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DIVERSIFY

SPECIAL EXTENDED ARTICLE:

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New species for EU aquaculture

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

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Figure 1: A group photo during the latest Annual Coordination Meeting, which was held at Palau Macaya, Barcelona Spain (17-19 January 2017).

The European Commission project DIVERSIFY (FP7-KBBE-2013, GA 603121) started in December 2013, with the objective of carrying out focused research in a number of new/emerging finfish species, in order to support the diversification of the European aquaculture industry and thus contribute to its sustainable expansion. The project has a total budget of 11.8 million € for its 5-year duration and it is coordinated by Dr. Constantinos C. Mylonas of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC), one of the three institutes of the Hellenic Center for Marine Research (HCMR). The DIVERSIFY consortium includes more than hundred senior scientists (**Fig. 1**) from twenty research and academic institutions, three Large Enterprises, eight Small and Medium Enterprises (SME), five Professional Associations and one consumer NGO (**Table 1**).

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The project DIVERSIFY (**www.diversifyfish.eu**) has identified a number of new/emerging finfish species, based both on their biological and economical potential, to cover the entire European geographic area and to stimulate different aquaculture types. Although the emphasis is on Mediterranean cage-culture, fish species suitable for cold-water, pond/extensive and fresh water aquaculture have been included as well *(Aquaculture Europe 39(1) March 2014)*. These new/emerging species are fast growing and/or large finfishes marketed at a large size and can be processed into a range of products to provide the consumer with both a greater diversity of fish species and new value-added products. The fish species studied include **meagre** (*Argyrosomus regius*) and **greater amberjack** (*Seriola dumerili*) for warm-water marine cage culture, **wreckfish** (*Polyprion americanus*) for warm- and cool-water marine cage culture, **Atlantic halibut** (*Hippoglossus hippoglossus*) for marine cold-water culture, **grey mullet** (*Mugil cephalus*) a euryhaline herbivore for pond/extensive culture and **pikeperch** (*Sander lucioperca*) for freshwater intensive culture using recirculating aquaculture systems (RAS).

In the three-and-a-half years that DIVERSIFY has been running, a number of research activities have been carried out in the scientific disciplines of Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health, Socioeconomics and Final product quality. As the project is approaching its conclusion in November 2018, significant progress has been made in all six species. To speed up the dissemination of these results to the interested stakeholders and the aquaculture industry, one-day species-specific workshops are being organized for 2018, to be carried out at different locations around Europe (see later for more information). Moreover, four promotional workshops are being organized in four European countries, to create awareness of the project findings in the area of socioeconomic, marketing and product development. These events are targeted for specialized audiences in the fish market sector, such as fish producers, processors and retailers, consumer organizations, and fisheries and aquaculture authorities.

A full-day special session for research results from DIVERSIFY is also planned during the Aquaculture Europe 2017 conference in Dubrovnik, Croatia (17-20 October 2017). In the present article, we are presenting some highlights of the major achievements of the project so far.

Participating organizations in DIVERSIFY

Greece: Institute of Marine Biology, Biotechnology and Aquaculture (HCMR/IMBBC); ARGOSARONIKOS FISHFARMS SA; AQUACULTURE FORKYS SA; IRIDA SA; Galaxidi Marine Farms S.A.; Hellenic Research House AE; VAS. GEITONAS & Co Ltd; Federation of Greek Maricultures.

Spain: Institut de Recerca i Tecnologia Agroalimentaries (IRTA-San Carles de la Rapita); Parque Científico y Tecnológico de la Universidad de Las Palmas de Gran Canaria; Centro Tecnológico de la Acuicultura de Andalucia (CTAQUA); Universidad de la Laguna; Instituto Español de Oceanografía; Asociación Empresarial de Productores de Cultivos Marinos-APROMAR; Consellería do Medio Rural e do Mar-Xunta de Galicia; Ayuntamento de A Coruña (Museos Científico Coruñeses); CANEXMAR SL; ANFACO-CECOPESCA.

France: French Research Institute for the Exploitation of the Sea (IFREMER); Université de Lorraine

Israel: Israel Oceanographic and Limnological Research-National Center for Mariculture; DOR AQUACULTURE Ltd

Norway: Institute of Marine Research, National Institute of Nutrition and Seafood Research; Skretting Aquaculture Research Center AS; Stirling White Halibut AS

The Netherlands: Stichting Wageningen Research (previously DLO/LEI); Eindhoven University of Technology

United Kingdom: The University of Aberdeen

Italy: Universitá degli Studi di Bari «Aldo Moro»

Belgium: Université de Namur; European Food Information Council; Fish2Be; S.A.

Denmark: Technical University of Denmark, Aarhus University (MAPP Center)

Germany: German Association of Fish Processors (Bundes Verband Fisch, BVFi E.V.)

Hungary: Hungarian Aquaculture Association (Mayar Akvakúltra Szövetség, MASZ)

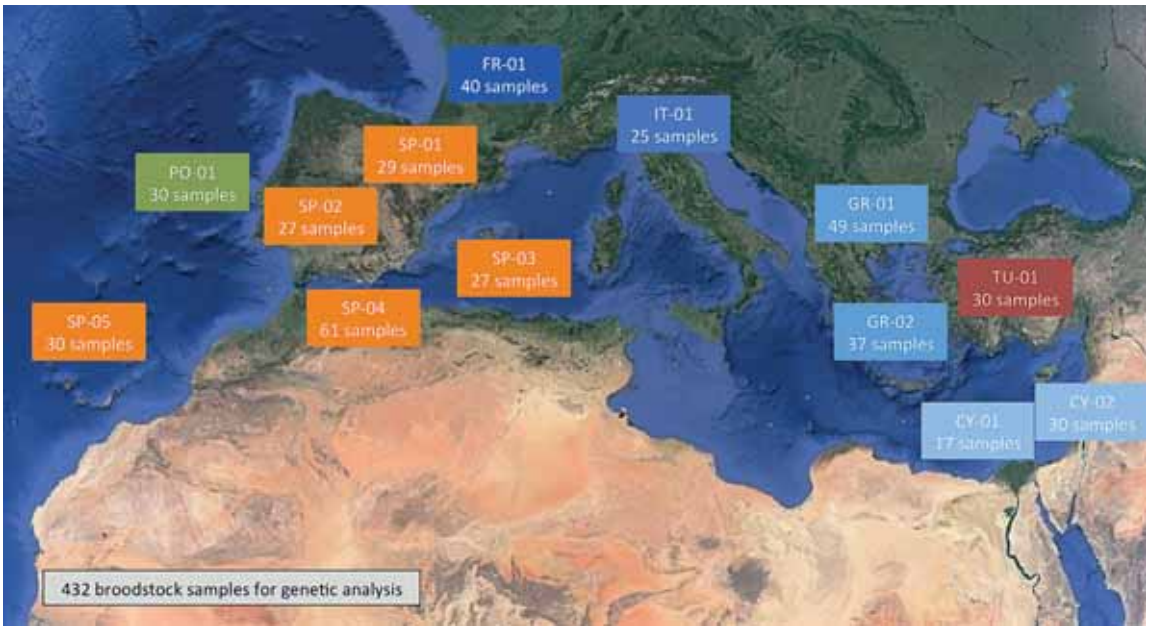

Figure 2: Geographic distribution of meagre samples used in the study of genetic variation in the available research and commercial broodstocks from the Canary Islands to Cyprus.

MEAGRE

Meagre is considered an emerging species that has been cultured increasingly in Europe in the last two decades, though in relatively limited quantities compared to gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Some of the attractive attributes of meagre include **large size, good processing yield, low fat content, excellent taste and firm texture** (Monfort, 2010). A survey of meagre producers carried out during the proposal stage of DIVERSIFY identified the principal bottlenecks to the expansion of the industry *(Aquaculture Europe 40(1) March 2015)*. These include **limited genetic variation of the available broodstocks**, **variable growth rates** and **fish health** related issues, such as the wide occurrence of **systemic granulomatosis** (Elkesh et al., 2012), which may stem from the fact that no specific diets have been developed for this fish. Also, socioeconomic factors have been identified as bottlenecks, including the need for a more expanded market and diversification of available products beyond the whole fresh fish.

To address the issue of limited genetic variation in the broodstocks, DIVERSIFY carried out a genetic characterization of different meagre broodstocks in Europe and evaluation of available variability. Eighteen microsatellite markers (Short Tandem Repeats, STRs) were used to genetically characterize 13 meagre broodstocks held in aquaculture facilities from seven countries, ranging from Gran Canaria in Spain to Cyprus (**Fig. 2**). The analyses indicated that the genetic variation in captive broodstocks is more than adequate to form a base population in breeding programs, even though some broodstocks could benefit from the addition of new breeders. The mean number of alleles and observed heterozygosity were estimated at 3.7 and 0.48, respectively, with the captive populations showing lower mean number of alleles and observed heterozygosity than wild populations (around 3 times and 18% lower, respectively). Population genetics analyses using AMOVA revealed that 18.2% of the variation was found among studied broodstocks, while the remaining 81.8% was located within populations. Moreover, a Factorial Correspondence Analysis showed two clusters; in the first cluster, there seems to be a correlation with the geographical distribution of populations (Atlantic Vs Mediterranean), while in the second there is only the population from Turkey.

The next part of the genetic work for meagre was to characterize for the first time the muscle and liver transcriptome in the species, in order to base future physiology performance. This was done through transcriptome sequencing and RNA-Seq; the assembled transcripts were assigned to a wide range of biological processes including growth, reproduction and behavior. The whole transcriptome has been scanned to identify thousands of markers that may have impact on the functional role of protein-coding genes. The marker search revealed a total of 48,526 high-quality Single Nucleotide Polymorphism (SNP) markers and 20,582 STR markers. The relatively low rate of polymorphism reported might be indicative of inbreeding in the particular broodstock used.

In order to construct a genetic linkage map in meagre and perform preliminary Quantitative Trait loci (QTL) analysis, we used the double-digest restriction-site associate DNA (ddRAD) methodology to genotype two full-sib families and constructed a genetic linkage map that included 731 markers organized in 27 linkage groups (LG), which means 3 LGs-chromosomes more than the haploid number determined in the karyotype of this species (n=24). Comparative genomic analyses through similarity searches revealed conserved synteny with more than one third of the loci having a region homologous to the European seabass genome. Lastly, we completed a genome scan for QTLs that affect body weight (BW) and total length (TL) in fish from five full-sib families using the markers developed for

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the linkage map of meagre distributed across 27 LGs. Model mapping from the two larger families identified 5 QTLs on only two LGs (11 and 20) that exhibited significant evidence of linkage at the genome level. Multiple QTLs on LG20 seem to affect both BW and TL, and were located at close positions, suggesting that the same genetic factors may control variability in these traits and are expected to be of great value in future Marker Assisted Selection (MAS) programmes.

