access activity, indicating the membership of the selection panel as well as the amount of access provided to the user groups, with the description of their work, and the names and home institutions of users.

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.

Project meetings

Annual Coordination Meeting for Year 5 (2018 February)

The Annual Coordination Meeting (ACM) for Y5 (2018) was hosted by Drs José Peréz and Covadonga Rodríguez (ULL), and Salvador Jerez and Virginia Martín (IEO). It was held at two venues between 23-25 January 2018 (**Fig. 2.3.1**). The task-specific presentations during Days 1 and 2 took place at the Faculty of Sciences, ULL. The Group Work Package (GWP) workshops took place at the IEO facilities in Santa Cruz. The 3-day meeting was attended by 79 persons: 78 coming from the DIVERSIFY consortium and only 1 invited guest from outside the consortium. No representative attended from Beneficiaries P25. DOR, P26. GEI, P27. FORKYS, P34. BVFi and 35. MASZ, the latter two having previous commitments that they could not modify, while the PI from P2. FCPCT was again missing (having missed four ACMs so far, out of a total of five).

As for all previous ACMs, information regarding the meeting was uploaded continually on the project's web site (**http://www.diversifyfish.eu/2018-annual-coordination-meeting-jan.html**) to ensure that all participants had access to the most updated information. The Agenda (**Tables 1, 2 and 3**) was developed with assistance from GWP leaders and consisted of:

(f) DAY 1 and 2: A common session for all participants presenting Task-specific presentations from various WPs,
(g) DAY 2: A presentation of the WP 31 Dissemination presenting the dissemination activities of the consortium, and organizing the preparation of Deliverables as well as of manuscripts for scientific articles,
(h) DAY 2: Presentations by the Species leaders of the organization of the Species-specific Knowledge-transfer Workshops that will be held during 2018,
(i) DAY 2: Presentation by the Project Coordinator (PC) dealing with the status of the 3rd Periodic Report, financial issues, the preparation of Amendment 4, various management issues and the preparation of two (2) books from DIVERSIFY research.
(j) DAY 3: The Group Work Package (GWP) workshops for each scientific discipline, for the coordination of the work during Y5 of the project.
(k)

The format of the task-specific presentations for DAY 1 & 2 (**Table 2.3.1** and **2.3.2**), as it was adopted also for the ACM 2017 meeting in Barcelona, Spain, allowed a large number of the RTD partners to present their work (some for the first time) –which in many cases was done in collaboration with the SMEs and Large companies participating in the project, as well as work to be presented from all Scientific Disciplines. In total, 21 presentations from 16 RTD partners were presented, representing collaboration with the two large companies and six SMEs from the DIVERSIFY consortium. A full report on the ACM 2018 has been submitted in Deliverable ***D1.10 Annual Coordination Meeting for Y5***. Therefore, only a brief description of the meeting is provided in this 4th Periodic Report.

At the end of the specific task presentations of Day 2, there was a presentation by the WP 31 Dissemination leader, Dr. Rocio Robles. As always, and to remind our partners of the great significance of this WP for our project, the presentation begun with a brief reiteration of the WP's many objectives, emphasizing the need for all Partners to participate actively in the preparation of dissemination materials and activities. Then there was a presentation of the various dissemination activities carried out during the 3rd Reporting Period (2016-2017), which included a special 21-page featured article presenting work from DIVERSIFY in all species published in September 2017, just before the annual EAS meeting in Dubrovnik. There, a special "DIVERSIFY" session was again held (Deliverable 31.20 EAS Special session). The Dissemination leader also discussed about the scientific articles that have been published so far and the partners were encouraged to submit their work for publication as soon as possible, in order to disseminate the work carried out.

Figure 2.3.1. The two venues of the Annual Coordination Meeting 2018, held in Tenerife, Spain. The Faculty of Sciences, University of La Laguna (left) and the IEO facilities in Santa Cruz (right).

Table 2.3.1. Agenda of DAY 1 of the Annual Coordination Meeting 2018, which took place on the 23-25 January 2018, at the Faculty of Sciences, University of La Laguna, Spain.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda **2018 Annual Coordination Meeting** **Tenerife 23-25 January 2018** **Uni La Laguna**

DAY 1		23-Ιαν	Tuesday (Open Day presentations)		
Start	End		Title	Presenter	Details
8,00	9,00		Registration		Pick up badges
9,00	9,30		Welcome-Logistics	Perez, Jose Antonio & Mylonas, Constantinos	HCMR/ULL
9,30	9,50	1	Induced gametogenesis in flat-head grey mullet using recombinant gonadotropins	Ramos, Sandra	IRTA
9,50	10,10	2	Annual cycles of gonadotropins and sex steroids in plasma of farmed and wild-caught female Atlantic halibut	Norberg, Birgitta	IMR
10,10	10,30	3	Spawning induction of F1 greater amberjack in eastern Atlantic	Jerez, Salvador	IEO
10,30	10,50	4	Broodstock management and spawning induction in greater amberjack in tanks and sea cages in Greece	Mylonas, Constantinos	HCMR
10,50	11,30		**Coffee**		
11,30	11,50	5	Comparison of progammed and auto-demand type feeding of meagre in tanks	Duncan, Neil	IRTA
11,50	12,10	6	Effect of dietary fatty acids on spawn quality in greater amberjack broodstock	Djellata, Adnane	FCPCT
12,10	12,30	7	Some insights in lipid metabolism of larvae from novel aquaculture candidate species	Rodriguez, Covadonga	ULL
12,30	12,50	8	How to achieve predictable and stable juvenile production in marine fish- an industrial approach	Erstad, Borre	SWH
12,50	13,10	9	Effect of of phospholipids on lipid metabolism in Atlantic halibut	Sæle, Øystein	NIFES
13,10	15,00		**Lunch on site (Faculty of Sciences), 10 euro**		
15,00	15,20	10	Designing weaning diets based on the ontogeny of digestive tract enzyme activity during the carnivorous-omnivorous transition in grey mullet juveniles	Koven, Bill	IOLR
15,20	15,40	11	Greater amberjack larval rearing in IEO: effect of live prey enrichments and feeding regime	Martin, Virginia	IEO
15,40	16,00	12	Requirements for n-3 HUFA of meagre fingerlings	Carvalho, Marta	FCPCT
16,00	16,20	13	Test of different feeding methods on growth performance and feeding behavior of meagre	Papadakis, Ioannis	HCMR
16,20	17,00		**Coffee**		
17,00	17,20	14	Identification and expression of type I interferons in meagre	Secombes, Chris	UNIABDN
17,20	17,40	15	Dietary use of prebiotics in greater amberjack juveniles: effects on growth performance, immune gene expression and disease resistance against *Neobenedenia girellae*	Fernandez Montero, Alvaro	FCPCT
17,40	18,00	16	Epitheliocystis disease; results and progress	Katharios, Pantelis	HCMR
20,30			**Dinner at REAL CASINO de Tenerife, Plaza de la Candelaria 12 (consortium dinner)**		

Table 2.3.2. Agenda of DAY 2 of the Annual Coordination Meeting 2018, which took place on the 23-25 January 2018, at the Faculty of Sciences, University of La Laguna, Spain.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda **2018 Annual Coordination Meeting** **Tenerife 23-25 January 2018** **Uni La Laguna**

DAY 2 Start	End	24-Ιαν	Wednesday (Open Day presentations & Consortium Management) Title	Presenter	Details
8,00	9,00		Registration		Pick up badges
9,00	9,20	1	Overview of consumer behavior-related affairs in the frame of DIVERSIFY and their key findings	Krystallis, Thanassis	HRH
9,20	9,40	2	The effect of message framing on consumers' attitudes and purchase intentions towards new DIVERSIFY products	Banovic, Marija	AU
9,40	10,00	3	Feasibility study, the contributions of partners needed	Stokkers, Robert / Tacken,Gemma	SWR/DLO
10,00	10,20	4	Business model and marketing strategy development	Nijssen, Ed / van der Borgh, Michel	TU/e
10,20	10,40	5	EU funded projects: why communication matters?	Abundancia, Carlos	EUFIC
10,40	11,30		**Coffee**		
11,30	12,30		Dissemination activities, articles and species leaflets	Robles, Rocio	CT-AQUA
12,30	13,00		Meagre one-day workshop	Estevez, Alicia	IRTA
13,00	13,30		Greater amberjack one-day workshop	Papandroulakis, Nikos	HCMR
13,30	15,00		**Lunch on site (Faculty of Sciences), 10 euro**		
15,00	15,30		Pikeperch one-day workshop	Fontaine, Pascal	UL
15,30	16,00		Atlantic halibut one-day workshop	Norberg, Birgita	IMR
16,00	16,30		Wreckfish one-day workshop	Alvarez, Blanca	IEO
16,30	17,00		Grey mullet one-day workshop	Koven, Bill & Corriero, Aldo	IOLR/UNIBA
17,00	17,30		**Coffee**		
17,30	18,15		Preparation of books on meagre and amberjack biology and culture	Mylonas, Constantinos	HCMR
18,15	19,00		3rd Periodic Report, Amendment 4, Budget and Deliverables - General Asssembly	Mylonas, Constantinos	HCMR
Dinner on your own, explore the city!					

The Dissemination leader then presented the planning for the six seminars/workshops during the last year of the project, one for each of the species of DIVERSIFY. This was 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). According to the DOW, these seminars will include 30 min presentations on selected aspects (*e.g.*, reproduction and spawning induction, final product diversification and quality, socioeconomic issues and marketing, etc.), given by DIVERSIFY Partners. Aquaculturists (mainly), but also European aquaculture support companies (feed, pharmaceutical, equipment, engineering, etc.), researchers and educators, government organizations and other important institutions (FAO, Globefish) would be invited to attend these meetings. The cost of the invited speakers and the registration of the participants would be covered by the project (max 50 participants). The seminars would be organized by the **Species Leaders** (HCMR, IRTA, UNIBA, IMR, IEO, UL) in countries where the particular species are

cultured --or has the potential to be cultured -- and/or is located centrally in a region with interested aquaculture operations. One seminar will be organized for each of the selected species. The result of this task has been submitted as Deliverables ***D31.29 to D31.34. Species-specific "Know-how transfer" seminars for the aquaculture industry, presenting the progress achieved through DIVERSIFY in the production technology***.

The final section of Day 2 was dedicated to a presentation by the PC on a number of coordination and management issues. The PC first reported on the status of the deliverables from the project, presenting a list of submitted and delayed deliverables according to partner (**Fig. 2.3.2**). It was stressed that some partners seem to have fallen behind in the submission of their deliverables, and they were encouraged to speed up the process of writing them up, to prevent being labelled as "underperforming" by the final review (as it has happened for one partner). Overall, more than 50% of the total number of deliverables had been submitted, with 38 being delayed. We expected that with some rare exceptions, all of these deliverables would be completed and submitted in time, according to their description in the DOW.

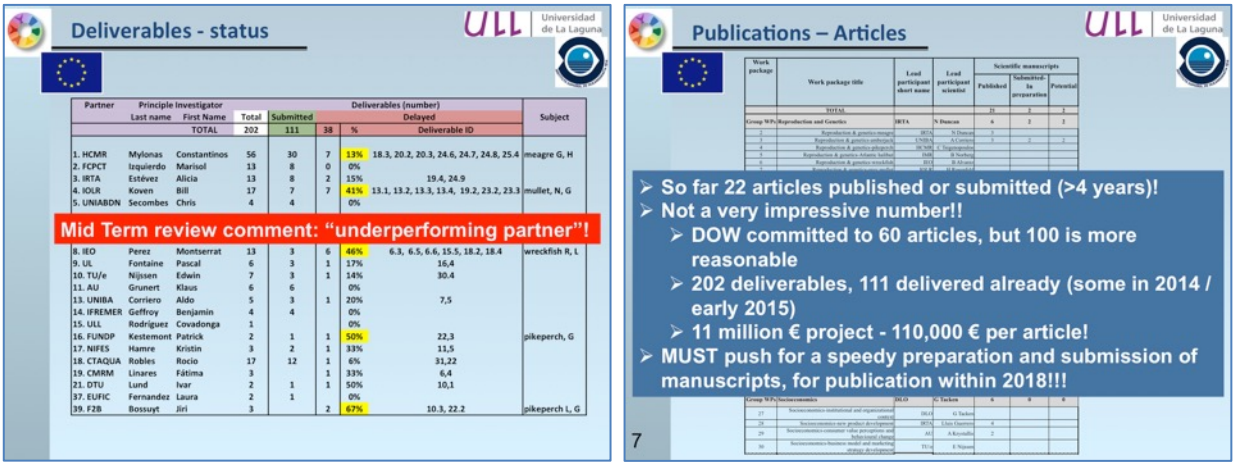


Figure 2.3.2. The status of the deliverables (left) and the publication of scientific articles (right) by January 2018, at the time of the ACM 2018.

Then, the PC presented the status of scientific manuscript preparation (**Fig. 2.3.2**). By early 2018, the consortium had published 22 articles, which was not a very high number for a consortium of this size, after 4 years of research! Obviously, the majority of the work is expected to be concluded and submitted for publication at the end of a project (and mainly afterwards). However, it was the belief of the PC that the consortium should publish more than 60 scientific articles before the end of the project in 2018. With more than 100 deliverables already completed and submitted at that time, the PC expected that a larger number of publications would have resulted so far.

The PC then reported on the submission of the final Amendment (4) and the budget modifications that it would include. Most of these budget transfers had been already approved by the Scientific Officer (Dr. Marta Iglesias) before the submission of the Amendment request, and they referred to transfer of work between partners.

