New species for EU aquaculture

Technical Manual – Meagre (*Argyrosomus regius*)

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QR Code

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Introduction

The meagre is found in the Mediterranean and Black Sea, and along the eastern Atlantic coast (Haffray et al., 2012). It has attractive attributes for the market that include large size, good processing yield, low fat content, excellent taste and firm texture (Monfort, 2010). The species also has the biological characteristics required for commercial aquaculture using well-established culture technologies (Papadakis et al., 2013). These characteristics include a fast growth of ~1 Kg per year (Duncan et al., 2013), a low feed conversion ratio of 0.9-1.2 –which is similar to the Atlantic salmon- relatively easy larval rearing (Roo et al., 2010; Vallés and Estévez, 2015) and established induced spawning protocols for the production of viable eggs (Manousaki et al., 2018; Mylonas et al., 2016). Meagre was first produced in 1997 in a commercial hatchery in France and since then it has exhibited annual production increases as high as 7 fold (FAO, 2012)(**Fig. 1**). In 2016, meagre European aquaculture production was 7,280 t, produced in Spain, Greece, Turkey, France, Portugal, Italy, Cyprus and Croatia (FAO, 2018).

The survey of meagre producers identified four principal bottlenecks to the expansion of the industry. Firstly, variable growth rates --whose exact cause is not obvious-- are reducing greatly yield (Duncan et al., 2013). A multidisciplinary approach is required in order to examine the role of genetics, nutrition --particularly dietary requirements during weaning, pre-ongrowing and in cage culture-- feeding behavior and grow out husbandry. Secondly, the distribution of this fish only in specific areas in the Mediterranean region has resulted in the acquisition of broodstocks from a limited number of sources (mainly a hatchery in France), resulting perhaps in a limited genetic variation of the available broodstocks. This will have significant negative implications for the future initiation of breeding selection programs, which are necessary to move the industry to the next level of efficiency and production. Thirdly, the industry must address issues in fish health, emerging diseases, parasites and the wide occurrence of Systemic Granulomas, which may stem from the fact that no diets have been developed for this fish. Finally, socioeconomic factors have been identified as bottlenecks, including the need for a more expanded market and diversification of provided products (Monfort, 2010) beyond the whole fresh fish. National initiatives for meagre domestication are underway in Spain and Greece, coordinated by Partners of the consortium, and DIVERSIFY will build on the acquired information by targeting specific issues recognized as bottlenecks for further production.

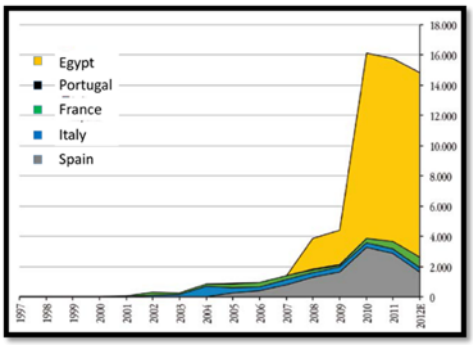


Figure 1. Evolution of meagre aquaculture production in Europe and Egypt from 1997 to 2012.

In the present Technical Manual, we provide information obtained during the 5-years of the DIVERSIFY project, relevant to the acquisition of new broodstock, broodstock management and control of reproduction, nutrition, larval rearing and grow out husbandry. This information is targeted towards commercial and research organizations, that are interested in investigating the potential of wreckfish for aquaculture. More information will become available in the near future, as research will reach the publication stage, at the website of the project (www.diversifyfish.eu).

1. Reproduction and Genetics

Evaluation of the genetic variation in captive meagre broodstocks

(led by FCPCT, Juan Manuel Afonso)

Genetic variation in the base population is essential for developing breeding programs, because genetic variation affects the selection response in the short and long term (Falconer and Mackay, 2001). Wild populations of meagre have been studied by Haffray *et al.* (2012), in terms of genetic differentiation along the Atlantic Ocean and Mediterranean Sea, using red drum (*Sciaenops ocellatus*) microsatellite markers (Renshaw *et al.,* 2006). These authors found an average of 13.2 and 0.57 for number of alleles and observed heterozygosity, respectively. Thus, the authors estimated that the meagre has at least two very distant genetic groups: the Atlantic and the Mediterranean, in a context of six independent spawning areas, where a lower allelic richness and effective sizes of the Mediterranean populations were reported. This structuration was mainly related to interglacial phases of the Quaternary. Haffray *et al.* (2014) used the same multiplex of microsatellites (Panels-A & -B) and reported a similar, but slightly lower, genetic variation in two domestic populations of meagre from France (LPDS and FMD), with mean estimations of 8.5 and 0.52 for number of alleles and observed heterozygosity, respectively, as expected.

The purpose of this study in DIVERSIFY was to determine the genetic variability of meagre in research centers and university institutions in Europe, to describe the status of the bottlenecks identified through the genetic characterization of captive broodstock, as a first step to starting meagre breeding programs.

A total of 432 meagre breeders were sampled from broodstock in 13 centers from 7 countries (**Fig. 2**), and studied using 18 microsatellite markers (STRI & SRTS).

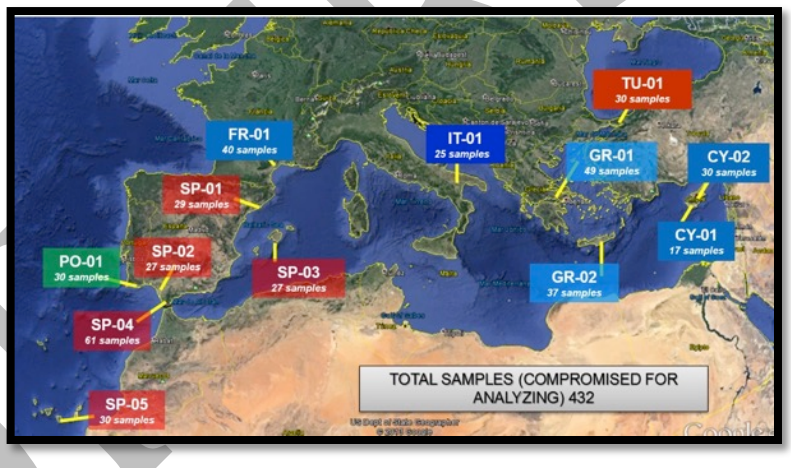


Figure 2. Geographic distribution of meagre studied samples in Europe, from the Canary Islands to Cyprus.

The arithmetic and weighted means of allele number were 3.7 and 4.13, respectively (**Table 1**). A positive relationship between number of alleles and population size was found. As heterozygosities, both arithmetic and weighted estimates were the same (0.48) for observed heterozygosity, while the values were similar for expected heterozygosity, 0.48 and 0.49, respectively. The captive European populations of meagre had mean number of alleles and observed heterozygosities that were lower than in wild populations (around 3 times and 18% lower, respectively). These numbers of alleles and heterozygosities indicated that the variation of the populations is very similar to wild populations or has declined. Essentially these broodstocks have adequate genetic variation for a breeding program, but the decline in variability and low mean number of alleles of some broodstocks clearly indicated that these broodstocks should be enlarged with new families and stocks to ensure an optimal base population for a breeding program. Estimates of effective sizes

(*Ne*) of each population ranged between 82 and 115, with a mean of 87 (**Table 2**), and was higher than the minimum recommended to minimize inbreeding depression (50), but lower than the minimum suggested for maintaining sufficient evolution capacity (500).