Reproduction is no longer considered a bottleneck in meagre aquaculture, since recent studies have produced efficient protocols for the control of reproduction and the induction of spawning in aquaculture (Duncan et al., 2013; Mylonas et al., 2015; Mylonas et al., 2016). Nevertheless, DIVERSIFY developed further techniques to assist in the implementation of breeding selection programs, such as (a) paired-spawning of fish in tanks, and (b) *in vitro* fertilization methods. For the first objective, paired-spawning experiments were completed to determine the potential of paired spawning inductions with male rotation to perform a dialed cross-mating design as the basis of a breeding program. The efficacy of spawning pairs with male rotation was high (76%) and across the three experiments a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success (**Fig. 3**). However, not all paired crosses with male rotation were successful, and a number of females after consecutive successful spawning inductions either failed to spawn or did not present vitellogenic oocytes, preventing their further induction as planned in the dialed cross design. This failure to spawn or maintain maturity status after successive successful spawning inductions appeared to represent a change in spawning kinetics from the prolonged (up to 17 weeks) induced spawning period observed in a previous same-pair experiment and other studies. This change in kinetics may be attributed to the stress of male rotation and consideration should be made that as the number of rotations increases, spawning pairs may fail or induced spawning may not be possible. However, together these experiments have shown that paired spawning of meagre is possible for the production of known families from parents with known phenotypes. Obtaining a large number of families with adequate fecundities that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite for a breeding program.

Since the development of strip spawning with *in vitro* fertilization methods is necessary for the meagre aquaculture industry (as an alternative to paired-spawning), in order to facilitate planned crosses between selected breeders, the following work was also undertaken in DIVERSIFY (**Fig. 4**). Females with advanced stages of maturity were induced to ovulate with a single injection of 15 μg kg^{-1} gonadotropin releasing hormone agonist (GnRHa). The injection was applied at 20:00-22:00 hours and the females held separate from males in darkness until being checked for ovulation. Checks for ovulation were made every 2.5 hours from 35 to 45 hours post GnRHa injection. When ovulated eggs were obtained, *in vitro* fertilization was made and egg quality assessed by determining

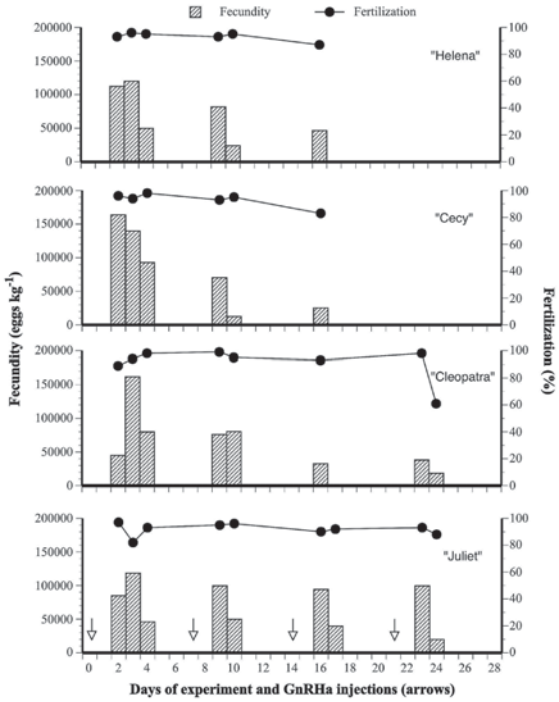

Figure 3. Daily batch relative fecundity and fertilization success of individual meagre females (n=4) induced to spawn with multiple GnRHa injections (n=4, once every week) and paired with four males (named Romeo, Cesar, Peri and Paris). At every GnRHa injection, the males were moved to a different tank, being paired with a different female so at the end all males were paired with all females.

Figure 4. Different phases of artificial fertilization experiments at the facilities of IRTA (Spain): Top left: sperm collection; Top right: egg collection; Bottom left: gamete mixing and activation; Bottom middle: view of 134 individual fertilized batches of eggs from a factorial cross with 3 females and 4 males, and 3 different types of sperm storage (fresh, chilled stored and frozen) in triplicates; Bottom right: devices for the assessment of embryo development after artificial fertilization.

the percentage of developing eggs. An injection of GnRHa was also applied to males, and sperm quality was assessed. Ratios of sperm to eggs were tested, from approximately 3,000 to 500,000 spermatozoa per egg. Ovulated eggs were observed from 35 hours onwards. Optimal egg quality was observed at 38-39 hours after the GnRHa injection. From 35 to 38-39 hours there was a slight increase in egg quality and the ease with which eggs could be stripped, indicating that after 35-38 hours there was a possibility that eggs were not fully ovulated. After 38-39 hours, there was a decline in egg

quality to 43-44 hours. Sperm quality was maintained without decline for up to 7 hours in Leibovitz medium and sperm quality did not appear to affect fertilization success. The *in vitro* fertilization was made by rapidly mixing eggs, sperm and seawater simultaneously, and the first 30 seconds after activation were identified as the optimal period for fertilization. The optimal ratio of sperm to eggs to obtain high percentage of fertilization was above 200,000 spermatozoa per egg. The protocol was used successfully in a large factorial cross of 120 *in vitro* fertilizations using either fresh or cryopreserved sperm.

One of the most important bottlenecks of meagre production is the occurrence of systemic granulomatosis (SG), a pathological condition affecting the majority of farmed populations. Systemic granulomatosis is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. The aetiology of the disease is unknown, however it is suspected that it is related to nutritional factors. One of the objectives of DIVERSIFY is to identify potential nutritional causes of SG via several feeding trials. These included the effect of vitamin D, calcium (Ca) and Phosphorous (P) and Ca/P ratios, and the effect of fishmeal (FM) substitution of the diets with plant proteins (PP) and P supplementation.

The development of SG was not prevented by vitamin D_3 (**Fig. 5**). The organs that seemed to be affected first from SG were the kidney and the liver. The other soft tissues exhibited granulomas after the second month of the feeding trial, mainly visible by microscope. Although the addition of vitamin D_3 did not prevent the development of SG in meagre, the study provided a significant lead concerning the pathophysiology of SG that will further assist the detailed description of this peculiar disease. In terms of the effect of Ca and P, although the fish of all groups exhibited granulomas, high P content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis. Fish fed this diet exhibited a significantly lower percentage of liver and kidney calcification, and there was a significantly higher percentage of fish with no granulomas, compared to those fed the low and medium content of P. As before, the organs mostly affected by granulomatosis were the kidney, the liver and the spleen. This result is in accordance with the hypothesis that granulomatosis could be a metabolic disorder or a nutritional disease. Accordingly, PP in the diets of meagre were found to affect negatively SG, while P supplementation in the PP diets did not affect the overall condition, but had a positive effect in the liver of the fish. Fish fed 60% FM were in a significantly better state regarding the total score of granulomas in all tissues. Furthermore, fish fed this diet exhibited a significantly lower percentage of liver and spleen calcification, and there was a significantly higher percentage of fish with no granulomas in these organs compared to those fed the PP, PP+Medium P and PP+High P diets. Phosphorus supplementation in the PP diets did not affect the overall condition of the fish (as assessed by the granuloma scoring system), but had a positive effect in the liver of the fish. Specifically, fish fed the PP+High P diet exhibited lower percentage of liver calcification and liver with macroscopically visible granulomas compared to those fed the PP and PP+Medium P diets.

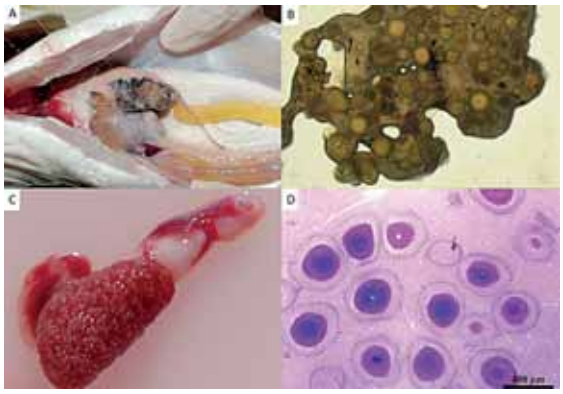

Figure 5. Systemic granulomatosis (SG) in meagre. A. Liver calcification in SG-affected meagre. B. Squash preparation of liver tissue with multiple granulomas. C. Heart of SG-affected meagre with multiple granulomas. D. Histological section of the heart showing the typical appearance of the granulomatous lesions with necrotic center and concentric lamellation.

Despite the fact that the fish of the FM group were in a better state, they also exhibited granulomas in all examined tissues. Some of plant derived ingredients such as wheat and wheat gluten are used as pellet binders in extruded feeds. Thus, FM-based diets also contained small amounts of those, which may be responsible for the appearance of granulomas in meagre. Moreover, the results obtained in our study showed that a reduction of FM from 60% to 14% is possible for juvenile meagre in terms of growth performance, but only in combination with high levels of P supplementation (15 g kg^{-1}).

As it is anticipated that future management of disease issues in meagre will require vaccines as part of the arsenal of approaches used, another objective of DIVERSIFY was to attempt to understand completely the chronology of events that occur –within the context of the immune system- during grow-out. Therefore, work was undertaken towards the characterization of the immune system to identify key immune molecules, as potential markers of immune system development and induction of antiviral and antibacterial responses. For markers of the adaptive immune system, a number of key genes were chosen for cloning, including RAG1/2, Ig and TcR genes, to determine when to vaccinate as the immune system matures. In addition, marker genes of inflammation (IL-1β, TNFα), antibacterial responses (antimicrobial peptides, such as piscidins and defensins) and the antiviral response (interferon, Mx) genes were also chosen. All of the genes initially targeted for this work have been isolated with the exception of transferrin and the expression assays have been established for these genes. Immune markers are now established for the innate, adaptive and inflammatory responses of the immune system as originally proposed. In total, we have 28 assays developed for genes of interest for the study of immune function in this species, and this will be of interest also to other groups and researchers studying this species outside of the DIVERSIFY consortium.

Finally in meagre, trials were conducted in order to improve the fatty acid or micronutrient contents of meagre weaning diets and to achieve early weaning on artificial diets (Campoverde and Estevez, 2017). Despite the fact that meagre larval rearing techniques

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have been studied extensively, weaning to dry diets remains an important bottleneck for this species, and it is thought to relate to the variable growth observed from early grow out. Our study has demonstrated the importance of supplementation of meagre weaning diets with 2.4 mg kg^{-1} of vitamin K, since the absence of this vitamin reduced markedly larval survival. However, meagre seemed to be very sensitive to hypervitaminosis D and only mildly sensitive to hypervitaminosis A, since supplementation with these vitamins lead to growth reduction. On the contrary, taurine supplementation did not have any effect in meagre larval performance. Also, based on the results of early weaning trials, meagre larvae can be weaned from live feed to artificial diets as early as 12 days post hatching (dph), but other important aspects for production success including larval performance and survival should be considered (Campoverde et al., 2017). Special care should be taken to avoid cannibalistic behaviour in the rearing tanks, by reducing the light intensity at the water surface and increasing larval feeding rate and daily doses. Early weaning did not affect the incidence of skeletal deformities in meagre, which is of special relevance in terms of assuring fry quality for further on-growing purposes.