Then the PC announced that following the suggestion of the Scientific Officer, the final meeting of the project would be held in Brussels. This would be a 2-day meeting, with the first one being open to the public and the second day dealing with coordination issues. In the first day, we would invite a number of

officers from relevant DGs as well as other organizations relevant to the aquaculture industry. This concluded the presentation of the PC and the General Assembly was called to a close.

During Day 3 of the meeting, six Workshop Sessions were organized according to Scientific Disciplines with the objective of (a) reviewing and evaluating the work carried out and (b) planning the work to be implemented in the various scientific WPs during the final fifth year (2018) of the project (**Table 2.3.3**).

Table 2.3.3. Agenda of DAY 3 of the Annual Coordination Meeting 2018, which took place on the 23-25 January 2018, at the IEO facilities in Santa Cruz, Spain.

DIVERSIFY
7FP-KBBE-2013-603121
Meeting Agenda

2018 Annual Coordination Meeting
Tenerife 23-25 January 2018

IEO, Santa Cruz

DAY 3	25-Iαν		Thursday (GWP Workshops)			
Start	End		ROOM 1	ROOM 2	ROOM 3	ROOM 4
9,00	9,30		GWP 2 Repro (wreckfish)	GWP 5 Grow out (amberjack)	GWP 3 Nutrition (mullet)	GWP 7 Socioeco
9,30	10,00		GWP 2 Repro (wreckfish)	GWP 5 Grow out (amberjack)	GWP 3 Nutrition (amberjack)	GWP 7 Socioeco
10,00	10,30		GWP 2 Repro (wreckfish)	GWP 5 Grow out (amberjack)	GWP 3 Nutrition (meagre)	GWP 7 Socioeco
10,30	11,00			GWP 5 Grow out (meagre)	GWP 3 Nutrition (pikeperch)	GWP 7 Socioeco
11,00	11,30		Coffee			
11,30	12,00			GWP 5 Grow out (meagre)	GWP 3 Nutrition (wreckfish)	GWP 7 Socioeco
12,00	12,30		GWP 2 Repro (amberjack)	GWP 5 Grow out (mullet)	GWP 3 Nutrition (halibut)	GWP 7 Socioeco
12,30	13,00		GWP 2 Repro(amberjack)	GWP 5 Grow out (pikeperch)	GWP 3 Nutrition	GWP 7 Socioeco
13,00	13,30		GWP 2 Repro (amberjack)	GWP 5 Grow out	GWP 3 Nutrition	GWP 7 Socioeco
13,30	15,00		Lunch on site (IEO), courtesy of IEO			
15,00	15,30		GWP 2 Repro (mullet)	GWP 6 Fish health (meagre)	GWP 4 Larval (wreckfish)	GWP 7 Socioeco
15,30	16,00		GWP 2 Repro (mullet)	GWP 6 Fish health (meagre)	GWP 4 Larval (wreckfish)	GWP 7 Socioeco
16,00	16,30		GWP 2 Repro (mullet)	GWP 6 Fish health (amberjack)	GWP 4 Larval (pikeperch)	GWP 7 Socioeco
16,30	17,00			GWP 6 Fish health (amberjack)	GWP 4 Larval (halibut)	GWP 7 Socioeco
17,00	17,30			GWP 6 Fish health (halibut)	GWP 4 Larval (mullet)	GWP 7 Socioeco
17,30	18,00			GWP 6 Fish health	GWP 4 Larval (amberjack)	GWP 7 Socioeco
18,00	18,30			GWP 6 Fish health	GWP 4 Larval (amberjack)	GWP 7 Socioeco
Dinner on your own, explore the city!						

As before, the workshops of DAY 3 were running in parallel in an attempt to minimize the potential time conflict for most Beneficiaries. In addition, the Workshops were organized in a way that the WPs dealing with the same species were planned at different times during the Workshops, to allow all scientists attending all the WPs of the same species. This was also achieved, to a degree, by the participation to the ACM 2018 of more than one scientist from some of the beneficiaries that are involved in many GWPs. Unfortunately, as last year, P2. FCPCT that has the third largest budget in the project was represented only by a single scientist (Dr. Daniel Montero, the GWP leader for Nutrition), while the PI of the organization was not present at this ACM either. More problematic was the absence of the lead beneficiary of WP 9 Nutrition – greater amberjack (P2. FCPCT) and the Principle Investigator of P28. CANEXMAR, as Task 9.2.2 includes work in the testing of a new feed for grow-out of greater amberjack that has not been reported so far, with only a year to go in the project. Unfortunately, there was nobody in the meeting to

provide the necessary information. This issue prompted an investigation in the following weeks by the PC of the status of the work, and a notification of both the Scientific Officer (Dr. Marta Iglesias) and the Financial Officer (Mrs. Annemie Van Vaerenbergh).

Final Annual Coordination Meeting (2018 November)

The Final ACM was hosted by Matthias Keller (P34. BVFi) and Carlos Abundancia (P38. EUFIC) and was held in the Thon Hotel Brussels Center, Brussels, Belgium on the 22-23 November 2018 (**Fig. 2.3.3**). As proposed during the previous ACM 2018 held in Tenerife, (Spain) in January 2018, and according to the suggestion of our EU Scientific Officer Dr Marta Iglesias, this final ACM was held in Brussels (Belgium). This central location was decided in order to be able to invite a large number of relevant officers from the European Commission, the Parliament and relevant stakeholders that have their headquarters nearby. As we had already completed the six "Species-specific Knowledge Transfer workshops", which were organized between May and October 2018 with the objective of disseminating the acquired knowledge to the aquaculture industry (farmers, feed companies, veterinarians, etc.), we considered important to broadcast the results of the project to this different audience.

Figure 2.3.3. The venue of the Final Annual Coordination Meeting, held in Brussels, Belgium (November 2018).

Invitations went out to specific persons suggested by our EU Scientific Officer, and also to other people and organizations decided by the Project Coordinator and the Dissemination leader. These included officers from the EC such as DG RTD, DG MARE and DG ENVIRONMENT, REA, members of the European Parliament Fisheries Committee, the European Aquaculture Technology and Innovation Platform (EATIP), the Federation of European Aquaculture Producers (FEAP), the European Aquaculture Society (EAS), the Aquaculture Advisory Council (AAC), and the European Market Observatory for fisheries and aquaculture (EUMOFA) and the International Union for Conservation of Nature (IUCN). A total of 16 people from the above organizations accepted our invitation (**Fig. 2.3.4**), while a number of people excused their absence.

The 1-day meeting was attended by 58 persons: 43 coming from the DIVERSIFY consortium and 15 invited guests from the list. No representative attended from Beneficiaries P2. FCPCT, P5. UNIABD (PI was retired), P14. IFREMER (PI was retired), P23. ARGO (death in the family) P25. DOR, P26. GEI, P27. FORKYS, P28. CANEXMAR, P31. IRIDA (prior commitment). The PI from P2. FCPCT did not attend the meeting (as in the previous five occasions) and the GWP for Nutrition belonging to the same institution could not attend due to economical limitations of his institution. Nevertheless, once the Project Coordinator

was aware of the problem, he offered financing for the travel; however, nobody from the institution attended the final project meeting.

DIVERSIFY
7FP-KBBE-2013-603121
List of Participants - 2018 Annual Coordination Meeting
Brussels 22-23 November 2018

	Lastname	Name	Comments	Affiliation
1	Balmforth	Nigel		5M Publishing
2	Buckhout	Marc-Philip		Seas at Risk, Aquaculture Policy Officer
3	Cheilari	Anna		EC DG Environment, Policy Officer
4	De la Cruz-Iglesias	Lorella	afternoon	EU DG MARE 2
5	Fouquet	Cecile		Aquaculture Advisorty Council
6	Goode	Andy		Focusbiz
7	Hatziyanni	Eleni		Region of Crete
8	Hough	Courtney		FEAP
9	Iglesias	Marta		DG RTD, Program Officer
10	Lacey	Rob		Focusbiz
11	Lane	Alistair	late morning	EAS, Secretary
12	Myrseth	Bjorn		Vitamar
13	Neyts	Alexandra	afternoon	EATIP, General Secretary
14	Petralli	Nila		EC
15	Valcarcel	Germán		REA
16	Zampoukas	Nikos		EC DG RTD

Figure 20.3.4. The list of invited people from outside the consortium who accepted the invitation for the FinalACM**.**

A full report on the Final ACM 2018 has been submitted in Deliverable ***D1.12 Annual Coordination Meeting (Nov 2018 Final)***. Therefore, only a brief description of the meeting is provided in this 4th Periodic Report.

As it has been done for all previous ACMs, the information regarding the meeting has been uploaded on a continuous basis on the project's web site (https://www.diversifyfish.eu/2018-final-coordination-meeting-nov.html) to ensure that all participants had access to the most updated information. The Agenda (**Tables 2.3.4 and 2.3.5**) was developed with assistance from GWP leaders and consisted of:

I. DAY 1: A dissemination session open to outside guests, where summary presentations were made for the work achieved in each of the six species, in Fish health, Socioeconomics and New Product Development, and in Dissemination,
II. DAY 2: Presentation by the Project Coordinator (PC) dealing with the preparation of the 4th Periodic and Financial Report, and the preparation of the Final Report; and presentation by the WP31 Dissemination leader Dr. Rocio Robles discussing issues related to preparation of Scientific papers and to the continuation of dissemination activities beyond the end of the project.

The morning session of Day 1 started with a welcoming by the Project Coordinator Dr. Constantinos C. Mylonas who gave a brief presentation regarding the project and the consortium, some highlights of major achievements and a presentation of the Agenda for the meeting (**Table 2.3.4**). Then the Species Leaders continued with the presentations on the objectives and achievements in all six species included in the project.

Table 2.3.4. Agenda of DAY 1 of the Final ACM (Nov 2018), which took place on the 22-23 November 2018, in Brussels, Belgium.

DIVERSIFY 7FP-KBBE-2013-603121 Meeting Agenda	2018 Final Coordination Meeting	Brussels 22-23 November 2018	Thon Hotel

DAY 1		22-Nov	Thursday (Open Day presentations)		
Start	End		Title	Presenter	Details
8,30	9,30		Registration		Pick up badges
9,30	10,00	1	The DIVERSIFY project	Dr Constantinos Mylonas (HCMR, Greece), Project Coordinator	
10,00	10,30	2	The meagre (*Argyrosomus regius*): objectives and progress	Dr Alicia Estevez (IRTA, Spain), meagre leader	
10,30	11,00	3	The greater amberjack (*Seriola dumerili*): objectives and progress	Dr Nikos Papandroulakis (HCMR, Greece), greater amberjack leader	
11,00	11,30		Coffee		
11,30	12,00	4	The pikeperch (*Sander luciperca*): objectives and progress	Dr Pascal Fontaine (UL, France), pikeperch leader	
12,00	12,30	5	The Atlantic halibut (*Hippoglossus hippoglossus*): objectives and progress	Dr Birgitta Norberg (IMR, Norway), Atlantic halibut leader	
12,30	13,00	6	The wreckfish (*Polyprion americanus*): objectives and progress	Dr Blanca Alvarez (IEO, Spain), wreckfish leader	
13,00	14,30		Lunch on site		
14,30	15,00	7	The grey mullet (*Mugil cephalus*): objectives and progress	Dr Bill Koven (IOLR, Israel), grey mullet leader	
15,00	15,30	8	Perspectives of the DIVERSIFY species and the European aquaculture market	Dr Gemma Tacken (SWR/DLO, Netherlands) Socioeconomics leader	
15,30	16,00	9	Technical assessment of the DIVERSIFY species and new product development	Dr Lluis Guerrero (IRTA, Spain)	
16,00	16,30	10	Fish health	Dr Pantelis Katharios (HCMR, Greece)	
16,30	17,00		Coffee		
17,00	17,30	11	Dissemination	Dr Rocio Robles (CTAQUA, Spain), dissemination leader	
17,30	18,00	12	Future directions-discussion	Dr Constantinos Mylonas (HCMR, Greece), Project Coordinator	
20,00			Dinner at local restaurants		

The first species-presentation was given by Dr. Alicia Estevez (IRTA, Spain), who is the **meagre** leader (**Fig. 2.3.5)**. The highlights of the achievements in meagre include the identification of three different populations and the documentation on the sufficient genetic variation in a number of broodstocks around Europe; if managed properly there is sufficient genetic variation to apply breeding programs. Protocols for meagre paired spawning and for the acquisition of gametes for *in vitro* fertilization have been developed, as methods to implement breeding programs. A protocol for early weaning was developed and the role of essential fatty acids and vitamins C, E and K in weaning diets was identified. Feeding in sea cages can be carried out during day or night using programmed feeders with good results. Optical and mechanical stimuli can be used to improve feeding behavior in meagre. Immune markers have been established for the innate, adaptive and inflammatory responses of the immune system of meagre in order to develop vaccines in the future. Methods to prevent Chronic Ulcerative Dermatopathy, to ameliorate the extend of Systematic Granulomatosis and to address parasitic and bacterial infections have been developed.

Dr Nikos Papandroulakis (HCMR, Greece), species leader for **greater amberjack** reported that although spontaneous reproduction in captivity is still problematic, hormonal induction methods have been developed to induce spawning in broodstock maintained in tanks and sea cages, producing large numbers of eggs of good quality for commercial larval rearing purposes. Hatchery-produced (F1) individuals were

shown to undergo reproductive maturation in captivity. Significant breakthroughs were achieved in larval rearing, allowing the production of large numbers of juveniles adequate for commercial production. Husbandry practices were developed for successful transfer of juveniles to sea cages. On growing trials until commercialization resulted in important information on feeding patterns and stocking densities, while the species' temperature tolerance has been determined. Identification of immune markers and health management tools under aquaculture conditions were developed, including probes for the early detection of epitheliocystis, and methods to control infestations of the parasites *Zeuxapta seriolae* and *Neobenedenia gireliae*.