Table 1. Total averages for number of gene copies, alleles and observed and expected heterozygosities**.**

	AVERAGES			
POPULATION	Nº GENE COPIES	Nº ALLELES	HET.OBS.	HET.EXP
PO-01	27.8	1.8	0.261	0.259
SP-01	54.4	5.2	0.585	0.598
SP-02	45.8	6.3	0.548	0.625
SP-03	51.6	4	0.561	0.505
SP-04	101.5	7.1	0.532	0.595
SP-05	56.3	2.8	0.519	0.446
FR-01	59.5	5.3	0.433	0.507
IT-01	46.3	3.4	0.547	0.502
GR-01-F1	26.88	2.5	0.453	0.449
GR-01-F2	54.7	3	0.388	0.402
GR-02-FI	34.5	3.4	0.473	0.506
GR-02-F2	28.1	2.4	0.493	0.44
TU-01	57.8	2.5	0.375	0.375
CY-01	24	3	0.495	0.509
CY-02	35.3	2.7	0.469	0.455
Arithmetic mean		3.69	0.48	0.48
Weighted mean		4.13	0.48	0.49

Table 2. Effective size (Ne) and Theta values (H) per population.

POPULATION	Theta (H)	Ne
PO-01	2,10178	115,48
SP-01	1,58012	86,82
SP-02	1,63525	89,85
SP-03	1,50028	82,43
SP-04	1,57611	86,60
SP-05	1,52339	83,70
FR-01	1,50044	82,44
IT-01	1,50005	82,42
GR-01-F1	1,52021	83,53
GR-01-F2	1,57866	86,74
GR-02-F1	1,50039	82,44
GR-02-F2	1,5292	84,02
TU-02	1,6321	89,68
CY-01	1,5007	82,46
CY-02	1,51591	83,29

Therefore, the estimates of effective sizes (*Ne*) also indicated that the broodstocks probably originated from crosses between sufficient families, but the number of families in many broodstocks was at the lower limit for a base population, which again indicated that these broodstocks should be enlarged with new families and stocks to ensure an optimal base population for a breeding program. The AMOVA analysis revealed that 18.19% of the variation was found among studied populations (Fst), while the remaining 81.82% was located within populations (P<0.0001). A Factorial Correspondence Analysis showed two clusters correlated with the geographical distribution of populations (Atlantic and Mediterranean), and a third constituted for TU-01 population from Turkey (**Fig. 3**). The significant Fst reported is indicative of a low genetic flow among captive meagre populations studied, producing a fragmentation of populations and

increasing the effect of genetic stochastic processes. This high variation between population, low gene flow and fragmentation can perhaps be explained by the differences in the three groups or populations identified and associated to geographic area.

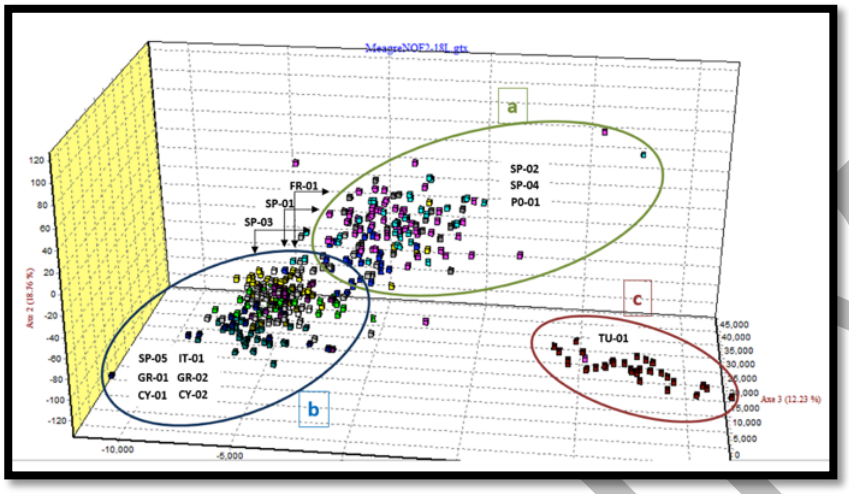


Figure 3. Factorial Correspondence Analysis from 18 loci and 376 fish (no F2) distributed in 13 Mediterranean populations of meagre.

All together these analyses indicate that generally the variation is adequate in captive broodstocks. However, some broodstocks are questionable as base populations and more in depth analysis would be required to determine the suitability of the broodstock for a breeding program. It is very clear that across all broodstocks there exists more than adequate genetic variation to form a base population. This potential genetic differentiation in quantitative genes and traits would be a magnificent tool for the constitution of the best available base population for a selective breeding program on a European scale, especially because meagre as a species is currently at a clear disadvantage, in terms of its genetic starting point, compared to other species in EU aquaculture.

Development of protocols for paired crossing in spontaneous spawning

(led by IRTA, Neil Duncan and HCMR, Constantinos Mylonas)

Breeding in pairs is one way to create families in breeding selection programs. However, some marine species such as gilthead seabream and European seabass do not spawn in pairs and artificial "strip" spawning is also complicated. For example, gilthead seabream spawning success was low when held in pairs or groups of 15 females with a single male (Gorshkov et al., 1997). Various authors have suggested large groups of breeders were required for successful spawning of gilthead seabream (Gorshkov et al., 1997; Duncan et al., 2013b) and Sparidae in general (Pankhurst, 1998; Mylonas et al., 2011). Spawning in large groups of breeders complicates the establishment of breeding programs that often require that specific breeders with desired phenotypes are bred together in many paired crosses to produce the required number of families. Therefore, an essential part to establishing a breeding program for meagre is to establish the control of reproduction that enable pairs to be selected and bred together. Since reproduction in captive meagre is almost always induced by exogenous hormone treatments followed by tank spawning, the objective of this work was to examine the potential of meagre to spawn repeatedly and reliably in pairs with male rotation.

1. Maximum number of spawns in response to weekly GnRHa injections (HCMR)

Single pairs of fish were transferred to 5,000-l tanks under simulated natural photoperiod, but controlled temperature of 19-20°C. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 µm (Mylonas et al., 2013b). Male fish were considered eligible for spawning induction, if they were releasing substantial amounts of sperm (Mylonas et al., 2013b). Injections of GnRHa were administered once a week using four pairs of fish per treatment (n=4). Females (mean ±SD body weight 9.7 ± 1.0 kg) were treated with a GnRHa injection of 15 µg kg^{-1}. Four males (7.9 ± 1.0 kg body weight) were treated at the start of the experiment with 43–57 µg kg^{-1} using a 450-500 µg GnRHa implant for an effective dose of ~50 µg kg^{-1} GnRHa, in order to enhance spermiation. GnRHa implantation of males was repeated at subsequent samplings, if sperm production was considered low. After treatment with GnRHa, fish were placed in tanks connected to overflow egg collectors and were allowed to spawn. When a cumulative total of two females (*i.e.*, 50%) failed to spawn in response to 2 consecutive injections, the experiment was concluded, and no further injections were given.

Eggs were collected into a 10-l bucket and their number (fecundity) and fertilization was evaluated by examining each of the eggs in this 10 ml sample for the presence of a viable embryo using a stereoscope. Eggs from each spawning were placed individually in 96-well microtiter plates (in duplicates). The microtiter plates were then placed in a controlled-temperature incubator and maintained for 5 days at 19 ± 0.5°C. Embryonic and early larval development was evaluated once a day, recording the number of live embryos 24 h after egg collection (or ~36 h after spawning), hatched larvae (examined ~60 h after spawning) and viable larvae on day 5 after egg collection (~ yolk sack absorption). At 18-20°C, hatching of meagre eggs takes place in 44-56 h.

Mean (± SEM) oocyte diameters at the onset of the study were 590 ± 10 µm, and throughout the study ranged between 550 ± 9 µm and 620 ± 6 µm, with some small, but statistically significant variations. Large numbers of vitellogenic oocytes, could be seen in the biopsies of all four females until week 5, of three females until week 16 and of two females until week 18 --one week after the last GnRHa injection (**Fig. 4**). One female failed to spawn in response to GnRHa injections on weeks 6 and 7, and was thus not used again for the study. Another female failed to spawn in response to GnRHa injections on weeks 16 and 17, at which time the experiment was concluded.