GREATER AMBERJACK

This is a cosmopolitan species of great interest to the aquaculture sector due to its **excellent flesh quality** and **worldwide market availability**. Its rapid growth and large size (3 kg in 2 years) makes this species **very suitable for product diversification and development of value added products**. In the Mediterranean (Lovatelli and Holthus, 2008), farming started with capture-based activities using wild juveniles (Crespo et al., 1994), but until recently a very limited commercial activity with hatchery-produced individuals existed in Europe, in spite of the existing interest and efforts by various aquaculture companies in the Mediterranean. The major bottlenecks for the incorporation of greater amberjack in the EU aquaculture industry include lack of **reliable reproduction**, **production of adequate numbers of juveniles** and **fish health** related issues, with monogenean parasites causing mass mortalities in farmed fish (Grau et al., 2003; Montero et al., 2004).

In DIVERSIFY, a major effort in greater amberjack has been channeled toward the study of its reproduction (in the wild and captivity) and the development of reproduction control methods that will allow the production, on demand, of viable eggs of adequate quantity and quality *(Aquaculture Europe 41(1) March 2016)*. This would enable the systematic research on the development of larval rearing methods for the species, and the production of juveniles to supply the aquaculture industry. Our work in greater amberjack reproduction begun with a comparative study looking into the reproductive function of fish in the wild and trying to identify the potential source of the reproductive dysfunctions observed in captivity (Zupa et al., 2017). Wild and captive-reared breeders were sampled in the Mediterranean Sea during three different phases of the reproductive cycle (**Fig. 6**): early gametogenesis (EARLY, late April-early May), advanced gametogenesis (ADVANCED, late May-early June) and spawning (SPAWNING, late June-July). Fish reproductive state was evaluated using the gonado-somatic index (GSI), histological analysis of the gonads and determination of sex steroid levels in the plasma, and correlated with leptin expression in the liver and gonad biochemical composition. The GSI and sex steroid levels were lower in captive-reared than in wild fish. During the ADVANCED period, when the wild greater amberjack breeders were already in spawning condition, ovaries of captive-reared breeders showed extensive atresia of late vitellogenic oocytes and spermatogenic activity ceased in the testes of half of the examined males. During the SPAWNING period, all captive-reared fish had regressed gonads, while wild breeders still displayed reproductive activity. Liver leptin expression and gonad proximate composition of wild and captive greater amberjack were similar. However, the gonads of captive-reared fish showed different total polar lipid contents, as well as specific lipid classes and fatty acid profiles with respect to wild individuals. This study underlines the need for an improvement in rearing technology for this species, which should include minimum handling during the reproductive season and the formulation of a specific diet to overcome the observed gonadal decrements of phospholipids, DHA (22:6n-3) and ARA (20:4n-6), compared to wild breeders.

Figure 6. Sampling of mature broodstock of greater amberjack in the wild (above) and at a commercial facility in Greece (below) at three different times during the reproductive season. The objective of the study was to understand the causes of reproductive dysfunctions that were commonly observed in captive broodstocks of this species.

For the acquisition of viable gametes, a number of different broodstocks were maintained in land-based facilities (Greece and Spain) and sea cages (Greece), and were monitored for reproductive maturation, implementing the principles of broodstock management established earlier. At the expected peak of the reproductive period, the breeders were examined for gonadal development (vitellogenesis and sperm production), and were selected to test different hormonal spawning induction methods. These were based on the use of GnRHa, either in the form of liquid injections or sustained-release delivery systems (implants). Three major experiments have been undertaken. The first (FCPCT,

Table 2. Number of greater amberjack females that spawned, number of spawns and time of natural spawns and latency period for GnRHa injections and implants (FCPCT, Gran Canaria, Spain).

Treatments	Number females that spawned	Number of inductions	Number of spawns	Spawns/ Induction	Latency period (h)
Natural (Control)	2	-	23	-	-
Injected	3	37	29	0.78 ± 0.53[b]	43.06 ± 2.49
Implanted	3	17	38	2.23 ± 1.85[a]	44.19 ± 7.44

* Results are expressed as means ± SD. Different superscripts in the same column indicate significant differences (P<0.01).

Table 3. Number of eggs obtained from greater amberjack broodstocks after treatment with GnRHa injections or implants, in comparison with spontaneously spawning fish (FCPCT, Gran Canaria, Spain).

Treatments	Number of eggs (x 10^6)	Number of eggs per female (x 10^6)	Number of eggs per spawn (x10^6)	Number of eggs per kg of female per spawn (x 10^4)
Natural (Control)	25.60	12.80	1.11±0.32[a]	5.67±1.66 [a]
Injected	12.90	4.30	0.44±0.27[b]	3.72±2.30[b]
Implanted	10.53	3.51	0.28±0.29[b]	2.52 ±2.73[b]

* Results are expressed as means ± SD. Different superscripts in the same column indicate significant differences (P<0.01).

Gran Canaria, Spain) examined the efficacy of different hormonal induction methods on wild-caught breeders from the eastern Atlantic Ocean stock, maintained in tanks. The second (IEO, Tenerife, Spain) examined the efficacy of different doses of GnRHa implants on F1 breeders of the eastern Atlantic Ocean stock. The third set of experiments (HCMR, Greece) examined the timing of application (early, mid, late season), the hormonal induction method (GnRHa injections vs implants) and the dose of GnRHa used, on wild-caught breeders from the Mediterranean Sea stock maintained in sea cages during the year.

For the experiments at FCPCT, breeders with an average weight of 10.7 ± 2.3 kg were distributed in three circular tanks of 40-m^3. In tank 1 (2♀y 5♂), the broodstock was not induced and was allowed to spawn spontaneously. In tank 2 (4♀ y 4♂), three individuals of each sex were selected and were injected with GnRHa at 20 µg kg^{-1} BW. In tank 3 (4♀ y 3♂), three individuals of each sex were also selected and were induced with 500 µg GnRHa implants. Natural spawns started on 1 June 2014 and ended on 18 October, obtaining a total of 23 spawns at temperatures of 21.5-24.5ºC (**Table 2**). The first GnRHa injection was given on 3 June and the last one that resulted in spawning was given on 21 October. The first GnRHa implant was given on 3 June and the last one that resulted in spawning was given on 14 October. The number of spawns per induction was significantly higher in females treated with the GnRHa implants. The number of eggs per spawn in the natural spawnings was greater than those obtained with GnRHa injections and implants (**Table 3**). The quality of the spawns was the best when the fish spawned spontaneously, with a percentage fertilization, hatching and larval survival 1 dph of 84 ± 21, 96 ± 6 and 69 ± 16%, respectively. With GnRHa injections the percentages for the three parameters were, respectively, 58 ± 26, 91 ± 25 and 58 ± 23% and with the GnRHa implants they were 32 ± 34, 77 ± 34 and 49 ± 27%. This study concluded that in the conditions of photoperiod and water temperature of the Canary Islands, it is possible to obtained natural spawnings of a small percentage of wild-caught greater amberjack maintained in captivity during three years in tanks with a volume of 40 m^3. These spawns were better than those obtained by induction with GnRHa injection or implants. However, the majority of breeders would require a hormonal therapy to complete maturation and undergo spawning.

For the experiments at IEO, the broodstock consisted of 14 fish born in captivity (F1 generation) between 2005 and 2009 from a wild-caught broodstock. Fish were maintained during the year in two outdoor covered 50-m^3 tanks, supplied with well water (10 renewals day^{-1}) at natural water temperature and ambient photoperiod. The fish were sampled four times during the spawning season (May, June, July and September) of two consecutive years (2015-2016), and each time (except in September) they were treated with implants at a dose of 25 or 75 µg kg^{-1} GnRHa, respectively, for the two years, in order to examine the effect of treatment dose on spawning performance and egg quality (**Fig. 7**). Using the 75 µg GnRHa dose, a total of 52 spawnings were obtained during a period of 72 days. The number of spawns obtained in the successive post GnRHa-treatment periods decreased, as well as the number of eggs released (**Table 4**). After the 1st treatment, a period of 31 days between the first and the last spawning was observed, while the eggs were collected almost daily (29 spawning events). However, after the 2nd treatment, a total of 15 spawnings were recorded during the first 16 days and no eggs were collected the later days. The eggs released after the 3rd GnRHa treatment were collected after 8 spawning events during the 9 days following treatment. Mean fertilization changed through the spawning period after each treatment, reaching its highest values after the 1st and 2nd GnRHa treatment, and a significant decrease was observed after the 3th treatment. On the other hand, no significant differences were observed in hatching, 1-day embryo survival and 3-day larval survival after successive GnRHa treatment. The study of the lower dose (25 µg kg^{-1} GnRHa in 2016) produced similar results in terms of reproductive performance and egg quality.

The third set of experiments was undertaken at a number of tank and sea cage facilities in Greece,

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Figure 7. Induction of spawning of F1 greater amberjack breeders at the IEO facility in Tenerife, Spain. (left) weighing the breeders and (right) evaluating the diameter of the oocytes prior to the administration of the GnRHa implants.

Table 4. Spawning and egg fecundity parameters (mean ± SEM) of greater amberjack broodstock induced to spawn using three consecutive GnRHa implant treatments. No statistically significant differences were observed (P<0.05) between different GnRHa treatments.

Treatment	Spawns (n)	Eggs spawn^{-1}kg^{-1}	Total eggs kg^{-1} (x1000 eggs)	Total eggs (x10^6 eggs)
1	29	2087±218	60.54	7.05
2	15	2679±398	40.18	6.55
3	8	1361±594	10.89	1.35

including the research facilities of HCMR, and the commercial operations of Galaxidi Marine Farms, Argosaronikos Fishfarms S.A. and Aquaculture FORKYS S.A. The first major finding of these studies was that maintaining greater amberjack breeders in land-based facilities supplied with the typical borehole water of commercial hatcheries does not allow full gametogenesis of the fish. This means that males do not spermiate adequately and females do not complete vitellogenesis to the stage of being able to be induced for spawning using GnRHa therapies. On the contrary, fish maintained in sea cages during the year completed gametogenesis adequately, and it was possible to induce them to spawn using exogenous hormones, after moving them temporarily to tanks for spawning (**Fig. 8**).