Figure 21.3.5. Representative slides from the presentations of the six species leaders, highlighting the objectives of the project and the accomplished work 5 years afterwards.

Dr Pascal Fontaine (UL, France), species leader of **pikeperch** presented the achievements in this species, which included the development of a genetic map comparing captive and wild broodstock using microsatellite markers, which can be used for breeding programs. Studies have identified optimal combinations of environmental, feeding and population factors to improve survival and growth during larval rearing in RAS. Essential fatty acids must be supplied in larval diets for normal development and to reduce stress sensitivity. Low light intensity and red-light spectrum have proven to be less stressful and the effect was confirmed in RAS farm conditions. Domestication level was shown to influence stress responsiveness and immune response.

Then, Dr Birgitta Norberg (IMR, Norway), the species leader for **Atlantic halibut** explained that the use of GnRHa implants advanced and synchronized spawning, resulting in improved egg production in F1 females, though egg quality remains highly variable. Larvae fed well and had good survival when dry feed was introduced 28 days post first feeding in small systems. Full-scale systems are needed to evaluate and improve these results in an industrial context. First feeding of larvae in RAS systems resulted in improved growth and development compared to flow through systems. Metagenomic analyses of the microbial communities in the water and larvae of the two systems revealed interesting differences, which will be useful in industrial applications. Finally, a range of systems for expression of a capsid protein from nodavirus were tested for use in the development of a vaccine against VNN.

Dr Blanca Alvarez (IEO, Spain), the species leader for **wreckfish** reported that the reproductive cycle of wild-caught fish was completed in captivity. Spontaneous spawning takes place in the spring, with a periodicity of 3-5 days. Males may be in full spermiation throughout the year. Based on evaluation of mature wreckfish from the fishery, the nutrient requirements for an appropriate broodstock diet have been determined. The commercial broodstock diet produced resulted in successful maturation and production of high-quality eggs. The ontogeny of the digestive and vision system has been described. Successful larval rearing was finally achieved in the last year of the project, resulting in the production of a small number of hatchery-produced juveniles, which is very encouraging for the efforts to incorporate this species in the aquaculture industry.

The last species-specific presentation was given by Dr Bill Koven (IOLR, Israel) who is the species leader for **grey mullet**. He reported that spontaneous reproduction in captivity remains a problem, but spawning was achieved using GnRHa and metoclopramide therapies, producing millions of fertilized eggs. Optimization of the hormone-based reproduction control protocol is still necessary. Algal addition during larval rearing provides beneficial effects in terms of rotifer consumption, and larval survival and growth. After metamorphosis, commercial feeds for juveniles should be designed for the omnivorous feeding of this species and include higher levels of starch or other low cost amylolytic energetic compounds. Larvae have a high taurine requirement during rotifer feeding, and the benefit of this nutrient during early feeding was still apparent during juvenile growth. Taurine is essential not only for promoting growth in larvae, but also for other physiological pathways such as muscle function. Diets with low fishmeal content can be used successfully for on-growing without any detrimental effect on growth performance.

Following the species-specific presentation, the leader of the Socioeconomics WPs Dr Gemma Tacken (SWR/DLO, Netherlands) gave a presentation on the “Perspectives of the DIVERSIFY species and the European aquaculture market”, and Dr Lluis Guerrero (IRTA, Spain) presented the “Technical assessment of the DIVERSIFY species and new product development” (**Fig. 2.3.6**). Then, Dr Pantelis Katharios (HCMR, Greece) gave a presentation on the Fish health work carried out in the project.

The presentations closed with the Dissemination leader Dr Rocio Robles’ who presented a summary of the dissemination activities carried out during the project. Special emphasis was given to the Promotional Workshops and the Species Workshops (Know-how transfer for the project species).

Figure 2.3.6. Representative slides from the presentations of the Socioeconomics WPs in the Final ACM.

The Promotional Workshops were one-day workshops organized in specific countries where fish production and processing are relevant economic activities. The workshops were designed for specific audience, such as fish producers, processors and retailers, consumer organizations, and fisheries and aquaculture authorities. Four Promotional Workshops have been successfully organized in Germany, Spain, Italy and Greece with the collaboration of the fish processors' associations and fish producers' associations of the project. Project achievements with special emphasis on the Socioeconomical research area were presented to the audience.

The "Know-how Transfer" seminars for the aquaculture industry, presenting the progress achieved in DIVERSIFY in the production technology of each of the six project species. The seminars included presentations on selected aspects of the culture given by DIVERSIFY Partners, but also from some authorities in the species, whose work was considered relevant. Fish farmers, feed companies, European aquaculture support companies (feed, pharmaceutical, equipment, engineering, etc.), researchers and educators and government organizations have been part of these meetings. Six Species Workshops have been organized in Italy (for grey mullet), France (pikeperch), Spain (wreckfish and meagre), Norway (Atlantic halibut) and Greece (greater amberjack). A Technical Manual for each species has been uploaded in the website of DIVERSIFY (**Fig. 2.3.7**). In collaboration with the producers' and processor associations, the Technical Manuals have been translated to other EU languages: Spanish (APROMAR and ANFACO) and German (BVFi). Translated Manuals are also available in the project web.

All the presentations from the Promotional workshops (https://www.diversifyfish.eu/promotional-workshops.html) and the Species Workshops (https://www.diversifyfish.eu/species-workshops.html) have been uploaded on the project's website and have been available for downloading by any interested party.

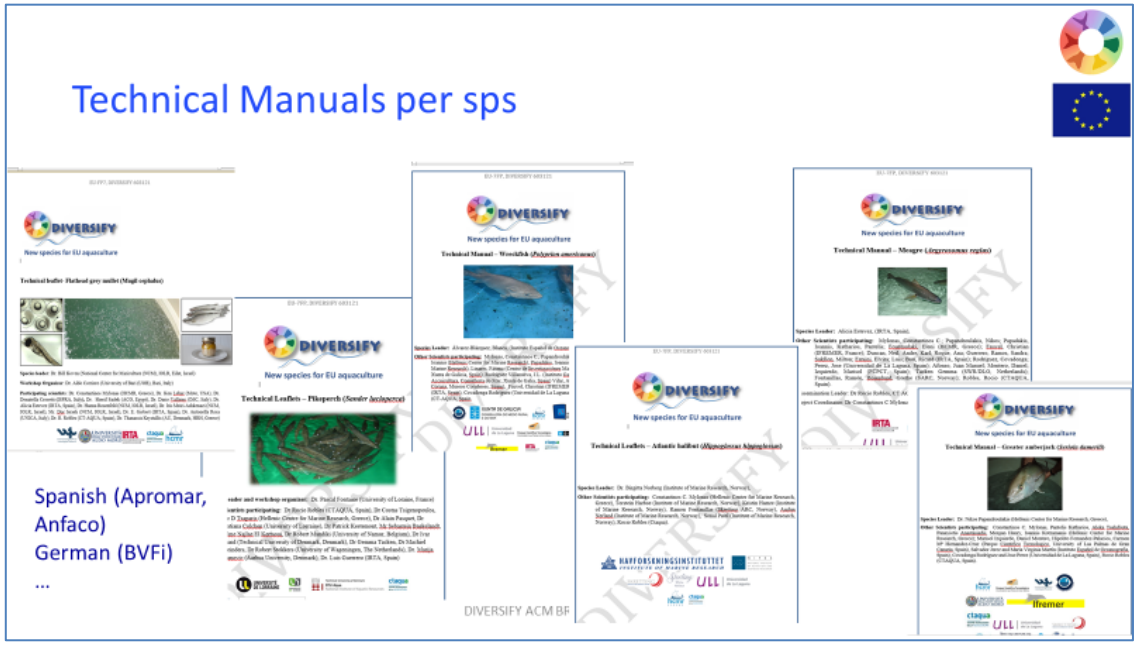


Figure 2.3.7. Slide presenting the Technical Manuals developed for each species of the project.

Just prior to the Final ACM, we have completed the design and production of the second DIVERSIFY folder (partners HCMR, CTAQUA and EUFIC), where the major achievements per species has been included. The second folder (**Fig. 2.3.8)** has been also distributed among the audience of the final Coordination and Dissemination meeting in Brussels.

Figure 2.3.8. Presentation of the second and final DIVERSIFY folder which was distributed to the attendees of the final meeting in Brussels.

The preparation of the 4th Periodic Report and the Final Report was the focus of the presentation by the PC during the Day of the meeting (**Table 2.3.5**), which was only for consortium members. Unfortunately, a key Partner in the consortium (P2. FCPCT) did not attend the meeting, neither with the Principle Investigator Dr Marisol Izquierdo, nor with the GWP leader for Nutrition, Dr Daniel Montero.

Table 2.3.5. Agenda of DAY 2 of the Final ACM (Nov 2018), which took place on the 22-23 November 2018, in Brussels, Belgium.

DIVERSIFY 7FP-KBBE-2013-603121 Meeting Agenda			2018 Final Coordination Meeting	Brussels 22-23 November 2018	Thon Hotel
DAY 2		23-Nov	Friday (Consortium Management)		
Start	End		Title	Presenter	Details
8,00	9,00		Registration		Pick up badges
9,00	10.00		Scientific and Financial Reporting	Constantinos Mylonas, PC (HCMR)	
10.00	11.00		Dissemination activities after the end of the project	Rocio Robles, (CTAQUA)	
11.00	11.30		Coffee		
11.30	13.00		Future actions for the promotion of DIVERSIFY II	Constantinos Mylonas, PC (HCMR)	
13.00	15,00		Lunch at a local restaurant for the ones staying the day in Brussels		

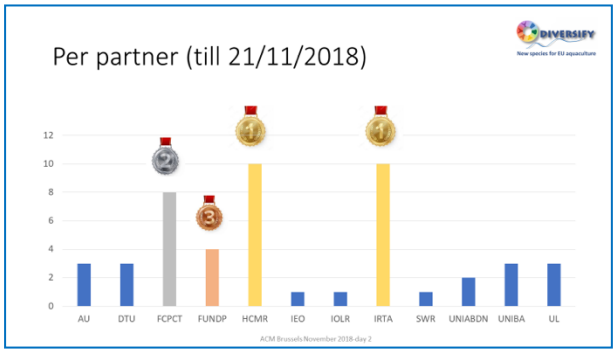
As before, the partners were given instructions as to the process that was going to be followed for the preparation of the 4th Periodic Report, which covered the last 12 months of the project (**Fig. 12**). The PC would prepare the format documents for all WPs, which would include summaries of the worked done in previous reporting periods, so the partners would only have to include the data obtained in the last reporting period. The time schedule for the conclusion of the various steps was agreed.

Then the PC dealt with the preparation of the final report, which is very different in format from the Periodic Reports with which the partners are very well familiar. Again, the PC would send the partners a format document with all the information needed to be filled by each partner, so that the contribution of each would be then consolidated by the PC.

Lastly, there was a mention of the financial situation of the project. The partners were informed of the possibility of some money remaining at the conclusion of the project, due to reductions in the budget of some SMEs. Therefore, they were encouraged to declare any additional expenses that they might have incurred during the project, due to additional work done (repetition of experiments, added analyses to improve the quality of the data, participation as speakers in the Species Workshops, etc.).

During Day 2, the presentation by the WP 31 Dissemination leader focused on the pending actions concerning mainly the scientific publications. At the time of the Final ACM, a total of 42 scientific articles had been published in peer-reviewed scientific journals and 7 more were under revision for publication. Moreover, a total of 26 articles were in preparation by the consortium (**Fig. 2.3.9)**. The scientific articles have been accessible in the "Scientific Publications" page in the main menu bar of the project web, (https://www.diversifyfish.eu/scientific-articles.html) so that visitors can have a more rapid and direct access to the scientific work of the Consortium.

Once again the Dissemination WP leader discussed the issue of uploading dissemination activities on the ECAS portal, as well as preparing the work done in DIVERSIFY for submission to scientific magazines.



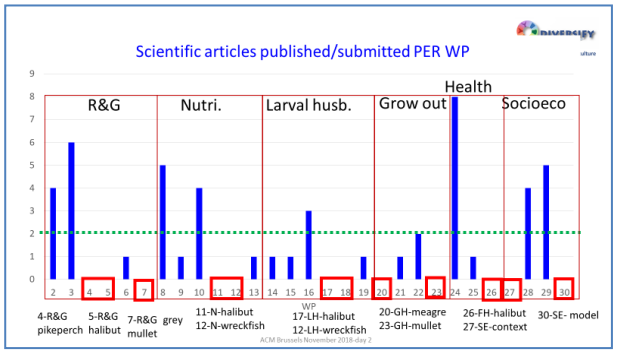


Figure 2.3.9. Slides from the presentation of the Dissemination leader showing the summary of the published articles.

The second day closed with some reflections of the work done in DIVERSIFY, and what the consortium thought was needed to be done in the future, based on the conclusions of the six Species leaders presented at the end of Day 1 (**Fig 2.3.10**). The partners agreed to maintain the communication among them, and in the coming months develop a document (s) identifying the needs for future research in the DIVERSIFY species in order to provide the EU DG RTD and EATIP with the bases for the potential opening of future calls for Species Diversification.