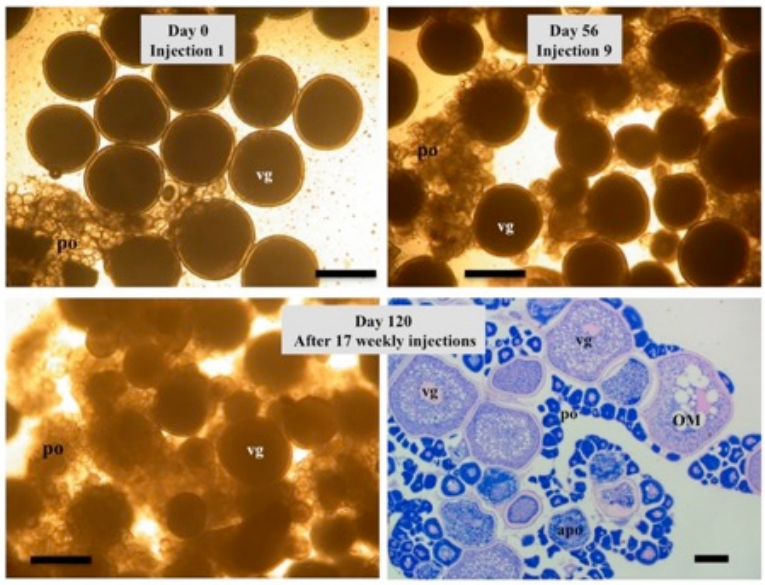


Figure 4. Wet mount and histological sections of ovarian biopsies from meagre injected weekly with GnRHa. apo = apoptotic, Vg = vitellogenic, OM = oocyte maturation, po = primary oocyte. The black bar in the wet mounts and histological sections are 500 and 200 µm, respectively.

The GnRHa injected females spawned for up to 17 consecutive weeks, most of the times spawning both on the 2nd and 3rd day after each weekly injection (**Fig. 5**). The first spawns obtained 2 d after each injection had significantly higher fecundity compared to the second spawns obtained 3 d after each injection. Overall, there was no significant effect of injection number on mean fecundity, but there was a slight negative linear correlation between 2nd spawn fecundity values and GnRHa injection number.

Fertilization success was high during the experiment (**Fig. 5**), without any significant effect of either GnRHa injection week or spawn number after each injection, and with the exception of the 2nd spawn of the last GnRHa injection (54%) it was always >80% and most of the times >90%.

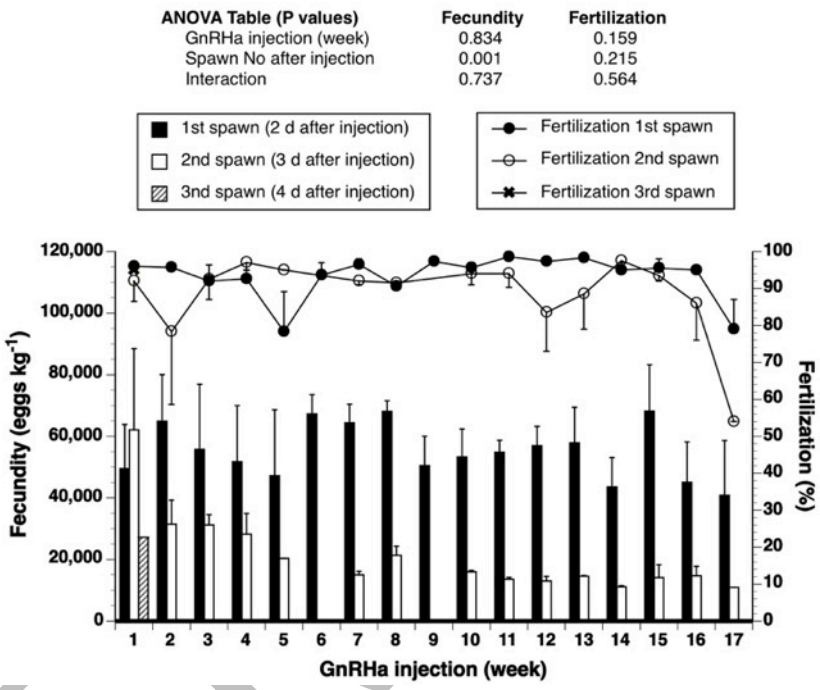


Figure 5. Mean (±SEM) daily fecundity and fertilization of meagre (n=1-4) induced to spawn with GnRHa injections (n=17, once every week).

Embryonic development was very high overall and there were no significant differences over the course of the study in response to the consecutive GnRHa injections, in terms of 24-h embryo survival, hatching or 5-d larval survival, even after 17 weekly injections of GnRHa.

In a recently published work, multiple injections of GnRHa were shown to induce up to 7 weekly spawning cycles in meagre (Mylonas et al., 2015). The present experiment corroborated and extended the results, demonstrating that meagre are able to spawn multiple times and in a predictable fashion for up to 17 weeks during the reproductive season to produce large numbers of high-quality eggs. It is not known if these results reflect the situation in the wild, since fish were maintained at a constant temperature of ~21°C during the trial, which was found to be optimal for spawning in earlier trials (Mylonas et al., 2013a; Mylonas et al., 2013b). Meagre in the wild has an asynchronous ovarian development (Gil et al., 2013) and depending on the location its spawning season extends from April to October. However, it is not clear what is the natural spawning kinetics of meagre, what is the inter-spawn interval and how many times a fish may spawn during a single season (Gil et al., 2013; González-Quirós et al., 2011). As meagre do not usually spawn spontaneously in captivity, it is also not possible to obtain reliable information from fish in captivity (Duncan et al., 2013). Nevertheless, the present study demonstrates that if the fish are maintained at the appropriate spawning temperature and are given a weekly GnRHa injection, they can continue to spawn for a very long period of time producing mean (±SD) relative fecundity values of 1.42±0.15 million eggs kg^{-1} body weight per year.

2. Paired spawnings with male rotation on a weekly basis, Experiment I (HCMR).

This experiment was run in order to develop a method that optimizes the number of families produced by a given number of individual breeders. Four pairs of fish (one male and one female) were transferred to 5,000-l tanks under simulated natural photoperiod and constant temperature (19 and 20°C). Injections of GnRHa (15 μg kg^{-1}) were administered once a week (every Monday) to the four females (named Juliet, Cleopatra, Cecy and Helena) of mean ±SD body weight 11.7 ± 2.6 kg, and the four males (named Romeo, Cesar, Peri and Paris) of mean ±SD body weight of 10.2 ± 1.2 kg body weight. Every week, the males were paired with a different female and after treatment with GnRHa they were placed in the separate spawning tanks and were allowed to spawn for a week. Eggs were collected and evaluated as described in the previous section 1 for fecundity and fertilization success, and then for embryo survival 24 h after egg collection, hatching success and larval survival on day 5 after egg collection.

After the first GnRHa injection, all females spawned on three consecutive days (**Fig. 6**), similar to what was observed in the previous year and presented above (**Fig. 5**). Similarly, after the second GnRHa injection all females spawned again for two consecutive days. However, from the third GnRHa injection, significant variations appeared among the four females. Some spawned only once at this time, while after the 4th injection only two fish continued spawning. These results are different than from the previous year (**Fig. 5**), but also from other published work from our laboratory (Mylonas et al., 2015). Examination of the ovaries of the two females that did not respond at the 4th injection ("Helena" and "Cecy") indicated that the females did not contain any more vitellogenic oocytes that could be induced to mature and spawn.

So, apparently these two females had matured and ovulated all the initially available vitellogenic oocytes in response to the first three GnRHa injections, and did not recruit any more oocytes, as was the case with the other two females ("Cleopatra" and "Juliet") and in earlier experiments (**Fig. 6**) and published work (Mylonas et al., 2015). The only difference between this experiment and the previous ones is the fact that males were changed and rotated among females after each injection, and one can assume that this disruption in the breeding behavior of the fish might have affected negatively some of the females. It is interesting to note, nevertheless, that the overall fecundity of the four females was not significantly different among them (**Fig. 7**), suggesting that the females that spawned fewer times produced more eggs per batch.