In one of the trials, wild-captured breeders (mean weight 17.0±2.6 kg) were kept in Argosaronikos Fishfarms S.A. in a 1,000-m^3 cage during the year and were fed with a broodstock diet (Skretting, Vitalis Cal, 22 mm). Females were treated with either a GnRHa injection (20-25 μg kg^{-1}) or GnRHa implant, with an effective dose of 49-69 μg GnRHa kg^{-1}. To enhance spermiation and ensure adequate sperm production, all males were treated at the start of the induction with a GnRHa implant at a dose of 45-70 μg kg^{-1}. After being treated for spawning, fish were transferred to inland facilities, into four 23-m^3 flow-through round tanks (n=3-4 females), at a 1:1 sex ratio. Females in the injected group were given a GnRHa injection every week for 3 weeks, whereas the implanted group was given a second implant after two weeks (a total of 3 GnRHa injections and 2 implants). Tank overflow egg collectors were examined three times a day, and fecundity and fertilization success were estimated immediately after egg collection. Spawning started 1 d after the 1st GnRHa treatment, as some females had oocytes already undergoing maturation (**Fig. 9**).

Figure 8. Acquisition of ovarian biopsies from greater amberjack (top) maintained in sea cages during the year, and evaluation of the stage of development of the obtained oocytes (bottom), in order to select fish for treatment with hormones to induce spawning. After treatment, fish were moved to tanks for spawning and egg collection.

Implanted fish spawned 9-10 times after the 1st implant and only 4 times after the 2nd implant. Injected fish spawned 7, 3-5 and 1-3 times after the 1st, 2nd and 3rd injection, respectively. The highest daily or batch fecundity was produced by the GnRHa implanted fish and was 4,242,000 eggs tank^{-1} 2 days after the

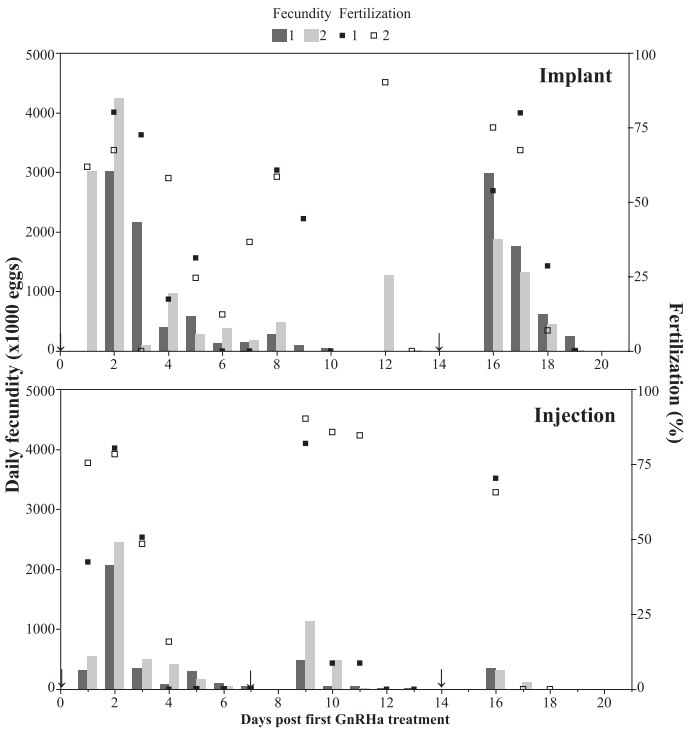

Figure 9. Egg production (per tank, n=2) and fertilization success (%) of greater amberjack maintained in sea cages in Greece during the year, and placed in tanks (numbered 1 and 2 for each treatment) for spawning after treatment with GnRHa injections or implants (arrows on the x-axis).

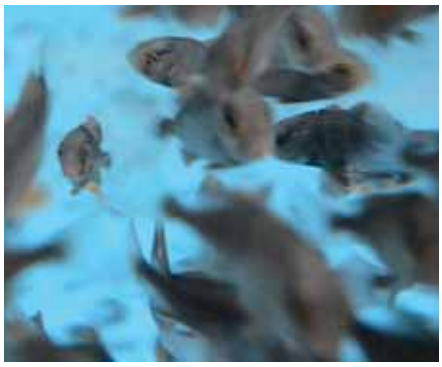

Figure 10. Greater amberjack juveniles produced at HCMR, Greece (above) and transferred to sea cages for grow-out studies (below).

1st treatment, while in the injected fish the highest fecundity was 2,454,000 eggs tank^{-1}. The GnRHa implanted fish produced 2.5X more eggs than the injected fish, with the same number of spawns. On the other hand, no differences were observed among the two treatment methods in terms of fertilization success, embryo survival, hatching or 5-day larval survival. The study demonstrated that GnRHa implants were more effective in this stock to induce spawning, contrary to what was observed for the eastern Atlantic Ocean wild-caught population (see earlier).

In the area of greater amberjack larval rearing, significant breakthroughs have been achieved, allowing the production of large numbers of juveniles for stocking into research and commercial sea cages (**Fig. 10**). The main objective of DIVERSIFY in this area was to improve the survival, growth and performance of greater amberjack larvae by defining the appropriate environmental and feeding conditions adequate for the species. Eggs from the different broodstocks of DIVERSIFY used for the spawning induction experiments, were provided to the larval rearing partners of the consortium to undertake a number of trials.

In one study that has been completed, optimum levels and ratios of essential fatty acids in relation to taurine (Tau) and combined Poly Unsaturated Fatty Acids (PUFA) and carotenoids in enrichment products were examined (**Fig. 11**). A list of the optimum levels and ratios of essential fatty acids and carotenoids that should be included in enrichment products for rotifers to be fed to greater amberjack larvae have been established. The results included the effects of essential fatty acids and carotenoids on (a) larval performance,

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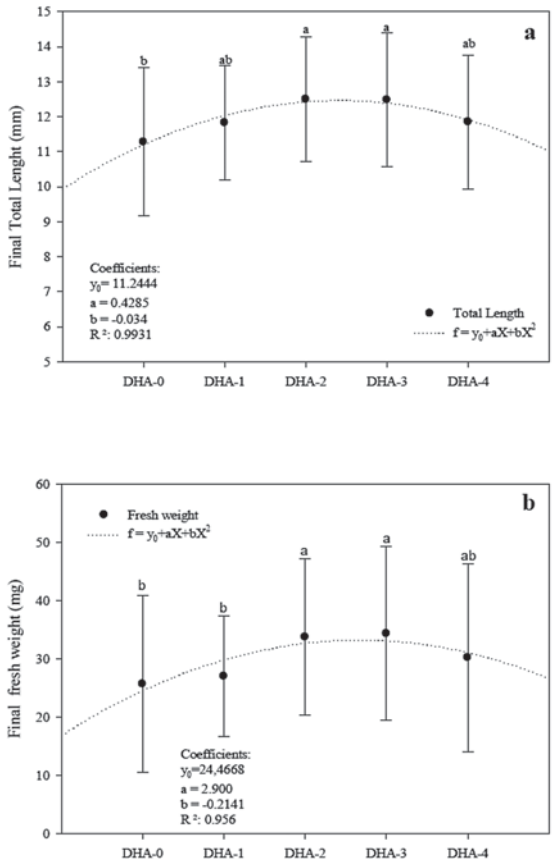

Figure 11. Relationship between (a) total length (mm) and (b) fresh weight (mg) and different levels of dietary *Artemia* DHA (22:6n-3) content in greater amberjack larvae 35 dph (mean ± S.D., n=3). Data are fitted to a quadratic regression analysis (f=y0+ax+bx2).

Figure 12. The mesocosm facilities of HCMR (Greece) where the studies on the effect of semi-intensive rearing of greater amberjack larvae were undertaken.

(b) welfare and (c) fatty acid analysis, lipid classes, and carotenoid profiles of enrichment products, live preys and larvae.

Another study compared the semi-intensive and intensive larval rearing systems **(Fig. 12)**. The gene expression of growth hormone (GH) releasing hormone (GHRH), GH, insulin-like growth factor(IGF) -I and –II, and IGF binding protein (BP)s were not affected by the rearing method. However, there was a gradual increase in their mRNA levels as development proceeded, with statistically significant differences observed at 20 dph with peak levels at 25 and 30 dph of IGF-I and GHRH, respectively. In addition, IGF-II was higher at 5 dph compared to 2 and 10 dph, while GH exhibited higher mRNA levels at 5 and 15 dph. The study determining the effect of stocking density on larval performance showed the optimum egg density for the larval rearing of greater amberjack is between 25 and 50 eggs l^{-1}. There was a marked appearance of a number of different anomalies in the larval stage that could lead to a lower survival. The study of the ontogeny of the greater amberjack larval digestive system showed that the average enzyme activity measured for a particular age range was independent of the larval rearing conditions. In general terms, the pancreatic enzymes amylase and alkaline protease were more active in the youngest larvae compared to the 30 dph larvae, whereas pepsin followed the opposite trend, displaying almost no activity at 12 dph. Intensive rearing seemed to favour amylase, alkaline protease and pepsin activities in the older larvae. Amylase was highly active in the eggs, decreasing at 0-5 dph, while increasing from 5 to 10 dph. From 10 to 30 dph, carbohydrates displayed a less relevant role in larval metabolism. Lipase and alkaline protease activities showed an increasing trend from 0-5 to 5-10 dph. However, although lipase decreased similarly to amylase activity after 10 dph, alkaline protease activity was still high at 10-15 dph, and increased further in the oldest larvae (20-30 dph). Amylase activity was also higher at 12 dph for the intensive system larvae, whereas the opposite trend was observed for alkaline protease and lipase activities. According to the results, greater amberjack seem to use dietary proteins effectively from 20-30 dph.

In another study, the effect of tank hydrodynamics on larval performance was studied by estimating the hydrodynamic field in tanks of 2,000 and 40,000 l. The applied water exchange rates (as % of total water volume) were 10% and 4% per hour, while the airflow was set at 350 and 1400 ml min^{-1} for the 2,000 and 40,000 l tank respectively. The conditions regarding water exchange and airflow were similar to the ones applied during larval rearing. The results showed differences between the conditions, as the higher currents occurred in the 2,000 l tanks followed by the 40,000 l tanks. Significant differences, in total length and body weight, were observed between treatments, with the individuals from the 2,000 l tanks being larger. Results showed higher survival at the end of the experiment in 2,000 l tanks, independent of egg stocking density. This was particularly apparent in 2,000 l tanks stocked with 10 eggs/l^{-1}.

The photoperiod study showed a good performance with the survival of the larvae varying between 6% and 13.6%. The mean survival for the 18L:06D photo phase was slightly higher (10.6±4.2%), than the 24L:00D (8.2±3.1%). In terms of total length, larvae grew with an exponential rate of 0.310 d^{-1} independent of photo phase. The trials revealed that the photoperiod affected the mRNA expression levels of IGF-I with higher levels for the 18L:06D group at 17 and 25 dph compared to the 24L:00D group. Additionally, there was a significant gradual increase in mRNA levels as development proceeded, which was observed only in the 18L:06D group with peak values at 25 dph. The mRNA levels of IGF-BP1 appeared to be generally stable during development, and increased expression levels were observed in the 18L:06D group compared to the 24L:00D group. The IGF-BP2 expression showed a gradual increase throughout development with statistically higher levels at 25 and 30 dph. Additionally, at 30 dph an effect of the photoperiod regime was observed with higher expression levels in the 18L:06D group compared to the 24L:00D group.