For example, in meagre we are still facing the issue of the lack of a specialized diet, and the occurrence of Systemic Granulomatosis. The results obtained with wreckfish reproduction and larviculture during the last year of the project need further research to finally offer this fish species to the industry. Grey mullet is another species that would require further research. Reproduction and larviculture, including nutrition requirements, have been documented well in the project, but further work is needed in addressing the monoculture cultivation of the species and the effect of density of the grow out phase. Greater amberjack has been one of the most successful species in the project, providing more than half million fingerlings to the industry in the Mediterranean to be grown in cages. However, the control of parasitic infestations still needs much further and deeper investigation.

As a general remark during the second day of the meeting, the necessity was mentioned to include recirculation aquaculture systems (RAS) in the culture of the species, specially to carry the nursery phase in more controlled conditions on land and move the fish once they have reached a size and developmental status that could guarantee a high survival in the sea cages.

The full report on the Final ACM 2018 has been submitted in Deliverable ***D1.12 Annual Coordination Meeting (Nov 2018 Final)***.

Figure 2.3.10. Future needs for research in the six DIVERSIFY species identified at the Final ACM 2018..

Amendment 4 and Consortium Modifications

As already discussed in the 3rd Periodic Report, another amendment was requested in July 2018 (no 4) due to (a) the universal takeover of two partners and (b) the need to make some reallocation of funds between partners and modifications in the implemented work. One Partner changed its name and status (P35.

MASZ became P35. MAHA), and another was taken over by an existing partner (P17. NIFES was taken over by P7. IMR).

Full name and legal form of the beneficiaries	Date of UTRO
HAVFORSKNINGINSTITUTTET (Institute of Marine Research, **P17. IMR/NIFES**) (PIC 999548432), with headquarters in Nordnesgaten 50, BERGEN, NORWAY is taking over NASJONALT INSTITUTT FOR ENAERINGS-OG SJOMATFORSKING (National Institute of Nutrition and Seafood Research, **P17. NIFES**) (PIC 963454441) with headquarters in Strangaten 229, BERGEN, NORWAY	1 January 2018
HUNGARIAN AQUACULTURE AND FISHERIES INTER-BRANCH ORGANISATION (**P35. MAHAL**) (PIC 905731293), with headquarters in H-1115 Ballagi Mór u. 8. fsz. 2, BUDAPEST, HUNGARY, is taking over HUNGARIAN AQUACULTURE ASSOCIATION (**P35. MASZ**) (PIC 9950307740) with headquarters in Anna Liget 8, SZARVAS, HUNGARY	12 June 2017

The reallocation of funds resulted from either savings from some partners, or transfer of activities from one partner to another. These transfers enabled us to implement all the work proposed in the DoW, but in some cases also enhanced the implementation of the DoW, resulting in more work being done and better results obtained. The proposed modifications are described below. As always, the majority of the modifications were already discussed and approved by the scientific office of the project (Dr Marta Iglesias) prior to the application for the amendment.

Budget reallocations/modifications

P1. HCMR. Increase of budget by 26,483 € (EU contribution). More work has been done in WP3 Reproduction-greater amberjack. This species has been quite a success for the project, and we have produced 100s of thousands of fingerlings and supplied to commercial farms for trials. This year, the first fish have been harvested and reached the European market! We want to do as much work as we can afford to provide a complete protocol to the farmers for the broodstock management and produce more eggs for giving to various farms for testing larval rearing protocols. Money will come from P27. FORKYS, who at the end could not implement some of the tasks originally allocated to them (see later).

P4. IOLR. This partner would like to remove the cost of subcontracting (15,000, €), which was originally planned for a company that produces ciliates as live feed items for marine larvae (See later under WP 19). Therefore, the cost for the subcontracting will be transferred to this partner for their additional cost in the

production of the new live feed items (4,591 € for personnel and 4,784 € for consumables) (EU contribution).

P8. IEO. Increase of budget by 2,500 € (EU contribution). This partner was not supposed to organize an ACM and was not allocated money for it. At the end they held the ACM 2018 meeting in Tenerife Spain together with P15. ULL, so some money will be transferred to this partner.

P8. 15 ULL. Increase of budget by 2,500 € (EU contribution). This partner was not supposed to organize an ACM and was not allocated money for it. At the end they held the ACM 2018 meeting in Tenerife Spain together with P8. IEO, so some money will be transferred to this partner.

P10. TU/e. Reduction of budget by 11,250 € (EU contribution). Some of the work originally planned to be carried out by this partner (WP 30, see later) will be done by P38. HRH. The two partners, and the WP leaders have agreed to this transfer.

P16. FUNDP and P21 DTU. It was agreed between the partners to transfer 7,500 € (EU contribution) between them to cover additional staff effort (5 PM). Work Package 10 (Pikeperch Nutrition) is under the responsibility of DTU and FUNDP is involved in several Tasks. Regarding Task 10.1, a large multifactorial experiment was conducted by DTU, but, due to excessive cannibalism and no feeding behavior, the experiment totally failed and was interrupted after about 10 days, despite the continuous and appropriate feeding of pikeperch larvae. A PhD student from FUNDP went to DTU for actively participating to this first experiment. Due to the failure of this multifactorial trial, it was decided to restart it in FUNDP facilities. This large experiment was conducted under the responsibility of FUNDP as well as most analyses (husbandry data, enzymatic activities, skeletal deformities, histology of selected organs) while DTU took in charge a part of the biochemical analyses (lipids and fatty acids) and ULPGC focused on the expression of some genes involved in skeleton development.

In addition to these experiments, FUNDP has been involved in two experiments conducted by DTU (HUFA and phospholipid effects on one hand, salinity effects on another hand) related to WP10.1, by taking in charge proteomics analysis and enzymatic activities. All these tasks largely exceeded the workload and budget initially allocated to FUNDP.

As a compensation, an agreement of budget transfer from DTU to FUNDP was set during a pikeperch progress meeting held in FUNDP in 2017, with the presence of all partners involved in pikeperch (UL, DTU, FUNDP and Fish2Be). The transfer of budget 7,500 € (EU contribution) will be used to cover 3 additional months of the PhD student involved, in order to finalize successfully all ongoing experiments and analyses conducted by FUNDP in WP10. There will be no negative impacts on the completion of the different WPs related to pikeperch, as defined initially in the DOW.

P23. ARGO. Increase of budget by 24,139 € (EU contribution). P27. FORKYS was not able to continue the work in WP21 Grow out of greater amberjack (phase II), so we decided to transfer this activity to P23. ARGO. Money will come from P27. FORKYS.

P25. DOR. Reduction of budget by 14,520 € (EU contribution). This SME partner has not attended many of the ACM meetings and have also not performed some of the allocated work, so the money allocated to them (mainly for travel) will be transferred to other activities of the project.

P26. GEI. Reduction of budget by 9,360 € (EU contribution). This SME partner has not attended many of the ACM meetings, so some of the money allocated to them (for travel) will be transferred to other activities of the project.

P27. FORKYS (EU contribution). This SME partner was supposed to work in WP 15 Larval rearing and WP21 Grow out of greater amberjack. Unfortunately, they were not able to implement the work (except

for phase I of their WP 21 commitment), and after repeated communications and warnings, we decide to transfer these activities to P23. ARGO and P40. GMF. The two partners, and the WP leaders have agreed to this transfer.

P38. HRH. Increase of budget by 21,250 € (EU contribution). Some of the work originally planned to be carried out by P10. TU/e will be done by P38. HRH (WP 30, see later). The two partners, and the WP leaders have agreed to this transfer. In addition, some money became available from P27. FORKYS, who could not implement some of the work originally allocated to them. An amount of 6,000 € subcontracting (EU contribution) is assigned for the platform development (for the e-shop) and consumables correspond to the provision of on line consumer panel in the 5 selected countries.

P40. GMF. Increase of budget by 48,336 € (EU contribution). All of the work originally planned to be carried out by P27. FORKYS in WP 15 Larval rearing – greater amberjack has been transferred to this partner as explained above. This partner joined the consortium in the 3rd Amendment in order to participate in WP 3 Reproduction – Greater amberjack to implement the spawning induction protocol produced by the consortium, and was willing to take over also the larval rearing implementation.

Most of the above modifications in the DoW (as indicated above in each item), both scientific and financial, were either first discussed and got the tentative approval of the EU scientific officer of the project (Dr Marta Iglesias) via email, or have been reported in (a) the 3rd Periodic Reporting. (b) Deliverable D1.10 Annual Coordination Meeting 2018 and (c) the Minutes of the Annual Coordination Meeting 2018 submitted to the EU Scientific Officer. We are now making an official request, through an amendment session in the NEFF platform of the ECAS.

Minor modifications of the DOW

WP 3. The Beneficiary for D3.5 and 3.6 was corrected to P13. UNIBA.

WP 19. The Beneficiary for D19.5 was corrected to P25. DOR. Also, P4. IOLR would like to remove the use of ciliates in the larval rearing of grey mullet, as it is no longer possible to obtain ciliates from the market (the company that used to produce them, ceased their operations). Therefore, the partner modified the larval rearing protocol including other live food items, such as copepod, which have became available I the last 5 years, and are the natural live food items of marine fishes. The new description of Task 19.3 has been include in the new DOW.

WP 28. The description of Deliverable 28.5 was corrected as it was erroneously written as the same as D28.6. It now reads: "The report will refer to the total proximate composition of the products (protein, lipid moisture, inorganic content and carbohydrates), the energy contents of the selected products and the quantitative nutritional value in aspects of fatty acids".

WP 30. *Sub-task 30.2.2 (TU/e) Testing 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).* This description of work suggests that a market test with physical products in physical stores will be done. However, the production and processing resources, capabilities, and processes needed for testing the Diversify species are not yet at the necessary level to conduct a real life market test with physical products. To date the production and processing of 4 of the 6 species only is executed in a research environment or at small scale in regular production environments. More specifically:

- There are no production facilities that can sell processed fish species from the Diversify project to regular stores, and/or
- The scale of the production is too small to do a physical market test, and/or
- Only fresh sales are possible in the current chain process (partly wild catch), what makes distribution in 5 countries expensive to reach the objected preservation time.

Given these drawbacks, we proposed to do either:

- virtual reality store test in which the participant has the impression that he/she is having a real shopping experience, with real products in a real fish shelve in an online environment (see https://www.incontextsolutions.com/ as an example), or
- an online shopping test, in which an online supermarket is imitated and in which consumers do their groceries.

Both approaches have the advantage over a market test with physical products that:

- they better mimic the trend for online grocery shopping).
- a larger test can be done than in a real shopping environment, making it possible to test more variables.
- Four species can be included, i.e. grey mullet, greater amberjack, meagre and pikeperch in four product types: i.e. fillets, fillets in olive oil, smoked fillets and hamburger for children. In this way the most important newly developed products can be considered, since creating awareness is much cheaper than in a real shopping environment.
- consumers exhibit more realistic choice behaviour than in a physical market test.
- Better manipulations are possible making new products easier to detect.

WP 30. The description of Deliverable 30.7 was corrected as it was erroneously written. It now reads: "D30.7 Feasibility study. In the feasibility study assessments will be presented on several themes: financial, return on investments, efforts needed, risks, technological, political (of potential risks of implementation) environmental impact, sociological and market impact and a stakeholder identification. These assessments will be based on the results of WPs 27, 28 and 20, and the previous tasks in WP 30".

W30. The description of Deliverable 30.8 was also corrected as it was erroneously written. It now reads: "Report on EU and international market development plans and recommendations: The diffusion studies show the effectiveness of alternative options of international market expansion and growth. Conclusions regarding the best options for internationalization help the industry/SMEs to share their international expansion strategies. Suggestions for policies that many stimulate growth/internationalization, based on potential bottlenecks identified".

Communications with the European Commission

As planned in the DOW, the EC's project Scientific Officer (Dr Marta Iglesias) was invited to all the project meetings and was sent the detailed minutes of the ACM 2018 within a month of the meeting (February 2018). Dr. Iglesias also attended the Final ACM held in Brussels, and provided us with a list of people from the Commission to invite to the meeting. Deliverables ***D1.10 Annual Coordination Meeting for Y5 (2018)*** and ***D1.12 Annual Coordination Meeting (Final 2018)*** reporting on the meetings were also promptly uploaded on the Participants Portal. As in the past, the PC has made every possible effort to keep the EU Scientific Officer informed in a timely manner, about any important developments, problems and major dissemination activities.

Problems with the consortium

Some problems were faced in the implementation of the work planned for one of the SME partners (P28. CANEXMAR). In general, there was very poor communication with this Partner, having missed many Coordination Meetings, and not interacting well with the scientific partner who invited them into the consortium (P2. FCPCT), was the WP leader of one of the two WP that CANEXMAR was involved (WP

9), and was responsible for overseeing the work of CANEXMAR -based on the DOW. This lack of communication resulted in the following issues in the two WPs that CANEXMAR was involved.

In **WP 9 Nutrition – greater amberjack**, a study was planned to examine the " Performance of grow-out diets for greater amberjack developed in order to maximize growth potential" (Deliverable 9.3). The deliverable was expected to describe the effects of the improved diet including a) detailed information about the on-growing rearing conditions, b) growth performance, c) survival, d) feed efficiency and e) significance for the industry. The efficiency of the developed grow-out diet on fillet quality was also to be determined. Unfortunately, the study was discontinued before the fish reached harvestable size, in less than a year after the juveniles were stocked in the sea cage. Although not explicitly stated in the Deliverable 9.3, the company seems to suggest that they discontinued the experiment because they noticed a parasitic infection in greater amberjack, which was also found in their gilthead seabream (*Sparus aurata*) stocks and it appeared to increase in time. There is no mention or description of the identity of the parasite, apart from belonging to the Monogenea group. It is important to note that, based on the available scientific literature, parasites are extremely species-specific and they do not usually infect fish other than their natural host. So, it seems extremely doubtful that the same parasite species infected both the greater amberjack and the gilthead seabream. However, without any proper description and documentation of the parasite, it is impossible to know what was the situation. Also, there was supposed to be an evaluation on fillet quality at the end of the rearing, which was not done, without any explanation by CANEXMAR.