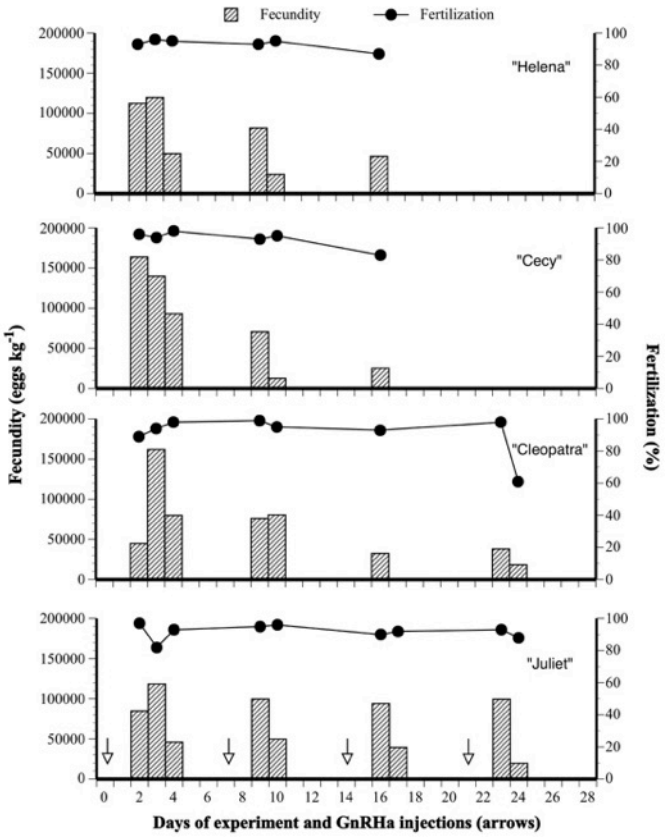


Figure 6. Daily batch relative fecundity and fertilization success of individual meagre females (n=4) induced to spawn with multiple GnRHa injections (n=4, once every week) during 2015 and paired with four males (named Romeo, Cesar, Peri and Paris). At every GnRHa injection, the males were moved to a different tank, being paired with a different female so at the end all males were paired with all females. The first GnRHa treatment was made on 4 May 2015.

Apart from the significant differences in the response to the 4th GnRHa injection and the overall number of spawns among the four females, there were no significant differences in the quality of the eggs produced (**Fig. 7**). These results suggest that female meagre is able to produce good quality eggs with different males and in response to weekly change of "partners", without any significant negative impact.

Similarly, there were no significant differences in the fecundity or egg quality of the eggs obtained when females were paired with a specific male (**Fig. 8**), again demonstrating that all males had equal potential of successful spawning and producing eggs of good quality. It has been demonstrated from in vitro fertilization experiments in other species, that there are some male-female incompatibilities when it comes to fertilization, resulting in lower fertilization success when specific males are crossed with specific females (Saillant et al., 2001). However, in the present experiment only a small number of individuals was used, so the possibility still exists that male-female fertilization incompatibilities may exist among some individuals of this species.

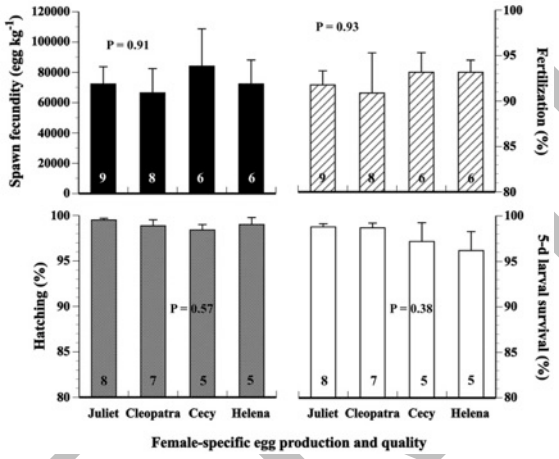


Figure 7. Mean (±SEM) daily relative fecundity, fertilization success, hatching and larval survival of individual meagre females (n=4) after each GnRHa injection (n=4, once every week)(see Fig. 1.5 for individual spawns). The numbers within the bars indicate the number of individual spawns.

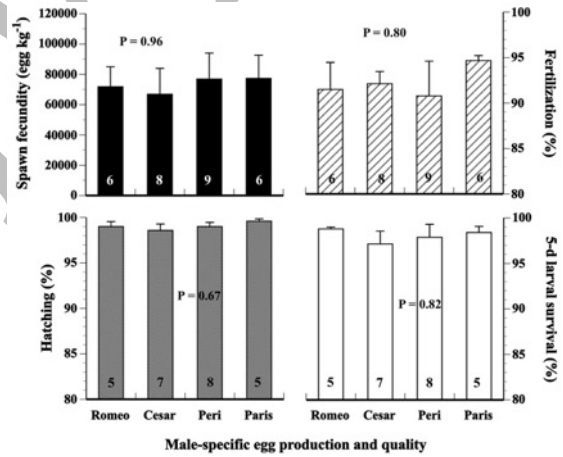


Figure 8. Mean (±SEM) daily relative fecundity, fertilization success, hatching and larval survival of eggs produced in the presence of different meagre males (n=4) after each GnRHa injection (n=4, once every week)(see Fig. 1.5 for individual spawns). The numbers within the bars indicate the number of individual spawns.

As expected based on previous published work (Mylonas et al., 2015), but not according to the experiments done earlier (**Fig. 5**) there was a significant reduction in the fecundity obtained after consecutive GnRHa injections, while there was no difference in the fertilization success (**Fig. 9**) and subsequent egg development. As far as seed production by a commercial hatchery, this result is considered undesirable, since a hatchery cannot expect to maintain a stable egg production over the course of the reproductive season of meagre. Similar results have also been reported for European seabass, where egg fecundity decreased by ~50% after each subsequent spawning induction with GnRHa injections (Mylonas et al., 2003).

However, from the point of view of the objectives of the present experiment, that is the production of adequate numbers of eggs from as large number of parents as possible in order to create multiple families for breeding selection programs, we believe the results can be considered successful. A total of 14 families (half-sib) were produced, out of a possible maximum of 16 (4 males x 4 females), each family consisting of at least 200,000 eggs of >80% fertilization success. So, this method of pairing male and female meagre with a weekly rotation of the males can be used by commercial hatcheries in order to produce multiple families and build a breeding selection program that can maintain genetic variability. Obviously, in a commercial situation a much larger number of males and females should be used, but the experiment was a successful "proof of concept" for this approach.

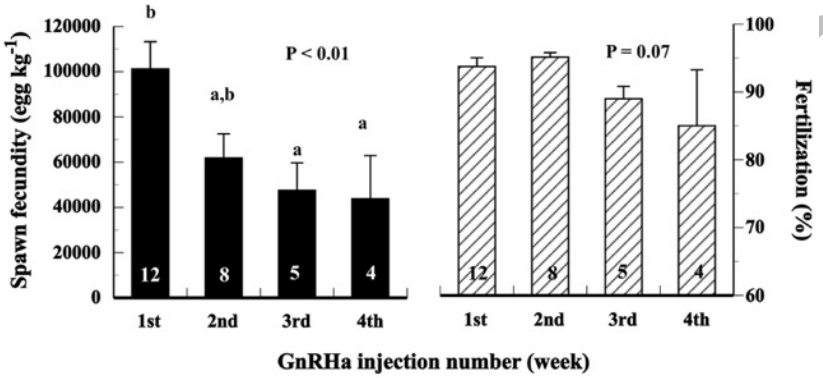


Figure 9. Mean (±SEM) daily relative fecundity and fertilization success, of meagre pairs (n=4) after each GnRHa injection (n=4, once every week) during 2015 (see Fig. 1.5 for individual spawns). The numbers within the bars indicate the number of individual spawns. Different letter superscripts indicate significant differences between means.