The study on the effect of tank color showed no significant differences in the growth of the larvae in terms of total length and body weight. Fish growth was exponential in terms of TL (black: 0.0481 d^{-1}, white: 0.0393 d^{-1}, green: 0.0355 d^{-1}) and wet weight (black: 0.1260 d^{-1}, white: 0.1970 d^{-1}, green: 0.171 d^{-1}). However, significant differences were observed in the survival of the larvae during the 2015 experimental period that were more profound during 2016. The white background resulted in a significantly higher survival rate (22.2±0.7%) compared to green (16.5±0.9%) and to black (8.2±3.1%) backgrounds. The analysis of the 2016 results showed that the fish reared in the white background showed increased levels of the genes implicated in the growth axis system compared to the fish reared in the black and green backgrounds. In particular, IGF-I showed generally higher levels of expression as development proceeds and also it appeared to be affected by the background color, as higher levels were observed in fish reared in the white background at 17 dph and 30 dph compared to fish reared in the black and green backgrounds. The IGF-II did not show a particular pattern during development. However its expression appeared up regulated in a statistically significant

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Figure 13. Examination of greater amberjack gills for the presence of monogenean parasites. This breeder was found with a large number of *Zeuxapta seriolae*.

way at 17 dph in fish reared in the white background compared to fish reared in the black and green backgrounds. This preliminary study provides for the first time information on the regulation of the various components of the IGF signaling pathway in greater amberjack and may serve for the better understanding of the complex relationship between background color and fish performance during early ontogeny. The results from the trials with the modified "light environment" improved survival an order of magnitude from any previous trial, reinforcing the validity of the tested hypothesis and indicate a clear technological step forward in the larval rearing of the greater amberjack.

In the area of fish health, progress has been made in all tasks included in the proposal. This included further mesocosm trials for the development of a rapid detection method for epitheliocystis, and screening of gill samples from different Greek fish farms (**Fig. 13**). Studies were undertaken on (a) the morphology and the incidence of monogenean parasites in greater amberjack skin, (b) the determination of environmental conditions that can modulate greater amberjack resistance to parasitic infection, (c) formulation of a diet supplemented with mucus stimulation products, and (d) standardization of monogenean cultures. Primers for the detection of 11 immune genes have been optimized for qPCR, ready for studies of mucosal defenses, with initial pathogen-associated molecular pattern (PAMP) stimulation *in vivo* revealing good induction at mucosal sites such as gills. Further grow out trials have been undertaken, to assess the relationship between monogenean parasite egg number and fish mortality, and the impact of several potential anti-monogenean treatments, with mannose looking promising. Diagnosis of bacterial and viral infections was performed with juveniles, and *Bacillus oceanisediminis* and *Aeromonas* spp. have been detected. Challenge trials 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.

One of the objectives of the research in this area was to identify the effect of dietary regime on mucus immune barrier and modulate the resistance to parasite infection by adding immunostimulants to the diet. Immune potential of mucus defenses has been studied from the systemic point of view (including lysozyme and bactericidal activities) and from the histological point of view. The utilization of mucus stimulatory substances, such as mannan-oligosaccharides (MOS) or concentrated mannan—oligosaccharides (c-MOS), has been also evaluated. A histological study of the effects of monogenean parasitization on greater amberjack juveniles was conducted, and the potential of immunostimulants to reduce parasitic infection has been also assessed. The study, completed recently, concluded that dietary regime could alter mucus immunological properties. The addition of mucus stimulating products, and especially those based on concentrated mannan-oligosaccharides, enhance mucus immune potential and resistance to the ectoparasite *Neobenedenia girelliae*.

A trial was also conducted in order to determine how aquaculture-associated stressful conditions are affecting selected parameters. The processes selected were manipulation (high and low) and stocking density (high and low), both related with aquaculture practices and are necessary to manage stocks of greater amberjack. At the end of the experimental period, a stress challenge test was conducted. The treatment with the highest manipulation and density had reduced growth performance when compared to the treatment with low manipulation and low density, denoting that greater amberjack is highly sensitive to aquaculture procedures. High stocking density induced a decrease in mucus lysozyme activity. A similar effect was obtained for bactericidal activity of the mucus, but no effects were found in peroxidase activity. These data are part of the results that have shown the immune potential of skin mucus of amberjack. Relative to other species, the mucosal surfaces of greater amberjack include a full repertoire of antimicrobial defenses that can vary with certain environmental conditions.

Pikeperch

This freshwater fish is considered to have the **highest potential in Europe for inland aquaculture diversification**. Pikeperch flesh has a neutral taste, thus lending itself to different forms of preparation, and the filets are without bones --unlike carp, which competes on the same market segment. Year-round production of pikeperch requires constant high temperatures (24-26°C) to ensure relatively high growth rates (*i.e.*, production of 1.2 kg fish in 15 -18 months from non-selected strains), which is only feasible in RAS. These RAS also allow high densities of 80-100 kg m^{-3}. Identified by a survey addressed to fish farmers in preparation for DIVERSIFY *(Aquaculture Europe 40(2) Sept 2015)*, the major bottlenecks for further expansion of pikeperch culture today include (a) **high sensitivity to stressors, handling and husbandry practices** that result in high and sudden mortalities, (b) **low larval survival** (typically 5-10%) and **high incidence of deformities**, and (c) **lack of knowledge of the genetic variability of the used broodstocks**. Identification of genetic relationships among different broodstocks, inbreeding phenomena and loss of heterozygosity is important in aquaculture, since it may result in subsequent reproductive and productive failure (reduced progeny survival, growth, food conversion efficiency and increased frequency of deformities). It is also important to know how the domesticated stocks differ from their wild counterparts, which could potentially be a future source of fish to include in breeding programs. Overcoming the above bottlenecks is very important to reduce production costs and, therefore, expand the aquaculture production of pikeperch in the EU (**Fig. 14**).

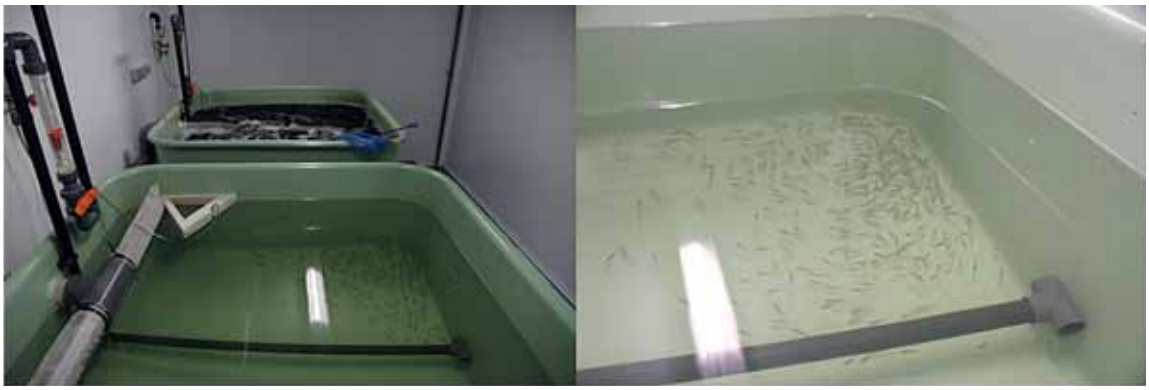

Figure 14. Rearing system for pikeperch larvae © Photos: Tatiana Colchen & Elodie Faux.

The first task of DIVERSIFY for pikeperch was to assess the genetic variability of captive broodstocks in commercial farms in Europe operating in RAS, and then compare this variability with that of wild populations. The results have indicated that some broodstocks have adequate genetic variation, but as some of them originate from few fish, attention should be paid in the future to establish breeding programmes. In general, there was agreement with the stock origin and our studies provided evidence that pikeperch populations in Europe are part of at least two genetically differentiated groups. The first group is found in northern Europe from the Netherlands/ Denmark to the West, Poland (at least) to the East, and Finland to the North. The second group comprises all remaining populations in Central Europe to as south as Tunisia (and probably Spain, Italy and northern Greece). Based on this grouping, it can be stated that most analyzed populations seemed to contain fish of a single origin; nevertheless, in few domesticated populations this ratio varied from 5-19%, possibly due to the mixing of fish from multiple sources. 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. A total of 21 populations/ broodstocks were sampled and analyzed, which included 13 captive broodstocks and eight wild origin populations. 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. For these broodstocks with reduced genetic variability, measures should be taken to introduce greater variation into the base population for future breeding programs.

In the area of pikeperch nutrition, trials have shown that pikeperch larvae require both high dietary inclusion levels of phospholipids and Long Chain (LC) PUFAs to perform optimally. A multifactorial screening trial of the importance of eight dietary factors (high or low levels) has been initiated and is still ongoing. Also, adding saline water to rearing does not improve growth, but can change the ability of pikeperch larvae to elongate and desaturate different fatty acids and phospholipids. An experiment investigating the consequence of various phospholipid levels and LC PUFAs on welfare indicators and stress physiology, behaviour and respiratory metabolism is currently ongoing. In the area of grow out, our studies identified the optimal conditions improving growth and welfare of pikeperch in aquaculture and characterized the effects of major husbandry and environmental factors on growth and physiological status of this species (Baekelandt et al., 2017). In a screening experiment, eight factors considered as relevant for the welfare of pikeperch were compared in two modalities using a fractional multifactorial design (2^{8-4}). Each experimental unit represented a combination of eight factors in two modalities, which included grading, stocking density (15 *vs* 30 kg/m^3), feed type (floating *vs* sinking), 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). Fish sampling occurred on days 36 and 63. Stress markers – glucose, cortisol and brain serotonergic activity – and changes in humoral immune activities and immune gene expression in kidney were assessed. Light intensity and the type of feed clearly appeared as directive factors for pikeperch culture. A strong effect of the feed type was observed on the final individual weight, the specific growth rate and the weight heterogeneity. High light intensity affected survival. The main influence on physiological and immune status was imposed by light characteristics, including intensity, spectrum and photoperiod, as well as temperature.