This situation first surfaced in the ACM 2018 (January 2018), but as neither of the researchers responsible for this Task from CANEXMAR or FCPCT were present at the meeting, it was not possible to get a clear idea of the status of the work. In a reply to my inquiry, CANEXMAR said that the reason the task has not been implement was because they never got any information from the scientific partners coordinating the task. The Project Coordinator confirmed that the Task was not implemented properly when the partner submitted the Deliverable in November 2018. No credible explanations on the deviation from the DOW were provided by CANEXMAR, and obviously there was no time available to request a repetition of the experiment. Therefore, ***D9.3 Performance of grow-out diets for greater amberjack developed in order to maximize growth potentia***l has been submitted incomplete (See "Deviations" in the Deliverable).

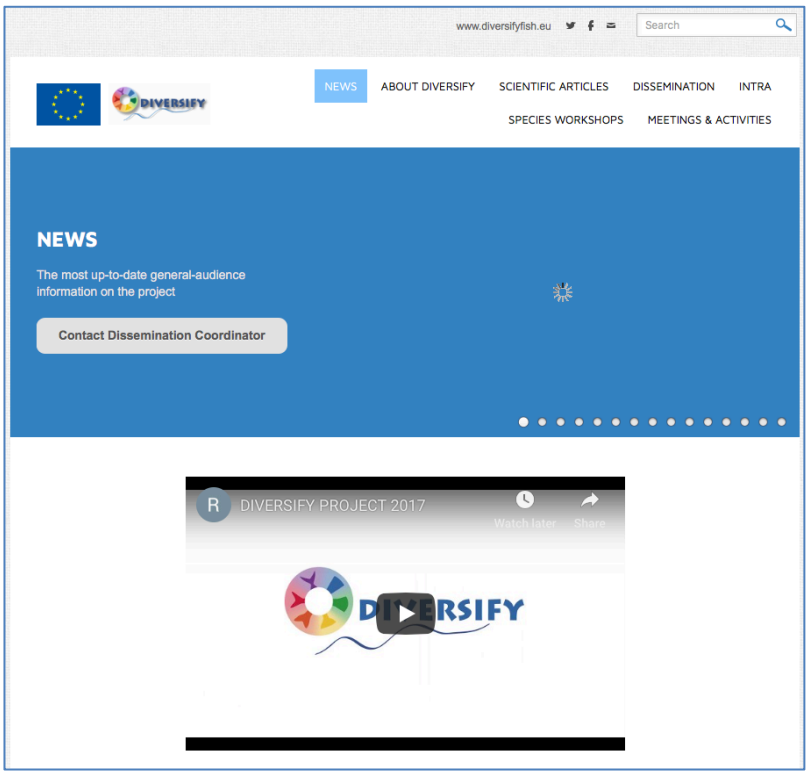
Another problem existed also in **WP 21 Grow out husbandry - greater amberjack**. The obligation of CANEXMAR in this WP (Action 21.1.2) was to examine the effect of rearing greater amberjack in floating vs submersible cages. The experiments planned in the DOW described the use of a floating and a submersible cage for two (2) consecutive growing periods (years). However, the work has not been undertaken in its full, and only a floating cage was employed and only for a single rearing period (January to November 2017). As with problem with WP 9 above, the situation first surfaced in the ACM 2018 (January 2018), but again as neither of the researchers responsible for this Task from CANEXMAR or FCPCT were present at the meeting, it was not possible to get a clear idea of problem. The company has not provided the Project Coordinator with adequate explanation, but it seems that the company faced some problem with licensing for the cage site, heavy mortalities of the juveniles during transfer from the hatchery to the cages, and breakage of the cages. Therefore, ***D21.2 Definition of optimum conditions for cage culture of greater amberjack has been submitted incomplete*** has been submitted incomplete (See "Deviations" in the Deliverable).

Based on the above, the Project Coordinator does not believe that this partner is justified to claim full costs for the work, as they failed to implement the work as planned in the DOW, have failed to communicate the issue to the Project Coordinator and have not provided adequate and timely explanations for these failure, so that the consortium could take corrective measure within the course of the project. These issues, and the decision not to accept the financial claims of P28. CANEXMAR have been communicated clearly and explicitly both to the

Scientific Officer Dr. Marta Iglesias and the Financial officer Mrs. Annemie Van Vaerenberg via emails and via the submission of the "Minutes of the Annual Coordination Meeting 2018 (for Y5)", submitted on 24 February 2018.

Project website

The project website continued to play an important role in the dissemination of the knowledge generated by the consortium, as well as in the communication within the consortium and management of the consortium (See Section WP 31 Dissemination in this report). Some major modifications to the appearance and organization of the website have been made, in order to better accommodate and exhibit the gathering number of scientific articles that were produced, as well as to promote the Species-specific Knowledge Transfer workshops and then disseminate the information presented in these workshops, which included all the scientific presentations given at the workshops, as well as the Technical Manuals for the production of each species and the Fish Health Manuals for the meagre and the greater amberjack.



It has been agreed also to maintain the project website active for a period of 3-4 years since it was considered a good tool to make available all the information on the culture of the six DIVERSIFY species generated during the five years of the project. Moreover, the conclusions of our project will be presented in the next EAS conference in Berlin (Germany), next October 2019. For more on the website of the project, please look at the section "Dissemination (WP 31)".

3 Deliverable and milestone tables

Deliverables

The project had a total of 202 Deliverables. All Deliverables have been submitted as proposed in the DOW, with the exception of

D9.3 Performance of grow-out diets for greater amberjack developed in order to maximize growth potential,

D19.2 Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production, *and*

D21.2 Definition of optimum conditions for cage culture of greater amberjack has been submitted incomplete.

These deliverables have been submitted incomplete (See "Deviations" in the submitted Deliverables).

TABLE 1. DELIVERABLE

Del. no.	Deliverable name	Version	WP no.	Lead beneficiary	*Nature*	Dissemination level[3]	Delivery date from Annex I (proj month)	Actual / Forecast delivery date Dd/mm/yyyy	Status Not submitted/ Submitted	Comments
1.1	Kick-off meeting and Annual coordination meeting for Y1	1	1	1	Other	RE	2	10/02/2014	Submitted	Due to the project starting in December, it was not possible to

[3] **PU** = Public
PP = Restricted to other programme participants (including the Commission Services).
RE = Restricted to a group specified by the consortium (including the Commission Services).
CO = Confidential, only for members of the consortium (including the Commission Services).
Make sure that you are using the correct following label when your project has classified deliverables.
EU restricted = Classified with the mention of the classification level restricted "EU Restricted"
EU confidential = Classified with the mention of the classification level confidential " EU Confidential "
EU secret = Classified with the mention of the classification level secret "EU Secret "

										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	Report	PU	30	22/11/2016	Submitted	
1.8	Periodic Report, including financial and administrative reports for Mo 13-30	1	1	1	Report	PU	32	01/08/2016	Submitted	
1.9	Annual Coordination Meeting for Y4	1	1	1	Report	PU	37	31/01/2017	Submitted	
1.10	Annual Coordination Meeting for Y5	1	1	1	Report	PU	49	31/12/2017	Submitted	

1.11	Periodic Report, including financial and administrative reports for Mo 31-48	1	1	1	Report	PU	50	31/01/2018	Submitted	
1.12	Annual Coordination Meeting (Final)	1	1	1	Report	PU	60	30/11/2018	Submitted	
1.13	Periodic Report, including financial and administrative reports for Mo 49-60	1	1	1	Report	PU	60	28/01/2019	Submitted	
1.14	Final Report	1	1	1	Report	PU	60	20/02/2019	Submitted	
2.1	SNP library and chip to genetically characterise meagre or to use in marker assisted breeding programs.	1	2	1	Report	PU	18	01/06/ 2015	Submitted	
2.2	Genetic characterization of different meager captive broodstocks and evaluation of available variability	1	2	2	Report	PU	12	15/12/2014	Submitted	
2.3	Protocol for paired spontaneous tank spawning of meagre.	1	2	3	Report	PU	21	22/09/2015	Submitted	
2.4	Identification of genetic markers related to growth for use in marker assisted breeding programs for meagre	1	2	1	Report	PU	36	28/11/2016	Submitted	
2.5	Genetic characterisation of fast and slow growing meagre	1	2	1	Report	PU	36	30/11/2016	Submitted	

2.6	Description of sperm characteristics and cryopreservation protocol of meagre sperm	1	2	14	Report	PU	36	08/11/2016	Submitted	
2.7	Protocol for the strip spawning of meagre females and in vitro fertilization	1	2	3	Report	PU	36	17/11/2016	Submitted	
3.1	Establishment of quantitative PCR assays to measure transcript levels of target genes in greater amberjack (i.e., LHβ, FSHβ, leptin, Vg and Vg receptor).	1	3	4	Report	PU	12	17/03/2015	Submitted	
3.2	Establishment of hormone specific ELISAs for measuring LH, FSH and leptin in greater amberjack	1	3	4	Report	PU	18	19/12/2017	Submitted	
3.3	Identification of possible reproductive dysfunction of gametogenesis of greater amberjack reared in captivity based on the comparative evaluation of fish sampled in the wild, in terms of proliferating	1	3	13	Report	PU	24	26/01/2016	Submitted	
3.4	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of	1	3	14	Report	PU	32	22/07/2016	Submitted	

	greater amberjack sperm									
3.5	Description of the process of oogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of ovarian development, (c) pituitary levels of FSH	1	3	13	Report	PU	46	06/12/2017	Submitted	
3.6	Description of the process of spermatogenesis in captive greater amberjack, including (a) aspects of growth and body indices, (b) histological evaluation of testicular development, (c) pituitary level	1	3	13	Report	PU	46	06/12/2017	Submitted	
3.7	Comparative effectiveness of a GnRHa injection vs GnRHa implant treatment for the induction of spawning of greater amberjack in the eastern Atlantic	1	3	2	Report	PU	48	31/10/2017	Submitted	
3.8	Development of a spawning induction therapy for captive reared broodstock in the Mediterranean Sea based on the use of GnRHa in the	1	3	1	Report	PU	56	16/07/2018	Submitted	

	correct mode of administration (hormone/implant), dose and timing of application									
3.9	Method for inducing spawning and collecting greater amberjack eggs in sea cages	1	3	1	Report	PU	59	20/10/2018	Submitted	
4.1	Genetic analysis of domesticated pikeperch broodstocks	1	4	9	Report	PU	12	19/11/2014	Submitted	
4.2	Population genetic analysis of wild and comparison with domesticated pikeperch populations to be applied in future breeding programs of the species	1	4	1	Report	PU	16	19/03/2015	Submitted	
5.1	Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut	1	5	7	Report	PU	30	27/9/2016	Submitted	
5.2	An optimised GnRHa therapy protocol to improve spawning performance of F1/F2 Atlantic halibut, and to increase availability of eggs of stable and predictable quality	1	5	7	Report	PU	30	12/05/2016	Submitted	
5.3	Identification of potential disturbances in reproductive development in F1/F2 Atlantic halibut females	1	6	14	Report	PU	24	10/05/2018	Submitted	

6.1	Computer Assisted Sperm Analysis (CASA) for wreckfish sperm	1	6	14	Report	PU	24	27/11/2015	Submitted	
6.2	Cryopreservation method for wreckfish	1	7	14	Report	PU	12	12/12/2014	Submitted	
6.3	Spawning induction methods with in vitro fertilization of wreckfish	1	7	8	Report	PU	24	30/11/2018	Submitted	
6.4	Establish reliable collection methods and protocols to form new wreckfish broodstocks	1	7	19	Report	PU	24	12/11/2018	Submitted	
6.5	Description of the reproductive cycle of wreckfish	1	7	8	Report	PU	48	30/11/2018	Submitted	
6.6	An in vitro fertilization protocol to be employed by the industry to spawn wreckfish	1	7	8	Report	PU	54	24/11/2018	Submitted	
6.7	Spawning induction method for spontaneous spawning of wreckfish in large tanks	1	7	1	Report	PU	59	21/10/2018	Submitted	
7.1	Establishment of a Computer Assisted Sperm Analysis (CASA) for the evaluation of grey mullet sperm	1	7	4	Report	PU	18	12/06/2015	Submitted	
7.2	Production of recombinant bioactive LH and FSH assay for grey mullet	1	7	4	Report	PU	24	27/11/2015	Submitted	
7.3	Comparative effectiveness of hormonal treatments for spawning induction in captive grey mullet	1	7	4	Report	PU	24	30/11/2015	Submitted	