Together, these experiments have shown that paired tank spawning of meagre is possible for the production of multiple families from parents with known phenotypes. The production of desired families forms the basis of a genetic improvement program {Duncan, 2013 #4442} and this has been a bottleneck in setting up breeding programs in some marine species such as gilthead seabream and European seabass. That paired spawning is possible in meagre confirms previous indications from communal spawnings, that paired spawning may be a natural phenomenon. For example, Duncan et al. {, 2012 #4363} demonstrated using microsatellite paternity assignment, that a number of spawns from groups of six breeders were from a single pair, indicating that at least when held in a small group a pair had spawned together. Also, Mylonas et al. (2015), set up pairs of breeders and induced the pairs to spawn each week for up to a total of 7 weeks.

The present studies have advanced our knowledge twofold. Firstly, paired spawning was achieved repeatedly for up to 17 weeks with no changes in the pair structure. Secondly, male fish were exchanged before each spawning induction to produce a different full or half-sib family with each induced spawning. The efficacy of spawning pairs was high 76% (Experiment 1, 14 pairs spawned

from 16 (87%); experiment II, 22 pairs from 37 (59%); experiment III, 25 pairs from 27 (93%)) and across the three experiments a total of 61 families (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success. Obtaining a large number of families with adequate quantities of eggs that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite of a breeding program.

Description of sperm characteristics and cryopreservation methods

(led by IFREMER, Christian Fauvel and IRTA, Neil Duncan)

The quality of sperm can be objectively described through different features accessible by field observations such as concentration, motility and fertility which were extensively described for different species in the literature and particularly in recent large reviews of applications to aquaculture (Cabrita et al. 2009, Bobe and Labbé, 2010, Fauvel et al., 1999, Fauvel et al. 2010). Fish spermatozoa are immobile in the genital tract and are activated to start swimming by the variation in osmotic pressure caused by contact with external medium at the moment of ejaculation (Cosson et al., 2008). If the dilution of sperm is adequate, this activation is global to activate all spermatozoa (Billard and Cosson, 1992) so that the maximal motility (% of motile spermatozoa) is usually observed in the first seconds. Due to a trade-off between weak respiration and the high energy cost of movement, the motility and the velocity of sperm rapidly decreases (Christen et al., 1987). The evaluation of concentration, initial motility, the velocity of sperm and the duration of progressive movement are interesting indexes of sperm quality, which must be confirmed by the fertility or ability of sperm to fertilize (Kime, 2001, Rurangwa et al., 2004).

In captivity, fish reproduction can be impaired by uncontrolled external or internal factors modifying the neuro endocrine control of reproductive process at different levels. These endocrine disruptions have been successfully overcome by the application of homologous or heterologous hormones (Zohar and Mylonas, 2001, Mylonas et al., 2016a). In the case of spermiation, chronic stress from repeated sampling can induce decreased sperm availability and in extreme cases the complete lack of semen in species such as European seabass (Fauvel et al., 1999). In the case of meagre, a treatment by analogs of LHRH induced males to recover sperm production after a short time laps (Mylonas et al., 2016b), but it remains interesting to evaluate the quality of this newly produced sperm through the standardized protocols developed in the DIVERSIFY project.

The analysis of sperm just at collection reveals the intrinsic quality of gamete as a consequence of paternal physiological input (spermatogenesis, spermiogenesis and spermiation) and also of proper sperm physiology such as ageing process, which progressively affects sperm along the reproductive season (Dreanno et al., 1999). After collection the quality of sperm decreases in a species-specific way. For example, undiluted rainbow trout sperm presented high motility after 34 days (Stoss and Holt, 1983) while high variability was reported after only some hours in seabass (Fauvel et al., 2012). In artificial fertilization, the collection of male and female gametes may not be concomitant. In that case, according to species, sperm must be conditioned for chilled or cryogenic storage. It was interesting to test the capacity of meagre sperm to tolerate chilled conservation in adapted media and to establish the effects of cryopreservation using an optimized medium. Since a modified cell culture medium Leibovitz L15 efficiently protected sperm of seabass *Dicentrarchus labrax* (Fauvel et al., 2012), wreckfish *Polyprion americanus* and greater amberjack *Seriola dumerili*, it was proposed to study its protective effect on meagre sperm.

For sperm collection from males that were in full spermiation, the genital pore was carefully cleaned and dried and a gentle pressure was applied to the testes in order to obtain sperm. Urine was avoided to prevent sample contamination. Sperm was directly collected in 2-mL syringes immediately before the first stripping of females. Sperm samples were maintained on ice. Milt was

diluted 1:4 (v:v) in Leibovitz cell culture medium modified; glutamine (0.3 mg/mL diluted Leibovitz), sodium pyruvate (6 mg/mL) and NaOH were added to the initially diluted medium of Leibovitz (350 mOSm and pH 7.3) to obtain a Leibovitz medium with pH 8 and 450 mOsm. In order to prevent sperm initial motility, the osmolarity was decreased to 250 mOSm by dilution in distilled water. Gentamycin sulphate (1 µL/mL) was added also to prevent any bacterial development and bovine serum albumin (BSA) (0.066 mL 30% BSA/mL dilution), to protect the plasma membrane and avoid sperm aggregation. For sperm sampling, positive displacement pipettes were used.

The results demonstrated that some of the measured parameters changed due to the hormonal induction of males (duration of initial motility and VAP along time) and others did not (density, duration, initial motility and initial VAP). No significant differences were observed between sperm density of sperm obtained before GnRHa injection and after it and neither in duration, initial motility or in initial VAP (**Table 3**). However, higher values in initial motility were obtained after GnRHa injection. The percentage of motility was decreased gradually after activation, exhibiting ~50% of motility until 55 s after activation in samples obtained before GnRHa injection. However, in samples obtained after GnRHa injection, ~50% of motility was still exhibited after 75 s from activation. After 75 s, a drastic decrease in motility was observed. The VAP exhibited the same pattern; initial values lasted longer after GnRHa injection (45 s) than before (35 s) (**Fig. 10**).

Table 3. Sperm quality parameters of sperm samples obtained before and after GnRHa injection. Data from 5 males during the whole experimental period were used when data before and after were available. Values are expressed as mean ± standard deviation. Different letters mean significant differences between before and after hormonal treatment.

Before/ after GnRHa injection	Sperm concentration (spermatozoa/ mL)	Sperm duration (min)	Initial motility (%)	Initial VAP (µm/s)
Before	$3.21 \cdot 10^{10} \pm 1.18^{a}$	1.71 ± 0.29^{a}	48.17 ± 2.80^{a}	90.69 ± 5.76^{a}
After	$2.76 \cdot 10^{10} \pm 0.62^{a}$	1.57 ± 0.50^{a}	66.76 ± 15.83^{a}	98.07 ± 11.68^{a}