In the area of larval rearing (**Fig. 15**), one of the objectives was to determine the effects of four environmental factors and their interactions on pikeperch production using a multifactorial approach. One of the most important results of the present study was that different behavioral traits were observed in very young pikeperch juveniles using several behavioural tests (cross maze, social and stressor tests). This implies that some personality traits appeared very early in the life of pikeperch, and could be inheritable characters. We know, for instance, that a gene mutation linked to growth factors may modify fish personality in zebrafish *Danio rerio* (Norton et al. 2011). It is also known that domestication may also act as a selection process for personality traits (Moretz et al. 2007). Most studies carried out on it recognized that personality is defined by behavioural traits consistent through time and/or contexts. In fish, most studies on personality were performed either on juveniles (aged between 6

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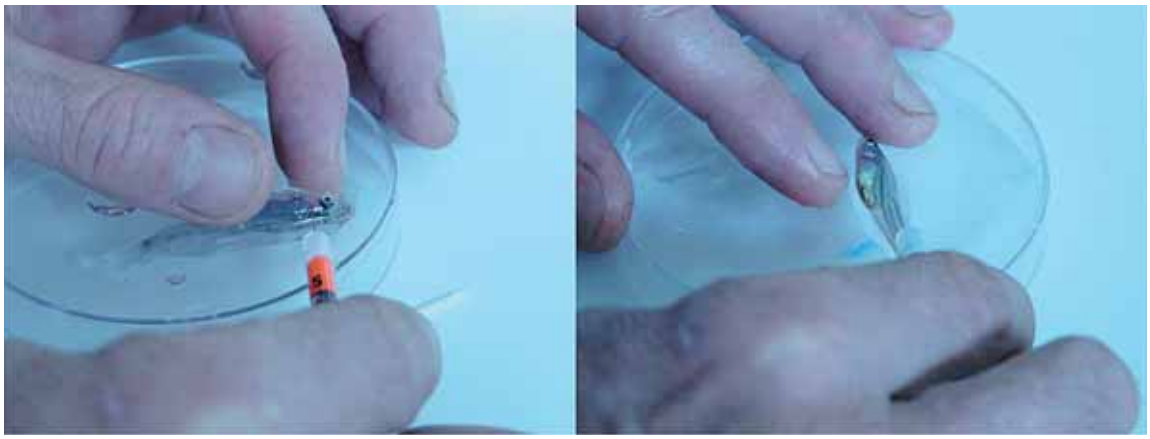

Figure 15. Fish tagged with Visible Implant Elastomers (VIE tag, Northwest Marine Technologies, USA). Two different colours (blue and pink) were used and tags were injected in the operculum and/or in flanks of each fish. © Photos: Tatiana Colchen.

months and 1 year) or adults, while very few focused on the early life stages. The main goal of this study in pikeperch was to characterize behavioural syndromes and to highlight the existence of a personality in young juvenile pikeperch. To study the consistency of behavioural responses of juvenile (50 and 64 days post-hatch) pikeperch (n = 41, TL =5.8 ± 1.0 cm and BW =1.6 ± 0.7 g), we performed three behavioural tests per fish: exploration (cross-maze), dyadic and restraint test. In the cross-maze test, exploratory fish were more active and bolder. In the dyadic test, fish with the highest number of contacts, showed also more approaches, orientations and avoidance behaviours. In the restraint test, bolder fish were more active and tried to escape more often. Consequently, the investigation of the different behavioural responses of each fish highlighted behavioural syndromes in this species. Furthermore, for the first time, we showed with a cross-context analysis that young juvenile pikeperch, responded in the same way to exploration and dyadic test, but their responses were opposite in the restraint test. Our results opened new opportunities for testing individual personality in very young fish that may help solving some aquaculture problems, such as the intra-cohort cannibalism.

Atlantic halibut

The **Atlantic halibut is the world's largest flatfish** and can attain a weight of over 300 kg. It is **highly prized at markets worldwide**, but availability of wild Atlantic halibut has been decreasing. The fish has been classified as endangered on the IUCN red list and a complete ban was imposed on Icelandic fisheries, while stocks along the Norwegian coast are declining and under strict regulation. This has led to a higher market demand for Atlantic halibut than can be met by fisheries alone. Cultured Atlantic halibut has an excellent reputation, but has been rarely available outside specialty restaurants due to low annual production. However, it is now the marine aquaculture industry that has the fastest growth in terms of production, with an increase of 20% from 2015 to 2016. The Atlantic halibut is a semi-fat fish rich in omega-3 fatty acids, with a characteristic flaky white meat with few bones. These characteristics led to the inclusion of Atlantic halibut in DIVERSIFY, as a great candidate for fish species and product diversification in European cold-water aquaculture *(Aquaculture Europe 41(2) Sept 2016)*. Despite a significant research effort between 1985 and 2000, the complicated life cycle of Atlantic halibut made aquaculture progress slow, and very little research funding has been allocated thereafter. The remaining bottlenecks for increased and stable production are related to a **steady supply of fry and a need to decrease the production time**. DIVERSIFY addresses these important bottlenecks with a coordinated research effort in **reproduction, and larval nutrition and husbandry**.

Advances have been made so far in all tasks in the area of Atlantic halibut reproduction (**Fig. 16**). Regarding the documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut, there were actually few differences between fecundity, fertilization, hatching, egg size and hormone content between eggs from wild-caught and farmed females. However, although there were no significant differences, wild-caught females appeared to be more predictable spawners and gave fewer, but larger batches of eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified correctly and stripping was carried out close to ovulation, thus reducing or eliminating over-ripening (**Fig. 17**). However, for commercial as well as breeding purposes, it is not practical to rely solely on wild-caught females. As at both the Institute of Marine Research and Stirling White Halibut AS (Norway) relatively few farmed females produced eggs with fertilization rates >80-85% consistently, it may be necessary to include also wild-caught broodstock in future breeding groups, in order to ensure a broad enough genetic material. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the considerable workload connected with artificial spawning and egg collection in Atlantic halibut. The other approach explored in the task included the use of GnRHa implant therapy as a means to improve reproductive performance, and ensure (and regulate) ovulation using GnRHa implants. The GnRHa implants used did ensure and synchronize ovulations

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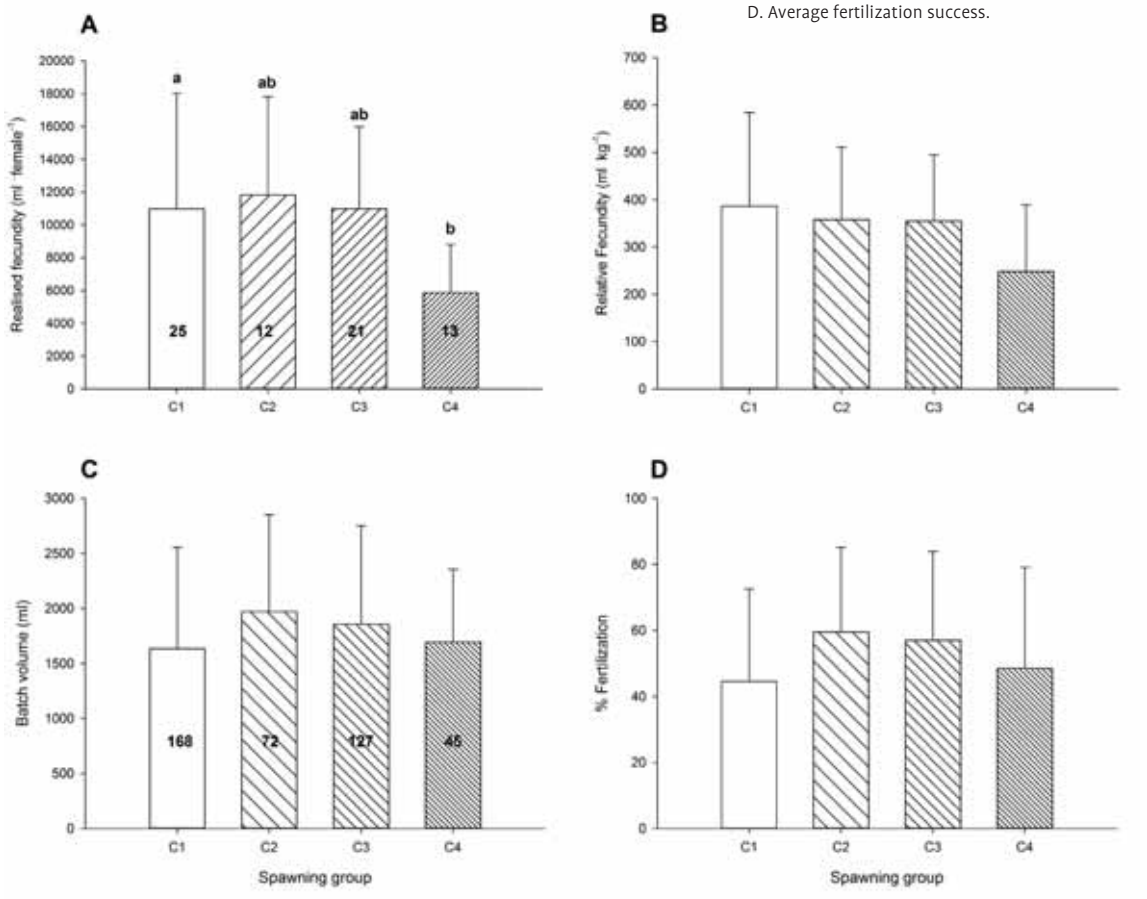
of the treated females and were found not to affect egg quality or quantity.

In the area of nutrition and larval rearing, a protocol for weaning of Atlantic halibut at 28 days post first-feeding (dpff) has been developed and almost 100% of the larvae fed Otohime diet (Japan) were filling up their guts with feed after a 5 day adaptation period. Gut fullness was lower in the morning than in the evening, possibly because the larvae were measured before and after hand feeding and clay addition in the morning and in the evening, respectively. According to the evening measurements, larvae fed *Artemia* were almost full after 1 day and stayed full for the rest of the experiment. Larvae fed Otohime showed increasing fullness over the whole period and on day 5 almost 100% of the larvae were full in the evening. The fraction of larvae with food in their gut increased more slowly on Gemma and Aglonorse feeds. On the evening of day 5, a total of 12 and 15 larvae, respectively, out of 28 had filled guts, while no larvae had filled guts on these diets.

Left: Figure 16. Atlantic halibut breeders being examined for reproductive maturation at the IMR facilities (Norway).

Below: Figure 17. Egg production results from four farmed Atlantic halibut broodstock groups with different spawning periods at Stirling White Halibut AS, Norway. A. Total fecundity (number in bars = n of females), B. Relative fecundity, C. Average batch size (ml of eggs; number in bars=n of batches) and D. Average fertilization success.



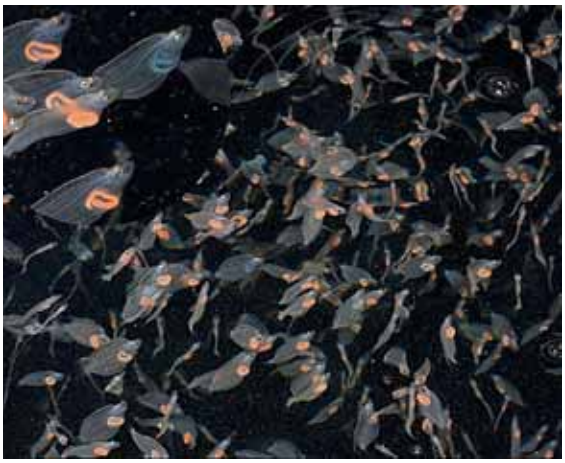


Figure 18. Atlantic halibut larvae at the stage of first feeding, at the IMR facilities (Norway).