7.4	Protocol for shipping grey mullet eggs	1	7	4	Report	PU	24	10/1/2017	Submitted	
7.5	Description of the process of oogenesis in captive-reared vs hatchery-produced grey mullet, including aspects of growth, body indices, and histological evaluation of ovarian development	1	7	13	Report	PU	48	24/07/2018	Submitted	
7.6	Culture procedure that identifies the ongrowing period for the production of grey mullet roe (bottarga) from wild and hatchery juveniles	1	7	4	Report	PU	61	12/12/2018	Submitted	
7.7	Development of a breeding protocol for captive reared grey mullet broodstock based on optimized hormonal treatment, group structure and photo-thermal regime	1	7	4	Report	PU	61	19/12/2018	Submitted	
8.1	Improvement of larval weaning diets	1	8	2	Report	PU	24	3/12/2015	Submitted	
8.2	Recommended essential fatty acids contents in diets to promote meagre growth, welfare and health	1	8	2	Report	PU	48	25/11/2017	Submitted	
9.1.	Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-	1	9	2	Report	PU	24	16/12/2015	Submitted	

	carotenoids									
9.2	Lys requirements of greater amberjack juveniles	1	9	1	Report	PU	36	20/02/2017	Submitted	
9.3	Performance of grow-out diets for greater amberjack developed in order to maximize growth potential	1	9	28	Report	PP	59	24/11/2018	Submitted incomplete	The fish were not grown for the full cycle to harvest size, and there was no fillet quality analysis
9.4	Recommended protein, carotenoids, Tau and EFA levels in greater amberjack broodstocks	1	9	8	Report	PP	59	12/11//2018	Submitted	
10.1	Recommended Ca/P, vitamins and phospholipids to improve larval development and reduce skeleton alterations in pikeperch	1	10	21	Report	PU	36	21/09/2018	Submitted	
10.2	Protocol for optimal early fatty acid enrichment to reduce stress sensitivity in pikeperch	1	10	21	Report	PU	36	21/12/2017	Submitted	
10.3	Formulation for a diet better adapted to pikeperch requirements	1	10	39	Report	PU	48	19/11/2018	Submitted	
11.1	Report on nutrient profile of Artemia nauplii and ongrown Artemia from IMR and SWH	1	11	7	Report	PU	24	28/11/2015	Submitted	
11.2	Report on optimal characteristics of feed particles	1	11	7	Report	PU	36	28/11/2016	Submitted	

	and feeding environment for early weaning of Atlantic halibut larvae									
11.3	Report on the nutrient retention and digestive physiology in Atlantic halibut larvae fed *Artemia nauplii* and on-grown *Artemia*	1	11	17	Report	PU	36	28/11/2016	Submitted	
11.4	Report on the nutrient retention and digestive physiology in Atlantic halibut larvae reared in RAS vs FTS	1	11	17	Report	PU	36	28/11/2016	Submitted	
11.5	Report on the effect of dietary phospholipids on Atlantic halibut juveniles	1	11	17	Report	PU	48	10/09/2018	Submitted	
12.1	Effect of live prey enrichment products on wreckfish larval performance	1	12	19	Report	PU	54	12/11/2018	Submitted	
12.2	Recommendations for wreckfish broodstock feeds	1	12	19	Report	PU	57	10/11/2018	Submitted	
13.1	Determine changes in the essential fatty acid requirement as a function of developmental stage and ambient salinity in grey mullet	1	13	4	Report	PU	59	25/11/2018	Submitted	
13.2	Determine a developmental stage ability to synthesize key enzymes in Tau and bile acid synthesis in grey mullet	1	13	4	Report	PU	51	23/02/2018	Submitted	
13.3	Determine the effects of	1	13	4	Report	PU	59	30/11/2018	Submitted	

	pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression accumulation									
13.4	Determine the effects of essential fatty acids and Tau in non-fish meal feeds on flesh and bottarga quality in grey mullet	1	13	4	Report	PU	62	19/01/2019	Submitted	
13.5	Evaluate and maximize the dietary incorporation of a non-GMO genetically selected soybean meal that will increase nutrient absorption and reduce DT inflammation	1	13	4	Report	RR	61	27/12/2018	Submitted	
14.1	Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles		14	3	Report	PU	30	16/05/2016	Submitted	
15.1	Effective greater amberjack larval stocking densities	1	15	2	Report	PU	16	09/05/2016	Submitted	
15.2	Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing	1	15	8	Report	PU	27	16/02/2017	Submitted	

15.3	Optimum hydrodynamics and light conditions during greater amberjack larval rearing	1	15	2	Report	PU	27	16/03/2017	Submitted	
15.4	Ontogeny of greater amberjack larval visual and digestive system	1	15	1	Report	PU	27	23/05/2016	Submitted	
15.5	An industrial protocol for greater amberjack larval rearing	1	15	8	Report	PU	58	17/10/2018	Submitted	
16.1	Determine effect of environmental factors on pikeperch larval rearing	1	16	9	Report	PU	12	28/7/2016	Submitted	
16.2	Determine effect of nutritional factors on pikeperch larval rearing	1	16	9	Report	PU	24	27/11/2016	Submitted	
16.3	Determine effect of population factors on pikeperch larval rearing	1	16	9	Report	PU	36	31/10/2017	Submitted	
16.4	Identification of optimal combinations of factors for pikeperch larval rearing	1	16	9	Report	PU	48	31/05/2018	Submitted	
16.5	Evaluation of selected rearing combinations for pikeperch on farm condition	1	16	9	Report	PU	60	05/11/2018	Submitted	
16.6	Proposition of an industrial protocol for pikeperch rearing	1	16	39	Report	PU	60	19/11/2018	Submitted	
17.1	Production protocol of on-grown Artemia	1	17	7	Report	PU	24	28/11/2015	Submitted	
17.2	Determine if RAS is a more effective protocol than FT for	1	17	7	Report	PU	57	30/08/2018	Submitted	

	Atlantic halibut larvae									
17.3	The effect of probiotics on Atlantic halibut larval microbiota and survival	1	17	7	Report	PU	58	30/10/2018	Submitted	
17.4	Comparison of feeding on-grown Artemia versus Artemia nauplii on Atlantic halibut larval performance	1	17	7	Report	PU	48	01/12/2016	Submitted	
17.5	Development of an industrial protocol for probiotic treatment of halibut larvae	1	17	7	Report	PU	56	26/07/2018	Submitted	
18.1	Development of the digestive system of wreckfish	1	18	1	Report	PU	36	31/10/2017	Submitted	
18.2	Determine optimum temperature conditions for rearing wreckfish larvae	1	18	8	Report	PU	59	12/10/2018	Submitted	
18.3	Develop a feeding protocol for wreckfish larvae	1	18	1	Report	PU	61	05/12/2018	Submitted	
18.4	Determine the most effective culture system (RAS vs flow-through) for wreckfish larvae	1	18	8	Report	PU	60	26/11/2018	Submitted	
19.1	Determine most effective type and concentration of algae used in grey mullet larval rearing	1	19	4	Report	PU	24	20/11/2016	Submitted	
19.2	Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production	1	19	4	Report	PU	61	27/12/2018	Submitted Incomplete	Although ciliates were produced in mass culture, it was not possible to transfer larvae to

										the testing aquaria due to complete mortalities, therefor no results were obtained on growth, survival and digestive tract maturation of larval grey mullet
19.3	Determine weaning time and type of feed according to the shift from carnivorous to omnivorous feeding	1	19	4	Report	PU	36	14/11/2017	Submitted	
19.4	Evaluate the effectiveness of replacing live algae with lyophilized algae during grey mullet larval rearing	1	19	4	Report	PU	56	26/07/2018	Submitted	
19.5	Evaluate an improved grey mullet larval rearing protocol in a commercial hatchery	1	19	4	Report	PU	56	23/07/2018	Submitted	
20.1	Methodology to avoid size variability in meagre juveniles	1	20	3	Report	PU	24	28/11/2015	Submitted	
20.2	Definition of the optimum conditions for cage culture of meagre	1	20	3	Report	PU	54	24/04/2018	Submitted	
20.3	Methodology for meagre feeding	1	20	3	Report	PU	57	25/07/2018	Submitted	
21.1	Definition of optimum feeding methods for greater amberjack grow out	1	21	8	Report	PU	45	28/07/2017	Submitted	

21.2	Definition of optimum conditions for cage culture of greater amberjack	1	21	2	Report	PU	60	05/11/2018	Submitted incomplete	The experiments planned in the DOW should have used floating and a submersible cage for two (2) consecutive growing periods (years). However, the work has not been undertaken in its full, and only a floating cage was employed and only for a single rearing period (January to November).
22.1	Effects of multiple variables on stress, immune response and growth performances and recommendations of optimal conditions for pikeperch grow out	1	22	16	Report	PU	24	17/5/2016	Submitted	
22.2	Validation of optimal rearing variables under commercial farm conditions	1	22	39	Report	PU	54	12/06/2018	Submitted	
22.3	Effects of domestication level and geographical origin on stress, immune response and growth performances and	1	22	16	Report	PU	54	20/06/2018	Submitted	

	strain recommendation									
23.1	Cost-effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status	1	23	3	Report	PU	18	1/10/2015	Submitted	
23.2	Stocking protocols for pond monoculture grow out of F1 and wild caught grey mullet	1	23	4	Report	PU	58	23/09/2018	Submitted	
23.3	Comparison of the project's improved grey mullet grow-out feed under the different environmental and water conditions in Israel, Greece and Spain	1	23	4	Report	PU	54	20/06/2018	Submitted	
24.1	The effect of vitamin D inclusions in diets in the development of Systemic Granulomatosis in meagre	1	24	1	Report	PU	20	7/1/2016	Submitted	
24.2	The effect of Ca/P ratio in the diet in the development of Systemic Granulomatosis in meagre	1	24	1	Report	PU	24	15/06/2016	Submitted	
24.3	Cloning of key marker genes of innate and adaptive immune responses in meagre	1	24	5	Report	PU	26	20/01/2016	Submitted	
24.4	Isolation and characterization of Nocardia from infected meagre	1	24	1	Report	PU	36	13/12/2016	Submitted	
24.5	The effect of high plant protein	1	24	1	Report	PU	36	25/05/2017	Submitted	

	diets in the development of Systemic Granulomatosis in meagre									
24.6	Experimental vaccine for Nocardia for meagre	1	24	1	Report	PU	60	30/11/2018	Submitted	
24.7	Diagnostics protocol for Chronic Ulcerative Dermatopathy in meagre, aetiological factors and solutions	1	24	1	Report	PU	53	26/04/2018	Submitted	
24.8	Report on the prevention/treatment of Chronic Ulcerative Dermatopathy in meagre	1	24	1	Report	PU	52	27/03/2018	Submitted	
24.9	Determination of effective treatments for common monogenean parasites in meagre	1	24	3	Report	PU	52	1/03/2018	Submitted	
24.10	Kinetics of antibody and cytokine production established post-pathogen exposure or stimulation with PAMPs	1	24	5	Report	PU	48	15/11/2017	Submitted	
24.11	Recommended levels of pro- and anti-oxidant nutrients to prevent Systemic Granulomatosis in meagre	1	24	2	Report	PU	54	18/05/2018	Submitted	
24.12	Determination of efficacy of vaccination of meagre against Nocardia	1	24	3	Report	PU	58	18/10/2018	Submitted	

24.13	Description of immune gene expression pre- and post-immunization of meagre with Nocardia	1	24	3	Report	PU	60	30/11/2018	Submitted	
24.14	Description of the causes of Systemic Granulomatosis in meagre	1	24	1	Report	PU	59	30/10/2018	Submitted	
24.15	Solutions to Systemic Granulomatosis	1	24	1	Report	PU	59	31/10/2018	Submitted	
24.16	Report of the major bacterial and viral diseases found in meagre, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided	1	24	2	Report	PU	58	23/09/2018	Submitted	
24.17	Diagnostic-recommendation manual for meagre fish health	1	24	1	Report	PU	60	18/11/2018	Submitted	
25.1	Marker genes of mucosal immunity in greater amberjack cloned and ways to increase their expression level determined	1	25	5	Report	PU	39	16/03/2017	Submitted	
25.2	Mucus defences of greater amberjack analysed and immune potential characterised	1	25	2	Report	PU	39	26/05/2017	Submitted	
25.3	Impact of dietary regime on parasite resistance and mucosal defences of greater	1	25	5	Report	PU	42	27/07/2017	Submitted	

	amberjack juveniles									
25.4	Protocol for early diagnosis of epitheliocystis during early stages of greater amberjack culture	1	25	1	Report	PU	56	02/07/2018	Submitted	
25.5	Impact of different dietary regimes in greater amberjack on immune marker gene expression determined	1	25	2	Report	PU	57	30/08/2018	Submitted	
25.6	Rearing protocol against monogenean parasites	1	25	8	Report	PU	60	05/11/2018	Submitted	
25.7	Report on the major bacterial and viral diseases found in greater amberjack, and where useful treatments have been developed, complete protocols for their implementation by the industry will be provided	1	25	2	Report	PU	58	05/10/2018	Submitted	
25.8	Diagnostic-recommendation manual for greater amberjack fish health	1	25	1	Report	PU	61	10/12/2018	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	Report	PU	24	13/11/2015	Submitted	
26.2	Testing of the delivery of vaccine candidates through *Artemia* to Atlantic halibut	1	26	7	Report	PU	53	27/04/2018	Submitted	

	larvae									
26.3	Determine immune response and effectiveness of orally delivered VNN capsid protein on protection of Atlantic halibut larvae	1	26	7	Report	PU	54	02/05/2018	Submitted	
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	1	27	6	Report	PP	14	22/05/2014	Submitted	
27.2	Report on current certification schemes and standards and their business dynamics in the fish supply chain	1	27	6	Report	PP	14	7/03/2014	Submitted	
27.3	Report on competitive analysis for the supply chains of meagre, greater amberjack, pikeperch, Atlantic halibut, wreckfish and grey mullet	1	27	6	Report	PU	12	15/01/2015	Submitted	
27.4	Report on trend mapping for the European aquaculture, seafood sector and protein market in the (near) future	1	27	6	Report	PU	12	5/12/2014	Submitted	
27.5	Report with results of international survey on industrial buyers' attitudes and perceptions regarding cultured	1	27	6	Report	PU	12	28/11/2014	Submitted	

	fish									
27.6	List of critical success factors for market acceptance	1	27	6	Report	PU	12	20/02/2015	Submitted	
27.7	Report on the analysis of the business models and supply chains of the participating SME's	1	27	6	Report	PU	12	28/11/2014	Submitted	
28.1	Report with results of focus groups with consumers and experts regarding ideas for new products	1	28	11	Report	PU	14	14/4/2015	Submitted	
28.2	List of ideas for new product development	1	28	1	Report	PU	16	21/7/2015	Submitted	
28.3	Report on product and process solutions for each species based on technological, physical and sensory characteristics	1	28	1	Report	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	Report	PU	26	19/4/2016	Submitted	
28.5	Report on results of quality evaluation study on basic quality characteristics of the developed products	1	28	1	Report	PU	55	12/06/2018	Submitted	
28.6	Report on results of sensory descriptive analysis of the developed products	1	28	1	Report	PU	54	15/05/2018	Submitted	