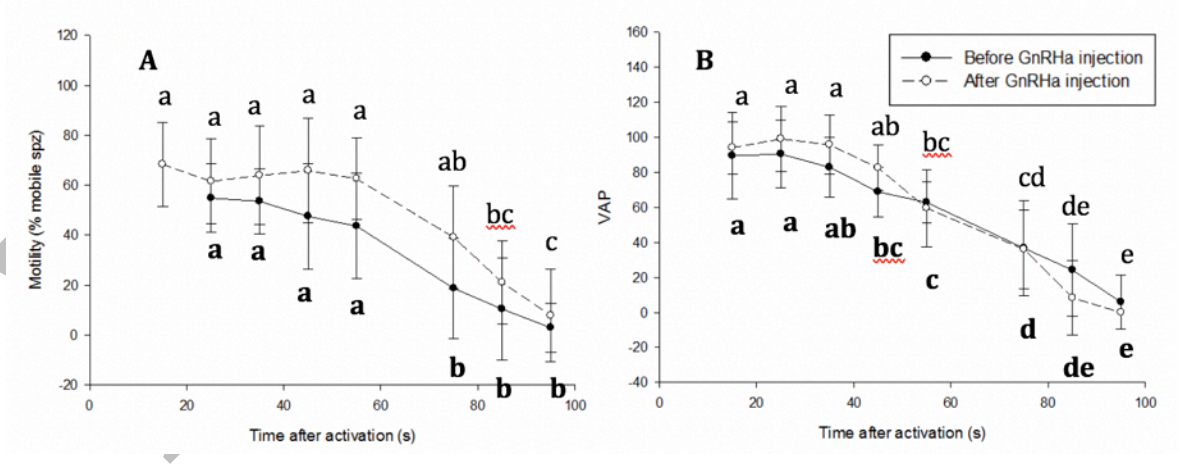


Figure 10. Effect of GnRHa treatment on (A) percentage of mobile spermatozoa (%) and (B) Average Path Velocity of spermatozoa (µm/ s) during the experimental period when data before and after treatment were available. Data are expressed as mean ± standard deviation. Different lowercase bold letters mean significant differences with time after activation before GnRHa injection. Different lowercase letters mean significant differences with time after activation after GnRHa injection. Data was from five different males with three repeated measures on different weeks for each male (n=15).

Effect of storage and cryopreservation on sperm quality parameters

The very simple system used to freeze sperm consisted in placing the straws at a distance 6 cm from the LN surface, which is a height where the vapors cool the sperm according to a relevant slope. The use of a 6cm high styrofoam device allowed obtaining an adapted cooling rate until -100°C after 10 min. After 10 min, the temperature was decreased down to -196°C by immersing the straws in LN (**Fig. 11**). The quality characteristics of sperm subjected to different conditions of storage are shown in **Table 4**. As regards analysis of sperm, there were no significant differences between the duration of motility nor initial VAP values from sperm subject to different kinds of storage, but initial motility of sperm stored in Leibovitz for 24 h was significantly the lowest.

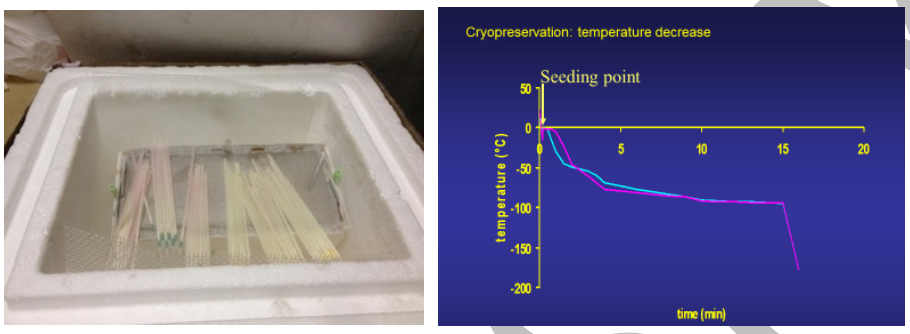


Figure 11. Cryopreservation device with straws on the floating device in liquid nitrogen vapors (left) and the temperature drop slopes measured by a thermocouple introduced in a filled straw i.e. in contact with sperm (right). An increase of temperature can be noticed at the time of crystallization of liquid phase, which is an exothermic reaction. This increase is called seeding point. The green curve corresponds to straw freezing and the purple one was obtained by placing 1.8 ml cryotube (Nalgene) at 2 cm over LN.

Table 4. Weekly sperm quality of sperm samples used in experiment 3 (comparison of fertilization by fresh, cryopreserved sperm and sperm stored in Leibovitz for 24h). Data from 5 males was used and values are expressed as mean ± SD. Different lowercase letters mean significant differences between each parameter from different storage types in the same week. Different capital letters mean significant differences between each parameter from fresh sperm used in fertilization among three weeks (read in columns).

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

The values of meagre sperm concentration recorded in the present study are similar to others published for meagre (1.89 – 3.15 $\cdot 10^{10}$ spzoa/mL) (Mylonas et al., 2013) and to other marine fish. The percentage of initial motility was also similar to others reported for meagre (53 - 74%) (Schiavone et al., 2012) (44 - 80%) (Mylonas et al., 2013). Sperm did not show a higher slope of motility decay and the percentage of motile spermatozoa followed a gradual decline and exhibited ~50% of motility until 55 s after activation. On the other hand, the initial VAP measured for meagre spermatozoa in the present study was not the same as those measured in other studies of meagre sperm (17 – 24 µm/ s) (Schiavone et al., 2012). However, the present studies values were similar to other marine fish, such as the seabass (150 µm/ s) (Fauvel et al., 2012) and the European eel (118 µm/ s) (Pérez et al., 2016). These values are related to fast sperm (>100 µm/ s) (Gallego et al., 2013), but initial VAP had a quick decrease so that the initial VAP values remained only 35 s after activation. Sperm motility is an important aspect in fish breeding and has been directly related to fertilization rates but spermatozoa velocities may also serve as a predictor of fertilization ability. In fact, in some studies the highest coefficients of correlation were found for VAP (Gallego et al., 2013). Thus, if VAP is highly correlated with fertilizing ability, the period of sperm fertility could be reduced to 35 s. The characteristics of the meagre sperm highlighted the need for an *in vitro* fertilization protocol to mix and activate sperm at the moment of contact between eggs and sperm, as activated sperm will quickly lose motility and VAP resulting in low fertilization.

This study showed that artificial induction of spermiation could affect the responsiveness of male fish and made a variation in quality parameters (Rurangwa et al., 2004) as observed in the duration of initial values of motility and VAP. Those values were longer after GnRHa injection than before injection, showing that hormonal treatment induced higher initial motilities and probably the fertilization success. Therefore, the application of GnRHa was a reliable method that should be recommended to induce males to extend sperm motility and velocity and facilitate sperm collection, especially towards the end of the spawning season.

As a conclusion, the standardized analysis of sperm quality using the open source ImageJ CASA provided a complete dataset for meagre sperm characterization that was used to estimate quality during the reproductive season and to implement protocols for sperm storage either chilled or cryopreserved. It reveals that it was possible to collect good quality sperm during a 2-month period and to store it for artificial fertilization. The modified Leibovitz medium is well adapted to meagre sperm conservation. However, it must be noticed that the use of this medium for chilled sperm storage must be refined in order to avoid uncontrolled result discrepancies. Finally, the current characteristics of sperm are in accordance with the fertilization success obtained from the different experiments developed in the DIVERSIFY project (setup of meagre artificial fertilization).

Development of in vitro fertilization methods for planned crosses

(led by IRTA, Neil Duncan and IFREMER, Christian Fauvel)

The development of strip spawning with *in vitro* fertilization methods is necessary for the meagre aquaculture industry, in order to facilitate planned crosses between selected breeders to aid the implementation of genetic breeding programs. Females with advanced stages of maturity were induced to ovulate with a single 15 µg/kg GnRHa injection. The injections were applied at 20:00-22:00 hours and the females held separate from males in darkness until being checked for ovulation. Checks for ovulation were made every 2.5 hours from 35 to 45 hours post GnRHa injection. When ovulated eggs were obtained, *in vitro* fertilization was made and egg quality assessed by determining the percentage of developing eggs (**Fig. 12)**. An injection of GnRHa was also applied to males, and sperm requirements and quality were assessed. Ratios of sperm to eggs were tested from approximately 3,000 to 500,000 sperm per eggs. Ovulated eggs were observed from 35 hours onwards. Optimal eggs quality was observed at 38-39 hours after the GnRHa injection. From 35

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

Figure 12. Different phases of artificial fertilization experiments in P3. IRTA (Spain): Top left) sperm collection; Top right) egg collection; Bottom left) gamete mixing and activation, Bottom middle) view of 134 individual fertilised batches of eggs resulting from a factorial cross with 3 females, 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.