A production strategy for on-grown *Artemia* has been established, which improves the nutritional value of *Artemia* with respect to protein, lipid and micronutrient contents. Growth and juvenile quality was excellent in larvae fed *Artemia* nauplii in this experiment, but not improved by feeding on-grown *Artemia* (**Fig. 18**). In the industry, the routine larval rearing method is to feed *Artemia* nauplii, with varying incidence of larvae with abnormal pigmentation and lack of eye migration, although the Atlantic halibut juvenile quality has improved in recent years. In this study, larvae fed the *Artemia* nauplii had perfect pigmentation and eye migration, so the juvenile quality could not be improved further by feeding on-grown *Artemia*. The nutrient concentrations of Atlantic halibut larvae fed *Artemia* nauplii and on-grown *Artemia* from 15 until 28 dpff were similar, except that the on-grown group had a slightly lower level of EPA than larvae fed nauplii, a difference that is probably biologically insignificant. This is another possible explanation of the lack of differences in growth and larval performance between the two treatments. It was very labor-intensive to produce the on-grown *Artemia* needed for the experiment, so on some occasions the on grown group had to be fed nauplii to get enough food. As the fish grow, more feed is needed and, due to capacity problems, the feeding period had to be shortened to last until 28 dpff instead of 45 dpff as was planned. These are all possible reasons that no differences were detected between the groups.

Figure 19. Wreckfish being sampled for ovarian biopsies and blood for the description of the annual reproductive cycle in Aquarium A Coruna (above) and HCMR (below).

Wreckfish

Wreckfish is one of the largest Serranid species, reaching a size of 100 Kg, and it is found in deep-waters almost throughout the world. Wreckfish is one of the most interesting new species for aquaculture, due to its **fast growth**, **late reproductive maturation**, **high market price and limited fisheries** landings. Its large size lends itself to **processing and development of value added products**, and its **cosmopolitan distribution may enable** EU exports. **Lack of reproduction control and established larval rearing protocols are considered major bottlenecks** preventing wreckfish aquaculture, and the clear biological and economical potential of this species justifies allocation of part of the effort of DIVERSIFY in bringing together almost all partners involved in Europe in wreckfish domestication.

Recently, an article has described the work and achievement of the DIVERSIFY project regarding wreckfish (Aquaculture Europe, 42(1) March 2017). Therefore, just a brief mention is made in this article of the latest information. Although significant progress has been made in the area of reproduction during the first 3 years of DIVERSIFY, development of effective larval rearing methods is still not at hand. The reproductive cycle of the species in captivity and the associated profiles of the sex steroids have been characterized recently, using a number of broodstocks from Spain and Greece (**Fig. 19**). Vitellogenesis begins in October and is completed between April-June, depending on geographic location and rearing temperature. Spontaneous spawning of viable, fertilized eggs has been accomplished in a number of broodstocks, but hatching success has been very limited (**Fig. 20**). Natural spawning occurs in a cycle of ~5 days. Males produced very good quality sperm during the reproductive season, and in some stocks they are spermiating for the whole year (under constant 15°C rearing conditions).

Egg quality and larval rearing continued to be problematic during the 2017 reproductive season, and we are still away from the development of a reliable larval rearing protocol for transfer to the industry. Efforts will continue in 2018, the last year of the project, and we hope for a better success.

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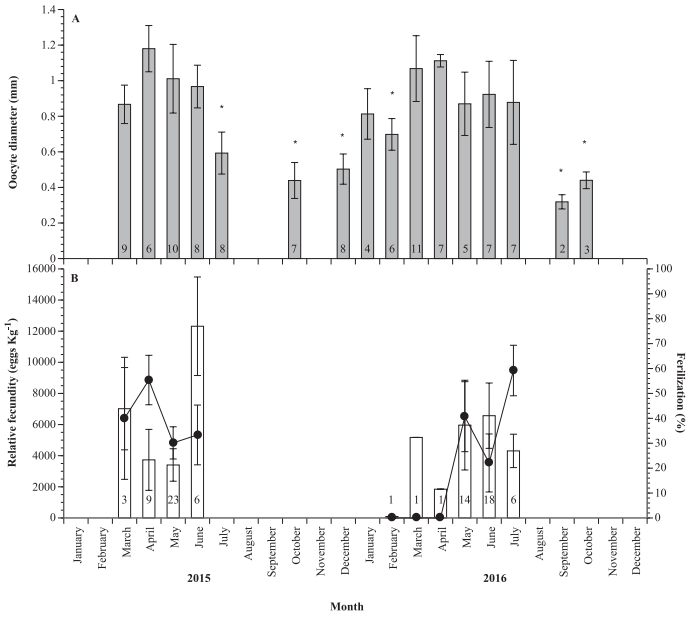

Figure 20. Mean (±SEM) oocyte diameter (A) of wreckfish broodstocks at four different sites in Greece and Spain during the annual reproductive cycles from March 2015 until October 2016. The numbers inside the bars indicate the number of females biopsied each month. Asterisks (*) denote significantly lower values than maximums observed each year (April 2015 and 2016). Mean (±SEM) monthly relative fecundity (bars) and fertilization percentage (lines, B) of natural and induced spawns of 4 wreckfish broodstocks in the reproductive periods of 2015 and 2016. The numbers inside the bars indicate the number of spawns obtained each month.

Grey mullet

Farming of grey mullet has been practiced for centuries, but production of this potentially invaluable source of animal protein in Europe has been small and non-intensive (Crosetti and Blaber, 2016). It is a **euryhaline species, found throughout the world** and is a **rapid-growing, herbivorous species** that can be reared over the wide geographical and temperature range of the Mediterranean basin (**Fig. 21**). As it is detritivorous in the wild, it has been stocked in fishponds to improve sediment quality and avoid oxygen depletion. Therefore, it can be an excellent candidate for the enhancement of aquaculture in **earthen ponds, coastal lagoons, "valli" and deserted Salinas** that exist throughout the EU Mediterranean countries. The development of FM-free feed will reduce the cost of aquaculture fish production, and will be **more sustainable and environmentally friendly**. In this way, grey mullet would be more acceptable to an increasingly aware consumer public that demands sustainability and lower environmental impact. Moreover, grey mullet aquaculture has the advantage of providing not only affordable whole fish and fillets, but also **fish roe or "bottarga" in Italian, a high value product (>100 € kg^{-1})**, whose market is expanding around the Mediterranean. Therefore, grey mullet has a great biological and economical potential for the diversification of fish species and product, and the development of value added products.

The future growth of the grey mullet aquaculture is limited by a number of bottlenecks, which are addressed in DIVERSIFY *(Aquaculture Europe 39(1) March 2014)*. Firstly, **controlling the reproductive cycle and improving egg quality** via broodstock management and nutrition is necessary not only for the production of robust larvae, but also for producing high value bottarga. Secondly, **development of a larval rearing protocol** is necessary to reduce early

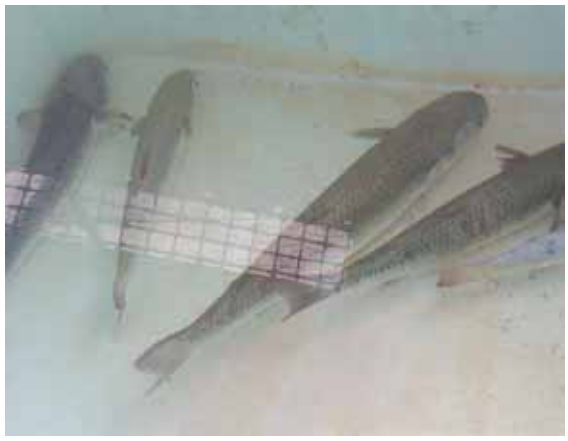

Figure 21. Grey mullet broodstock at the facilities of NCM-IOLR, Israel.

mortalities, size dispersion as well as increasing metamorphic synchrony, which will lead to a supply of high quality juveniles. Finally, development of a sustainable, economical, **FM-free grow out feed** is needed, which would perform well under different environmental conditions of temperature, pond type, and water quality, thus broadening the geographical range of grey mullet aquaculture in Europe.

Lacking the natural spawning environment, captive grey mullet fail to reproduce spontaneously, largely due to a failure to undergo complete gametogenesis (Aizen et al., 2005). Therefore, DIVERSIFY first evaluated the effectiveness of hormone-based treatments on synchronizing gonadal development (**Fig. 22**). 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. Once gonadal development was accomplished, we proceeded with the 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) the female's failure to ovulate in 5 out of 12 spawning induction trials and (ii) the episodic fertilization success ranging between 0 to 98%, underlining the need to fine-tune further and optimize the hormone-based breeding protocol for captive grey mullet.

In the area of larval rearing, the objectives in DIVERSIFY are to (a) investigate environmental and nutritional factors that affect larval rearing, (b) determine the effect of co-feeding copepods and rotifers on digestive tract maturation and enzyme production and (c) determine when to wean larvae and to feed weaning diet type according to digestive tract maturation and the shift from carnivorous to omnivorous feeding (**Fig. 23**). Some of our results so far indicate that the beneficial effect of "green water" in the rearing tanks for larval grey mullet was derived predominantly from the resultant turbidity on prey ingestion rate (within the turbidity levels measured in this study) and less so to the algal type or biochemical content (*i.e.* fatty acid profile). Nevertheless, ingested algae by the larvae may have stimulated and improved gut maturation in early developing larvae, resulting in markedly improved survival during the juvenile stage. The algal treatments given to 2-23 dph larvae did not have a significant effect on older larvae and juveniles in terms of pancreatic and digestive tract enzyme activities. On the other hand, diet composition may have influenced the lipase and total alkaline protease specific activities. Nevertheless, overall the ontogeny and activity of the pancreatic and digestive tract enzymes measured appeared to be genetically programmed. The enzymatic activity of Alkaline Phosphatase and leu-ala peptidase individually and

Figure 22. Acquisition of an ovarian biopsy from a grey mullet breeder, in order to evaluate reproductive stage of development (IOLR-NCMR, Israel).

Figure 23. The larval rearing facilities at IOLR-NCM, Israel, where the majority of the work with grey mullet is undertaken.

in ratio indicated gut maturation around 61 dph and an increasing amylase capacity to at least 79 dph. This suggests (a) the capacity to feed on micro- and macroalgae, as well as benthic organisms when the fish move to the lower saline and shallower waters of estuaries at this age and (b) when to include significant levels of low cost starch in prepared feeds in order to efficiently grow grey mullet following gut maturation. Also, studies on the taurine requirement at different stages of development during the larval rearing of grey mullet showed a significant effect of dietary taurine on larval growth and survival. This effect is strongest during rotifer feeding compared to *Artemia* feeding, which also influences significantly growth in later stages of larval development. Nevertheless, the results indicated that larvae fed both high taurine enriched rotifers and *Artemia*, survived and grew significantly better.