28.7	Report on correlation of technical quality with nutritional - rearing history	1	28	15	Report	PU	59	29/10/2018	Submitted	
28.8	Technical assessment of selected species	1	28	1	Report	PP	59	03/10/2018	Submitted	
29.1	Dataset of consumers' perceptions, attitudes, buying intentions, consumption, willingness to buy and pay, and value perceptions towards the selected species in the five	1	29	6	Report	PU	9	27/08/2014	Submitted	
29.2	Report on the segmentation analysis based on consumer value perceptions about the selected species in the five countries investigated (value-based segmentation task)	1	29	11	Report	PU	24	7/10/2015	Submitted	
29.3	Development of the actual product samples from the selected species for the sensory testing with consumers in the five countries investigated	1	29	3	Report	PU	28	4/4/2016	Submitted	
29.4	Report on the actual products' sensory profiling in the five countries investigated	1	29	3	Report	PU	29	27/7/2016	Submitted	
29.5	Development of the product mock-ups for use in the experimentation with consumers in the five countries	1	20	11	Report	PU	30	27/7/2016	Submitted	

	investigated									
29.6	Report on the experimentation with product mock-ups in the five countries investigated and identification of the optimal intrinsic-extrinsic product quality profiles for targeted segments	1	20	11	Report	PU	30	14/03/2017	Submitted	
29.7	Development of the stimulus (i.e. written and broadcasted information material) that will be used in the communication experiments in the five countries investigated	1	20	11	Report	PU	30	28/07/2017	Submitted	
29.8	Report on the experimentation with the communication stimulus and evaluation of their effectiveness in changing consumers attitudes and behaviour towards the products coming from the selected	1	20	11	Report	PU	30	31/10/2017	Submitted	
30.1	Report on value propositions for the producers and Partners	1	30	10	Report	PU	46	31/10/2017	Submitted	
30.2	Report on indications of resources for creating customer value for the specific products	1	30	10	Report	PU	46	31/10/2017	Submitted	

30.3	Guidelines to cultivate buyer-supplier relationships per species	1	30	10	Report	PU	48	05/12/2017	Submitted	
30.4	Revenue (pricing & costs structures) model per species	1	30	10	Report	PU	51	16/02/2018	Submitted	
30.5	New product marketing strategies per species and product	1	30	10	Report	PU	53	24/04/2018	Submitted	
30.6	Report on results of test markets per species	1	30	10	Report	PU	59	12/10/2018	Submitted	
30.7	Feasibility study	1	30	6	Report	PU	60	19/12/2018	Submitted	
30.8	Report on EU and international market development plans and recommendations	1	30	10	Report	PU	61	19/12/2018	Submitted	
31.1	Establishment of website (www.diversifyfish.eu)	1	31	18	Report	PU	4	02/04/2014	Submitted	
31.2	Project logo and brochure	1	31	18	Report	PU	6	24/06/2014	Submitted	
31.3	Publication of the first of two articles in Food Today	1	31	37	Report	PU	6	30/05/2014	Submitted	
31.4	Production and release of audio-visual material	1	31	18	Report	PU	6	24/06/2014	Submitted	

31.5	Collaboration agreement with food industry and consumer organization; linkage of websites	1	31	18	Report	PU	9	18/11/2014	Submitted	
31.6	Annual presentation of DIVERSIFY (Y1) at a relevant conference (mainly Aquaculture Europe, EU Forum (by the Project Coordinator)	1	31	1	Report	PU	9	27/10/2014	Submitted	
31.7	Production and release of audio-visual material	1	31	18	Report	PU	12	15/01/2015	Submitted	
31.8	Production and release of audio-visual material	1	31	18	Report	PU	18	31/08/2015	Submitted	
31.9	Annual presentation of DIVERSIFY (Y2) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	21	29/10/2015	Submitted	
31.10	Presentations of DIVERSIFY at the Aqua Europe meetings (Diversification Sessions) by the Species leaders (Y2)	1	31	1	Report	PU	21	16/11/2015	Submitted	
31.11	Scientific publications in relevant journals	1	31	1	Report	PU	60	30/11/2018	Submitted	
31.12	Production and release of audio-visual material	1	31	18	Report	PU	24	30/12/2015	Submitted	

31.13	Production and release of audio-visual material	1	31	18	Report	PU	30	25/6/2016	Submitted	
31.14	Annual presentation of DIVERSIFY (Y3) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	33	15/10/2016	Submitted	
31.15	Production and release of audio-visual material	1	31	18	Report	PU	36	09/02/2016	Submitted	
31.16	Promotional workshops for specialized audience in fish market sector (Spain, Greece, UK or Italy) (1st workshop)	1	31	18	Report	PU	37	28/07/2017	Submitted	
31.17	Production and release of audio-visual material	1	31	18	Report	PU	42	31/10/2017	Submitted	
31.18	Promotional workshops (2nd) for specialized audience in fish market sector (Spain, UK, Italy or Greece)	1	31	18	Report	PU	43	08/11/2017	Submitted	
31.19	Annual presentation of DIVERSIFY (Y4) at a relevant conference (mainly Aqua Europe meetings, EU Forum) by the Project Coordinator	1	31	1	Report	PU	44	02/11/2017	Submitted	
31.20	Presentations of DIVERSIFY at the Aqua Europe meetings (Diversification Sessions) by the Species leaders (Y4)	1	31	1	Report	PU	44	03/11/2017	Submitted	
31.21	Presentation of DIVERSIFY at the European SEAFOOD Expo	1	31	1	Report	PU	44	25/10/2017	Submitted	

31.22	Production and release of audio-visual material	1	31	18	Report	PU	48	17/7/2018	Submitted	
31.23	Promotional workshops for specialized audience in fish market sector (Spain, UK, Greece or Italy) (3rd workshop)	1	31	18	Report	PU	54	2/05/2018	Submitted	
31.24	Technical leaflets	1	31	18	Report	PU	59	30/10/2018	Submitted	
31.25	Audio-visual document with the project's activities and main achievements	1	31	1	Report	PU	60	30/11/2018	Submitted	
31.26	Audio-visual popularization document and publication of the second article in Food Today, electronic journal of EUFIC	1	31	37	Report	PU	61	24/12/2018	Submitted	
31.27	Promotional workshops for specialized audience in fish market sector (Spain, UK, Greece or Italy) (4th workshop)	1	31	18	Report	PU	59	2/10/2018	Submitted	
31.28	Annual presentations of DIVERSIFY at the Aqua Europe meetings (EU Forum) by the Project Coordinator (Y5)	1	31	1	Report	PU	59	18/10/2018	Submitted	
31.29	Meagre "Know-how Transfer" seminar	1	31	3	Report	PU	59	21/10/2018	Submitted	
31.30	Greater amberjack "Know how	1	31	1	Report	PU	59	12/10/2018	Submitted	

	Transfer" seminar									
31.31	Pike perch "Know-how transfer" seminar	1	31	9	Report	PU	58	21/09/2018	Submitted	
31.32	Atlantic halibut "Know-how Transfer" seminar	1	31	7	Report	PU	59	4/10/2018	Submitted	
31.33	Wreckfish "Know-how Transfer" seminar	1	31	8	Report	PU	59	2/10/2018	Submitted	
31.34	Grey mullet "Know-how transfer" seminar	1	31	13	Report	PU	55	12/06/2018	Submitted	
31.35	Production and release of audio-visual material	1	31	18	Report	PU	60	30/11/2018	Submitted	

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							
Mile-stone no.	**Milestone name**	**WP no**	**Lead benefit-ciary**	**Delivery date from Annex I dd/mm/yyyy**	**Achieved Yes/No**	**Actual / Forecast achievement date dd/mm/yyyy**	**Comments**
1	Kick-off meeting and Annual coordination meeting for Y1	1	1	31/12/2013	Yes	30/01/2014	P1. HCMR, Crete, Greece
2	Consortium agreement	1	1	31/01/2014	Yes	20/03/2014	
3	Annual coordination meeting for Y2	1	1	31/01/2015	Yes	6/11/2014	P13. UNIBA, Bari, Italy
4	Periodic Report (Mo1-12) to DG RTD, including financial and administrative reports	1	1	31/01/2015	Yes	30/12/14	
5	Annual coordination meeting (for Y3)	1	1	31/01/2016	Yes	04/02/2016	P9. UL, Nancy, France
6	Periodic Report (Mo13-30) to DG RTD, including financial and administrative reports	1	1	31/5/2016	Yes	29/7/2016	
7	Annual coordination meeting (Y4)	1	1	30/11/2016	Yes	18/1/2017	P3. IRTA, Barcelona, Spain
8	Annual coordination meeting (Y5)	1	1	30/1/2018	Yes	23/1/2018	P8. IEO and P15. ULL, Tenerife, Spain

9	Final coordination meeting	1	1	30/09/2018	Yes	22/11/2018	Brussels, Belgium
10	Periodic Report (Mo30-48) to DG RTD, including financial and administrative forms	1	1	30/11/2018	Yes	21/01/2018	
11	Periodic Report (Mo49-60) to DG RTD, including financial and administrative forms	1	1	30/11/2018	Yes	28/01/2019	
12	Final report to DG RTD	1	1	30/11/2018	Yes	20/02/2019	
16	SNIP library with candidate SNIPs potentially associated with growth in meagre	2	2	30/05/2015	Yes	30/05/2015	
17	Database of genetic variability of pikeperch	4	1	30/11/2014	Yes	30/11/2014	Excel database completed
18	Documentation of ovulatory cycles in wild and F1 halibut broodstock	5	7	31/05/2016	Yes	31/05/2016	
19	Basic diet formulation for meagre grow out studies	8	2	30/11/2014	Yes	30/11/2014	Established
20	Digestive utilization of experimental weaning diets for meagre	8	2	30/11/2015	Yes	30/11/2015	
21	Basic diet formulation for greater amberjack grow out studies	9	2	30/11/2014	Yes	30/11/2014	Established
22	Definition of reproductive quality parameters to be studied in amberjack	9	2	30/11/2014	Yes	30/11/2014	Literature search completed
23	Definition of parameters for skeleton study in pikeperch	10	21	30/11/2014	Yes	30/11/2014	Definitions and analytical parameters for skeleton studies has been included in the experimental protocols
24	Influence of salinity or temperature on LC-PUFAs synthesis in pikeperch	10	21	30/11/2016	Yes	30/11/2016	

25	Ranges of digestive enzymes activities in Atlantic halibut	11	7	31/08/2016	No		This was not planned for in the WP11 description, according to the WP leader, but it is not clear why it was written as a MS.
26	Obtain viable gametes (oocytes and sperm) for larvae production in wreckfish	12	19	31/08/2016	Yes	31/05/2017	
27	Definition of methodology to study cost-benefit of grey mullet weaning studies	13	4	30/11/2014	Yes	30/11/2015	
28	Protocol for weaning meagre larvae	14	2	31/5/2015	Yes	16/5/2016	
29	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack producing good quality eggs	15	2	31/5/2014	Yes	30/6/2014	Provision of eggs for larval nutrition and rearing experiments in Greece and Spain.
30	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2015	Yes	30/6/2015	Egg production has been achieved in both Mediterranean and Atlantic broodstocks, as well as in F1 broodstocks in Y2
31	Protocol for tank design, lighting and probiotics of larval rearing of greater amberjack	15	2	31/5/2015	Yes	30/7/2016	
32	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2016	Yes	31/6/2016	
33	Successful maturation and spawning of eastern Atlantic or Mediterranean Sea wild, F1 generation greater amberjack	15	2	31/5/2017	Yes	18/06/2017	

34	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2014	No	31/6/2014	Eggs were produced both in Greece and Spain, but their quality was poor and did not allow implementation of larval rearing experiments.
35	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2015	Yes, partly	31/6/2015	Eggs were produced both in Greece and Spain, and allowed a limited implementation of the larval rearing experiments.
36	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/04/2016	Yes, partly	31/5/2016	Eggs were produced in Spain, and allowed a limited implementation of the larval rearing experiments
37	Successful maturation and spawning of wreckfish to produce good quality eggs	6	8	30/06/2017	Yes, partly	06/03/2017	Eggs were produced both in Spain and Greece, but allowed only a limited implementation of the larval rearing experiments
38	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2014	Yes	31/10/2015	Millions of eggs of high quality were produced, allowing the start of larval rearing experiments.
39	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2015	Yes	31/10/2015	
40	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2016	Yes	30/09/2016	