A total of 14 females were induced to ovulate with some females being induced on more than one occasion to give a total of 24 different applications of GnRHa to induce ovulation. A total of 9 fish given a GnRHa injection did not ovulate within the period of 35-45 hours. These included 4 females that never responded to the GnRHa injection and 5 females that did not respond on the second application of GnRHa. A total of 15 GnRHa injections induced a complete or partial ovulation. Eleven ovulations from six different females were stripped in a time series every 2.5 hours to evaluate changes in egg quality after ovulation (over-ripening effect). Ovulation was detected at 35-39 h (2,100 – 2,340 min) after injection depending on the female. However, there was a high variability in the fertilization rate of eggs obtained from 35 to 36 hours between females (32.5 ± 43.50 %) while from 38 to 39 hours (51.11 ± 28.04 %) this variability was reduced (**Fig. 13**). This variability was still lower in the fertilization rate of eggs obtained from 40 to 41 hours (27.49 ± 16.39 %) and from 43 to 44 hours (5.72 ± 3.90 %) after GnRHa injection. The variability during the period 35-36 hours that represented the first revision for ovulation was clustered in two groups (bimodal) with very poor eggs (<20% fertilization) and good eggs (>60% fertilization). The poor eggs (<20% fertilization) appeared to be related to incomplete ovulation as in 5 of the 8 batches with low fertilization during the period 35-36 hours only small volumes of eggs could be

obtained. In all cases in subsequent revisions for ovulation, egg quality improved, indicating a period when egg quality improved to a maximum. If this initial variability is removed to consider only eggs that had completed ovulation to give an initial maximum value, the distribution of the averages of the fertilization rate of each group of eggs compared to the time between injection and stripping can be described by a Hill regression with $R^2 = 0.8167$. The Hill regression identified a linear decrease after 40 hours between injection and stripping in the fertilization rates of eggs from all the females.

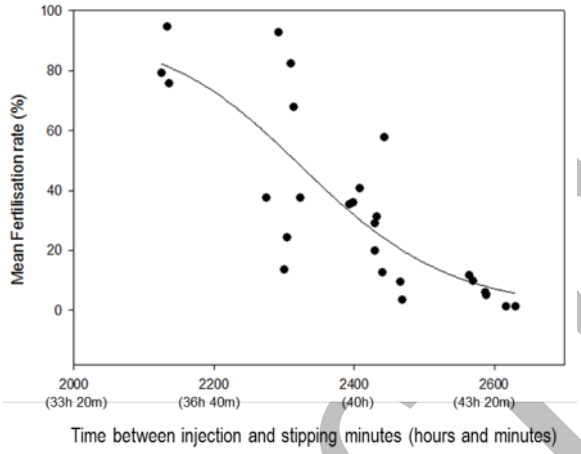


Figure 13. The fertilization rate (%) of each batch of eggs stripped from each female meagre (*Argyrosomus regius*) at different times after the GnRHa (15 µg/kg) injection. The line represents a Hill regression ($R^2 = 0.8167$). Data represents the decline from maximum fertilization rates of 10 ovulations from 5 different females i.e. the overripening period.

Eggs maintained at room temperature (approximately 20ºC) maintained viability for up to 2 hours (**Fig. 14**). There was an initial small decline in fertilization rate during the first 30-60 minutes, fertilization stabilized at approximately 80% until after 120 minutes when a rapid decline in the fertilization of the eggs was observed. Asynchronous divisions in many of the fertilized eggs were observed at 4 h.

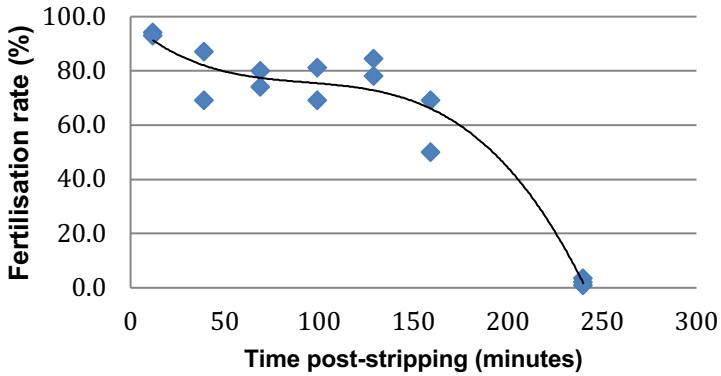


Figure 14. The fertilization rate (%) of stripped meagre (*Argyrosomus regius*) eggs held at room temperature for different times after stripping. The line represents a polynomial regression with an equation expressed: $y = -2E\text{-}05x^3 + 0{,}0058x^2 - 0{,}5891x + 97{,}447$ ($R^2 = 0.9523$).

The results obtained in the present study have established a protocol for the optimum artificial fertilization of meagre in the current state of knowledge. The broodstock should be examined at 38 h post-injection at 18ºC to obtain optimum egg quality. For conventional production, a minimum of 200,000 spermatozoa per egg is recommended to ensure high fertilization rates. Furthermore, it is recommended that eggs, sperm and water are mixed to coordinate activation with the sperm coming into contact with the eggs, and that meagre eggs should be fertilized within the first 50 min post-stripping at least to maintain a high capacity to be fertilized until more research is done to identify which delay in activation can be assumed not to affect fertilization success or until extenders that mimic the ovarian fluid are developed. The up scaling to a large factorial cross of 120 artificial fertilization using either fresh or cryopreserved sperm confirmed the feasibility of the protocol.

2. Nutrition

Advances in larval and juvenile nutrition

(led by FCPCT, Daniel Montero and Marisol Izquierdo and SARC, Fontanillas, Ramón and Rosenlund, Grethe.

To better define the nutritional needs of meagre during both pre-growing and on-growing phases to improve growth consistency and fish health and welfare is the general objective planned within this section. To reach this objective, two different tasks were planned: I) the improvement of larval weaning feeds and 2) the determination of nutritional requirements to promote feed utilization, growth and welfare.

2.1. The improvement of larval weaning diets:

Despite the fact that meagre larval development and larval rearing techniques have been extensively studied, weaning to dry diets remains to be an important bottleneck for this species. Thus, in a first step, the objective of was to better define the nutritional needs of meagre to improve the current larval weaning feeds for this species. In order to improve the weaning diets for meagre, different trials were conducted to determine optimum levels of both essential fatty acids and micronutrients, that are known to be determinant of fish performance at larval stages in other species (Izquierdo & Koven, 2011; Hamre et al., 2013).

2.1.1. Optimum essential fatty acids and related micronutrient levels in weaning diets for meagre

To determine the effect of weaning diets on larval performance, meagre larvae were obtained from an induced spawning broodstock from the GIA facilities (Grupo de Investigación en Acuicultura) at University of Las Palmas de Gran Canaria (ULPG) (Canary Islands, Spain) where the experiment was carried out. A trial was conducted to test six microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco; INVE, Dendermonde, Belgium) until 14 days after hatching (dah). Meagre larvae (initial total length 4.07±0.26 mm, mean±SD; dry body weight 0.06±0.01 mg) were randomly distributed into 18 experimental tanks at a density of 2500 larvae per tank and were fed one of the experimental diets tested in triplicates for 14 days, at an average water temperature of 23.2±0.20 °C. All tanks (200 L) were supplied with filtered seawater (37 mg L^{-1} salinity) at an increasing rate of 8% h^{-1} to guarantee good water quality during the trial. Average water dissolved O_2 reached 5.3±0.3 mg. Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights. Fish larvae were manually fed 14 times per day each 45 min from 8:00 to 18:00 hours. Daily feed supplied was 1.5 and 2 g per tank during the first and second week of feeding respectively. To avoid the nutritional contribution of *Artemia* with essential fatty acids and vitamins, this live prey was not added to the rearing tanks. Despite that complete weaning from 14 dah could reduce growth or survival, it was required to determine more accurately the effect of the levels of essential fatty acids and antioxidant vitamins in the weaning diets. Six isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm & 250-500 µm) were formulated using fish oil (peruvian anchovy) as source of high n-3 HUFA contents only for diets containing 3% n-3 HUFA (**Table 5**). Therefore six experimental diets (0.4/150/180, 0.4/300/180, 0.4/300/360, 3/150/180, 3/300/180, 3/300/360) were tested according to HUFA, vitamin E and vitamin C levels respectively. To determine larval performance was determined by measuring dry body weight and total length of 30 fish per tank at the beginning, at 24 (dah) and 20 fish per tank at the end of the trial.