Socioeconomics (including new product development)

Besides the technical improvement of the selected species, the socio-economic research in DIVERSIFY includes applied market development approach solutions on perception of aquaculture products, market demand, buyer preferences, new product development, value adding and market development (Banović et al., 2016; Grigorakis, 2017; Lazo et al., 2016; Reinders et al., 2016). These outcomes will help the EU aquaculture sector and the supply industry in targeted marketing and improvement of its international competitive position.

The combination of biological and socioeconomic research activities planned in DIVERSIFY *(Aquaculture Europe 39(1) March 2014)* are expected to **support the diversification of the aquaculture industry and help in expanding production, increasing aquaculture products and development of new markets**. Specifically, the socioeconomics work has three main objectives: (a) find out the consumer market opportunities for the six new species (*i.e.* greater amberjack, pike perch, meagre, wreckfish, Atlantic halibut and grey mullet), (b) examine the business-to-business market opportunities for the species above and (c) develop business models for the new species on the basis of an online market test. These insights are being generated for the five largest European fish markets:

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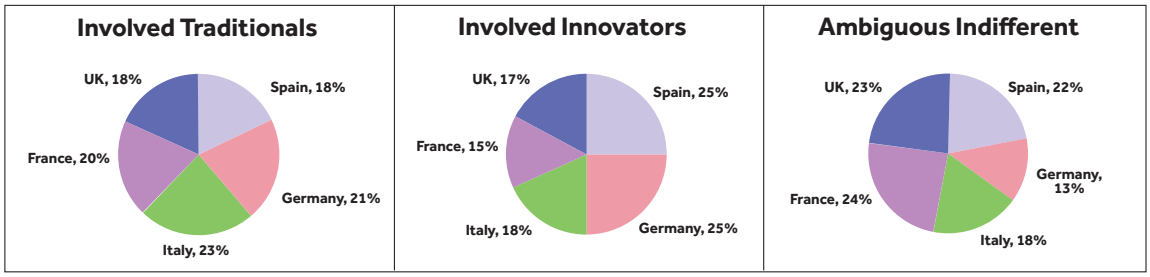

Figure 24. Consumer segments and country membership according to a DIVERSIFY study.

France, Germany, Italy, Spain and the United Kingdom. Not all activities are finished yet, but some general insights are visible already.

A quantitative online study with 2,500 consumers in the above five focal countries demonstrated that some consumer segments are open to try new species (**Fig. 24**). Especially in Germany and Spain, consumers from the segment «involved innovators» are very open to new fish species. On the other hand, French and Italian consumers are interested in new fish species although more traditional in their fish choices (*i.e.* the «involved traditional» segment) and British consumers are less involved and more ambiguous in the product choices they make (*i.e.* the «ambiguous indifferent» segment).

The market analysis demonstrated that buyers (*i.e.* retailers) in the five countries find it very difficult to position the six new species in relation to the current species in the market. Both as wild catch and aquaculture products, they are all fairly unknown. However the buyers are open to welcome new species under the following conditions: (a) the product must be cultured in a sustainable way, (b) the product should be available as a fresh product (southern-Europe) and as a frozen product (especially Germany), (c) the product must be easy to prepare and/or ready to eat, and (d) the product must be priced competitively. All these issues have been covered in DIVERSIFY. The feasibility study based on real cost prices of production is still going on. Sustainable production is covered in the reported biological research work packages (presented above), and in the socio economic work package convenience is included in the consumer oriented product development.

A qualitative study with 10 focus groups consisting of six participants each, undertaken across the five study markets (*i.e.* two focus groups per country) has identified the most promising product ideas for new fish products per investigated country (**Table 5**). 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 that are most relevant for consumers have been identified (**Fig. 25**).

On the basis of this study, a long list of product ideas has been developed for the different countries. However, not all products were practically possible with the different fish species. Therefore, only a selection of products has been sensory-tested in the five countries among regular consumers of fish. This sensory test showed that all the products were well accepted, except for fish pate. Products with a lower degree of processing were those who generated higher expected scores and higher acceptability in the blind test. It seems reasonable to infer that products having a higher degree of processing would be more appropriate for consumers who do not like fish because of its taste, presence of bones, odour, etc. In these cases, the existence of different processed alternatives could be a good solution for those individuals looking for a more convenient and less "fishy" product.

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Table 5. Preferred fish products per country, based on the six new/emerging fish species included in the DIVERSIFY project.

Country	Description of the created and best voted product ideas
France	Fresh fish Carpaccio that can be used as starter for a hot meal or as sandwich filling. This Carpaccio is seasoned with ginger and chili and presented as scales of the fish. The product is produced environmentally sustainable. The packaging is a plate that looks like a round box with the compartments and transparent wheel on the top that you can turn to reach different sections.
Germany	Fresh fish fillet covered with herbs and spices in the transparent packaging. Different fillet size in the packaging conveying the product message through images and voice: 'For him – Fish for the triathletes'; 'For her – vacation in Provence'.
Italy	Fresh fish steak for grilling in the pan. Transparent packaging with a label that guarantees the origin of the product and communicates its quality, signs and references to tradition and respect for the environment.
Spain	Fish sausages and fish burgers. The main advantage of this product is that the product has no bones. The seasoning is very mild and therefore this product is therefore suitable for children. The product is produced environmentally sustainable.
UK	Fresh fish fillet with different 'healthy' seasoning and marinades separately packed that consumer can choose and vary depending on the occasion. This product is sold with recommendation for the appropriate vegetables and wine to accompany the dish. Product message: 'Not two same dishes in a row; 'You have it ready for you, healthy but still have the hectic lifestyle.'

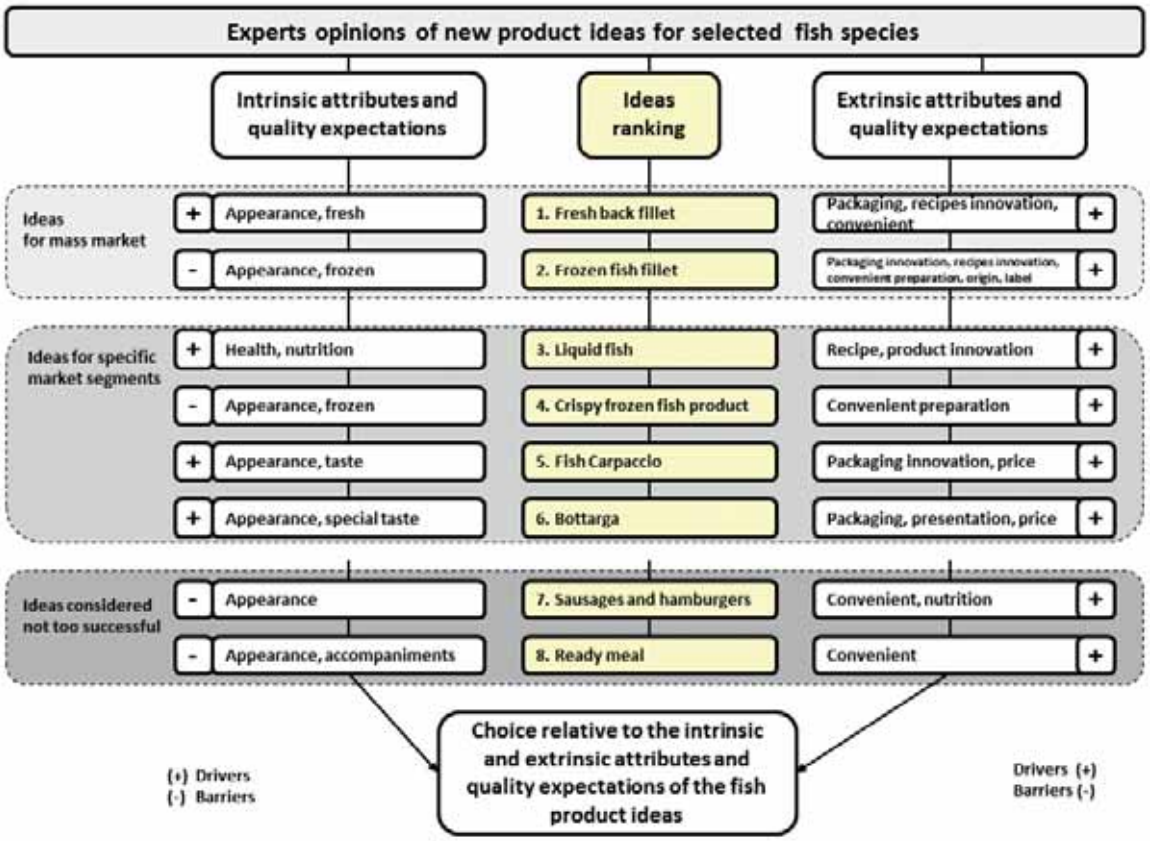

Figure 25. Drivers and barriers of new product ideas based on the six new/emerging fish species included in the DIVERSIFY project.

Upcoming "DIVERSIFY One-day Species-specific Workshops" for stakeholders

To speed up the dissemination of the project's results to the interested stakeholders and the aquaculture industry, one-day species-specific workshops are being organized for the Spring of 2018, to be carried out in different locations around Europe (Greece, Italy, France, Spain, Belgium, Norway). The location will be selected based on the aquaculture potential of each species and the Workshops will be will be hosted by the species leaders within the DIVERSIFY consortium, as follows:

1. Meagre, Alicia Estevez, IRTA, Spain
2. Greater amberjack, Nikos Papandroulakis, HCMR, Greece
3. Pikeperch, Pascal Fontaine, Uni. Lorraine, France
4. Atlantic halibut, Birgitta Norberg, IMR, Norway
5. Wreckfish, Blanca Alvarez, IEO, Spain
6. Grey mullet, William (Bill) Koven, IOLR-NCM, Israel

The workshops will be announced in the website of the project **(www.diversifyfish.eu)** at the end of 2017, and will be also advertised in relevant websites. In each workshop, researchers from within the DIVERSIFY consortium will present a summary of the work carried out and the production methods developed, in the different areas (Reproduction and Genetics, Nutrition, Larval and Grow out husbandry, Fish health, Final product quality and Socioeconomics). Relevant researchers from outside the consortium will also be invited to present their work. This will ensure that the participants are provided with the State-of-the-art of the scientific knowledge for each of the species, coming not only from DIVERSIFY, but also from other European or National initiatives. The workshops will be **free of charge** and will be open to any interested researcher, farmer or regulator, on a **first come-first served basis**.

An online experimental choice study with product mock-ups developed from the created product ideas from the qualitative study (**Fig. 26**) was conducted in the five study countries to identify the optimal intrinsic-extrinsic product quality profiles for earlier identified consumer segments (*i.e.* the involved innovators and the involved traditionals). This study showed that country-of-origin and price come first when choosing new fish products, followed by quality certification (*i.e.* Aquaculture Stewardship Council – ASC logo), while nutrition and health claims appear to have varying and minimal impact, which is highly dependent on the type of product and level of processing, and country. Thus, a certain degree of customisation is needed for certain products, depending on the level of processing and countries.

Figure 26. Different products developed by DIVERSIFY for testing with consumers.

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