41	Successful maturation and spawning of grey mullet broodstock to produce good quality eggs and larvae	19	4	30/08/2017	Yes	20/09/2017	
42	Results on feeding stimuli of meagre	20	3	01/06/2015	Yes	01/06/2015	
43	First cage trials (different volume and light conditions) with meagre implemented	20	3	30/11/2015	Yes	01/12/2015	
44	Results on feed distribution method in cages with meagre	20	3	30/11/2015	Yes	01/12/2016	
45	Feeding pattern of greater amberjack fry available	21	1	31/8/2015	Yes	31/8/2015	
46	First results on optimum husbandry practice (thermal ranges, stocking density) of greater amberjack	21	1	31/3/2016	Yes	31/3/2016	
47	First experiment on cage culture condition (net volume, cage type) of greater amberjack implemented	21	1	31/5/2016	Yes	01/09/2016	
48	Experiment on the definition of optimal conditions for pikeperch on growing implemented	22	16	31/5/2016	Yes	31/5/2016	
49	First trial with different strains of pikeperch implemented	22	16	30/11/2017	Yes	31/05/2018	
50	Experimental trials of grey mullet in the three locations implemented	23	4	28/2/2015	Yes	31/5/2016	
51	Design of primers for amplification of meagre target gene DNA sequences	24	5	30/11/2014	Yes	30/11/2014	
52	Grow-out of larvae and collection of samples from immune ontogeny time-line	24	5	30/11/2015	Yes	30/11/2015	

53	Amplification and sequencing of target gene sequences from stimulated tissues	24	5	31/5/2016	Yes	31/5/2016	
54	Completion of challenge and collection of samples for study of immune gene modulation	24	5	30/11/2016	Yes	30/11/2016	
55	Complete preparation of cDNA synthesis from all meagre samples	24	5	31/05/2017	Yes	31/05/2017	
56	Complete gene expression analysis of immune ontogeny	24	5	31/05/2017	Yes	31/05/2017	
57	Complete genes analysis for immune stimulus/response	24	5	31/10/2017	Yes	31/10/2017	
58	Design of primers for amplification of greater amberjack target gene DNA sequences	25	5	31/5/2015	Yes	31/5/2015	
59	Successful Chlamydia screening and sequencing	25	5	31/5/2016	Yes	1/5/2016	
60	Samples collected from stimulated primary cultures/explants, ready for immune gene expression analysis	25	5	31/5/2016	Yes	30/11/2015	PhD student Douglas Milne of P5. UNIABDN visited P1. FCPCT in November 2015 to undertake the work
61	Ideas for new products	28	1	31/5/2015	Yes	21/7/2015	
62	Optional physical new products	28	1	31/3/2016	Yes	19/4/2016	
63	Insights in the consumer and B2B market for cultured fish	29	1	30/11/2014	Yes	30/11/2014	
64	Selection of new products, with good sensory perception	29	1	31/5/2016	Yes	30/7/2016	
65	Intrinsic and extrinsic attributes related to the new products	29	11	30/11/2016	Yes	30/11/20116	

66	Communication concept for behavioral change to cultured fish	29	11	31/08/2017	Yes	31/08/2017	
67	Business models to market the new products	30	10	30/11/2017	Yes	31/10/2018	
68	Business models to market the new products	30	10	31/5/2018	Yes	31/10/2018	
69	Marketable new products	30	10	30/11/2018	Yes	30/11/2018	
70	Agreement on project logo for website and publications, this will provide a recognizable image of DIVERSIFY	31	1	01/06/2014	Yes	01/06/2014	
71	Design and printing of project brochure (hard-copy) including the project logo, inserts with project	31	18	01/06/2014	Yes	24/06/2014	
72	Agreements with food industry and consumers associations for web linkage	31	18	31/08/2014	Yes	20/11/2014	Considerable difficulties have been faced in reaching an agreement with organizations proposed in the DOW.
73	Agreement on the Promotional workshop (1st) program	31	18	01/07/2016	Yes	01/07/2016	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events

74	Agreement on the Promotional workshop (2nd) program	31	18	01/07/2016	Yes	01/06/2016	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events
75	Agreement on the Promotional workshop (3rd) program	31	18	31/5/2017	Yes	1/11/2017	It was agreed by the association partners to organize the workshop during the spring /autumn seasons (out of high sales periods) to have more audience for the events
76	Agreement on the Promotional workshop (4th) program	31	18	30/11/2017	Yes	10/05/2018	
77	Agreement on the one-day State-of-the-art seminar program for meagre	31	3	31/12/2017	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting
78	Agreement on the one-day State-of-the-art seminar program for greater amberjack	31	1	31/12/2017	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting
79	Agreement on the one-day State-of-the-art seminar program for pikeperch	31	9	31/1/2018	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting
80	Agreement on the one-day State-of-the-art seminar program for Atlantic halibut	31	7	31/1/2018	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting

81	Agreement on the one-day State-of-the-art seminar program for wreckfish	31	8	28/2/2018	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting
82	Agreement on the one-day State-of-the-art seminar program for grey mullet	31	4	31/3/2018	Yes	18/1/2018	The dates were confirmed during the ACM 2018 meeting

4 Explanation of the use of the resources and financial statements (Staff effort only)

The financial statements have to be provided within the Forms C for each beneficiary (if Special Clause 10 applies to your Grant Agreement, a separate financial statement is provided for each third party as well) together with a summary financial report which consolidates the claimed Community contribution of all the beneficiaries in an aggregate form, based on the information provided in Form C (Annex VI of the Grant Agreement) by each beneficiary. The "Explanation of use of resources" requested in the Grant Agreement for personnel costs, subcontracting, any major costs (ex: purchase of important equipment, travel costs, large consumable items) and indirect costs, have now to be done within the Forms (user guides are accessible within the Participant Portal)[4].
When applicable, certificates on financial statements shall be submitted by the concerned beneficiaries according to Article II.4.4 of the Grant Agreement.

The use of the resources is explained in detail in the submitted Forms C from each Beneficiary. However, for the convenience of the potential reviewer we include the staff effort in the tables below. **Tables 4.1a-b** show the staff effort for the 4th Reporting period.

[4] In the past, the explanation of use of resources requested in the Grant Agreement was done within a table in this section. The merge of this table within the Forms C was a measure of simplification aimed at avoiding duplication and/or potential discrepancies between the data provided in the table 'Explanation of use of resources' and the data provided in the Forms C.

Table 4.1a Staff effort per Partner (P1-22) and WP, for the 4th Reporting Period (49-60 Mo).

DIVERSIFY	4th Periodic Report (49-60 months)																							
KBBE 2013.1.2.09. Diversification of fish species and products in aquaculture																								
Calculation of staff effort per parter and sub WP																								
						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
			226.56	29.35	3.20	27.55	26.00	3.26	12.46	8.61	13.925	2.60	9.87	7.05	3.010	4.60	0.00	3.767	10.00	0.00	13.50	0.80	3.82	2.46	0.25
WP1 Management	Management	1	20.88	14.00		0.56		0.80	0.22	0.10	0.100	0.28		0.15	0.104	0.10		0.285			0.20	0.10	0.18		0.15
WP2 Reproduction and Genetics			29.46	5.70	0.00	2.73	4.00	0.00	0.00	2.53	4.000	0.00	0.00	0.00	0.000	0.70	0.00	1.314	0.00	0.00	0.00	0.20	0.00	0.00	0.00
2.1	Reproduction - meagre	2	1.29	0.10		1.19																			
2.2	Reproduction - amberjack	3	12.00	4.00																					
2.3	Reproduction - pikeperch	4	0.10	0.10																					
2.4	Reproduction - halibut	5	2.53							2.53															
2.5	Reproduction - wreckfish	6	8.27	1.50		0.22	2.00				4.000							0.059				0.20			
2.6	Reproduction - mullet	7	5.28			1.32	2.00									0.70		1.255							
WP3 Nutrition			37.37	0.00	3.20	1.57	9.00	0.00	0.00	0.15	2.850	0.00	0.00	0.00	0.000	3.30	0.00	0.000	10.00	0.00	5.38	0.10	1.82	0.00	0.00
3.1	Nutrition - meagre	8	0.00																						
3.2	Nutrition - amberjack	9	2.22								0.400												1.82		
3.3	Nutrition - pikeperch	10	10.00																10.00						
3.4	Nutrition - halibut	11	0.15							0.15															
3.5	Nutrition - wreckfish	12	5.75		3.20						2.450											0.10			
3.6	Nutrition - mullet	13	19.25			1.57	9.00									3.30					5.38				
WP4 larval husbandry			19.94	0.75	0.00	0.88	5.50	0.00	0.00	5.09	4.200	1.64	0.00	0.00	0.000	0.00	0.00	0.000	0.00	0.00	0.00	0.40	0.00	1.21	0.10
4.1	Larval husbandry - meagre	14	0.00																						
4.2	Larval husbandry - amberjack	15	0.75	0.75																					
4.3	Larval husbandry - pikeperch	16	2.85									1.64												1.21	
4.4	Larval husbandry - halibut	17	5.19							5.09															0.10
4.5	Larval husbandry - wreckfish	18	4.77								4.200											0.40			
4.6	Larval husbandry - mullet	19	6.38			0.88	5.50																		
WP5 Grow out husbandry			34.78	1.50	0.00	4.25	7.50	0.00	0.00	0.00	0.400	0.00	0.00	0.00	0.000	0.00	0.00	1.729	0.00	0.00	1.00	0.00	0.00	1.25	0.00
5.1	Grow out husbandry - meagre	20	2.54	0.25		2.29																			
5.2	Grow out husbandry - amberjack	21	20.38	1.25							0.400							1.729							
5.3	Grow out husbandry - pike perch	22	1.25																					1.25	
5.4	Grow out husbandry - mullet	23	10.61			1.96	7.50														1.00				
WP6 Fish health			19.58	4.40	0.00	9.96	0.00	2.46	0.00	0.74	0.200	0.00	0.00	0.00	0.000	0.00	0.00	0.000	0.00	0.00	0.00	0.00	1.82	0.00	0.00
6.1	Fish health - meagre	24	16.39	3.40		9.96		1.21															1.82		
6.2	Fish health - amberjack	25	2.45	1.00				1.25			0.200														
N/A	Fish health - halibut	26	0.74							0.74															
WP7 Socioeconomics			46.42	1.00	0.00	6.81	0.00	0.00	12.24	0.00	0.000	0.00	9.87	6.90	2.442	0.00	0.00	0.439	0.00	0.00	1.12	0.00	0.00	0.00	0.00
7.1	Institutional and organizational context	27	0.00																						
7.2	New Product Development	28	7.23	1.00		5.09												0.439			0.70				
7.3	Consumer value perceptions and behavioral change	29	10.83						6.18					1.46							0.12				
7.4	Business model and marketing strategy development	30	28.36			1.72			6.06				9.87	5.44	2.442						0.30				
WP8 Dissemination	Dissemination	31	18.13	2.00		0.79					2.175	0.68			0.464	0.50					5.80				

Table 4.1b Staff effort per Partner (P23-40) and WP, for the 4th Reporting Period (49-60 Mo).

DIVERSIFY	4th Periodic Report (49-60 months)																			
KBBE 2013.1.2.09. Diversification of fish species and product																				
Calculation of staff effort per parter and sub WP																				
			SME partners									New Partners								
Work Package (Proposal)	Title	DOW WP	23. ARGO	24. ITICAL	25. DOR	26. GEI	27. FORKYS	28. CANEXMAR	29. ASIALOR	30. CULMAREX	31. IRIDA	32. MC2	33. FGM	34. BVFi	35. MASZ	36. ANF	37. EUFIC	38. HRH	39. F2B	40. GMF
			26.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.51	0.60	0.44	0.440	1.95	4.43	5.81	14.30	12.59
WP1 Management	Management	1	1.00								0.15	0.05	0.10	0.04	0.221		1.78	0.21	0.82	0.14
WP2 Reproduction and Genetics			8.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.000	0.00	0.00	0.00	0.00	4.69
2.1	Reproduction - meagre	2																		
2.2	Reproduction - amberjack	3	8.00																	4.69
2.3	Reproduction - pikeperch	4																		
2.4	Reproduction - halibut	5																		
2.5	Reproduction - wreckfish	6										0.29								
2.6	Reproduction - mullet	7																		
WP3 Nutrition			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.00	0.00	7.34	0.00
3.1	Nutrition - meagre	8																		
3.2	Nutrition - amberjack	9																		
3.3	Nutrition - pikeperch	10																	7.34	
3.4	Nutrition - halibut	11																		
3.5	Nutrition - wreckfish	12																		
3.6	Nutrition - mullet	13																		
WP4 larval husbandry			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.000	0.00	0.00	0.00	5.56	7.76
4.1	Larval husbandry - meagre	14																		
4.2	Larval husbandry - amberjack	15																		7.76
4.3	Larval husbandry - pikeperch	16																	5.56	
4.4	Larval husbandry - halibut	17																		
4.5	Larval husbandry - wreckfish	18										0.17								
4.6	Larval husbandry - mullet	19																		
WP5 Grow out husbandry			17.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.000	0.00	0.00	0.00	0.09	0.00
5.1	Grow out husbandry - meagre	20																		
5.2	Grow out husbandry - amberjack	21	17.00																	
5.3	Grow out husbandry - pike perch	22																	0.09	
5.4	Grow out husbandry - mullet	23									0.15									
WP6 Fish health			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.00	0.00	0.00	0.00
6.1	Fish health - meagre	24																		
6.2	Fish health - amberjack	25																		
N/A	Fish health - halibut	26																		
WP7 Socioeconomics			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.00	0.00	5.60	0.49	0.00
7.1	Institutional and organizational context	27																		
7.2	New Product Development	28																		
7.3	Consumer value perceptions and behavioral change	29																3.07		
7.4	Business model and marketing strategy development	30																2.53	0.49	
WP8 Dissemination	Dissemination	31											0.50	0.40	0.219	1.95	2.65			

This is the end of the 4th Periodic Report.