Table 5. Variable ingredients and proximate composition (g 100 g^{-1}dw) of early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels fed to meagre (*A. regius*) larvae from 14 to 28 dah.

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

As a result of the experimental period feeding the different diets, it could be observed that the utilization of 3% of n-3 HUFA in larval diet improved larval growth (**Fig. 15**) and the supplementation of antioxidant vitamins significantly ($P<0.05$) improved larval growth, irrespectively of the level of n-3 HUFA in the diet (**Fig. 15**)

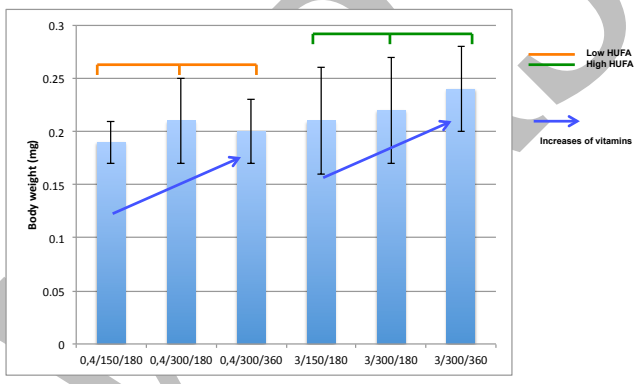


Figure 15. Meagre larvae growth after 10 days feeding experimental diets.

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

Following similar protocols described above, meagre larvae (initial total length 7.2±0.7 mm; dry body weight 0.5±0.1mg) were randomly distributed in 15 experimental tanks at a density of 2100 larvae per tank and were fed one of five experimental diets tested in triplicates for 14 days, at an average water temperature of 24.5±0.5 °C. Experimental conditions were equal as described above. Daily feed supplied was 2 and 4 g per tank during the first and second week of feeding respectively. Five isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm & 250-500 µm) were formulated using squid powder non defatted as source of protein and lipid, and were completed with Krill-PL as source of marine phospholipids, and level of vitamin E (1.500 mg kg^{-1}), vitamin C (3.600 mg kg^{-1}), gelatin (3.0), mineral premix (4.5 g/100 g), vitamins premix (6.0 g/100g) without menadione, ergocalciferol and retinol acetate (Table 6). Additional, menadione as source of vitamin K was added to the vitamin mix (175 mg kg^{-1}) in all diets except to C-Vit K (diet without vitamin K supplementation diet), ergocalciferol as source of vitamin D was added to the

vitamin mix (37 mg kg^{-1}) in all diets except to C-Vit D (diet without vitamin D supplementation) and retinol acetate as source of vitamin A was added to the vitamin mix (3 mg kg^{-1}) in all diets except to C-Vit A (diet without vitamin A supplementation). Taurine (2.000 mg kg^{-1}) was added only to C+Taurine diet (diet with taurine addition diet). The diet with vitamin K, D and A supplementation and without taurine addition was considered as a control diet (C) (**Table 6**). To determine the welfare status a stress resistance test was conducted at the end of the trial with 30 larvae that were handled out of the water in a scoop net for 30 sec. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment.

Table 6. Ingredients and proximate composition of early weaning diets fed to meagre (*Argyrosomus regius*) larvae from 20 to 33 dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

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

After feeding period, the supplementation of Vit A or D (diets C and C+Taurine) induced a reduction of growth. The lack of supplementation of vitamin K (Diet C-vit K) induced a high reduction of larval survival (**Table 7**).

Table 7. Total length (mm), dry weight (mg) and survival of meagre larvae fed early weaning diets from 20 to 33 dah (initial total length 7.2±0.7 mm and dry body weight 0.5±0.1mg). (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

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

At 33 days after hatching, high incidence of granulomas was found in larvae fed without supplementation of vitamin K (C-Vit K) (12.5%) followed by larvae fed without supplementation of vitamin A (C-Vit A) and without supplementation of vitamin D (8.3% and 3.3%, respectively) (**Table 8**). Larvae from control and taurine diets did not show granulomas. Larvae with granulomes were stained with Ziehl-Neelsen technique for Mycobacteria detection being negative for all cases.

Table 8. Percentage of total larvae affected with granuloma and percentage of larvae affected with granuloma in stages I and II.

	33 dah		
Diets	Total	Stage I	Stage II
C	0.0±0.0	0.0±0.0	0.0±0.0
C+Taurine	0.0±0.0	0.0±0.0	0.0±0.0
C-Vit K	12.5±10.6	5.0±7.1	7.5±3.5
C-Vit D	3.3±2.9	1.7±2.9	1.7±2.9
C-Vit A	8.3±10.4	6.7±7.6	1.7±2.9

"Stage I" was considered those granulomas of 0.5 mm composed of a central cluster of voluminous macrophages (**Fig. 16a**) and late granulomas or "Stage II", granuloma of 1.8 mm were composed of an eosinophilic necrotic central area surrounded by several concentric layers of macrophages that presented vacuolized cytoplasm, observing more flattened in the outer layers. Hepatic cells around of the granulomas were compressed (**Fig. 16b**).

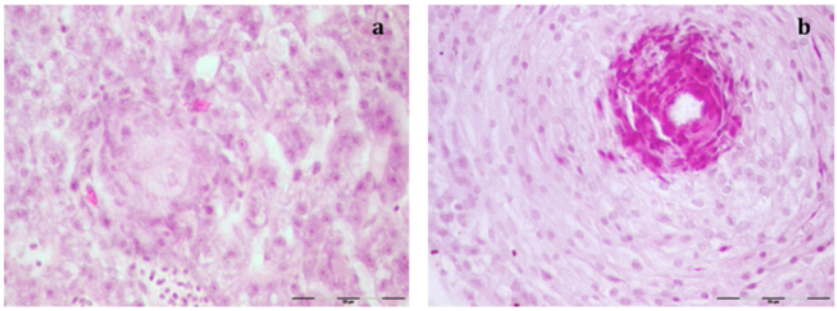


Figure 16. a. Initial or "Stage I" granuloma in the liver (40x). b. "Stage II" granuloma in the liver (40x).

2.1.3.- Improving meagre fry quality with vitamin K supplementation

As vitamin K was shown to be crucial in the development of systemic granulomas and lack of vitamin K in diet is related with a higher incidence of deformities in the vertebrae and caudal skeleton (Udagawa 2001), causing the formation of thin and weak bone, and inducing bone structure abnormalities such as vertebral fusion and row irregularity, both in early development and during later growth, an experiment with graded levels of vit K (in form of menadione sb) supplementation was conducted. Five different levels of supplementation were assayed, 0, 4.3, 8.6, 17.3 and 35 mg menadione/kg diet, diets K0, K25, K50, K100 & K200 respectively. Results showed that the supplementation of menadione increased larval survival, except for the highest level of supplementation, in which larval mortality increased (**Fig. 17**). Larval severe deformities decreased as menadione supplementation increased in diet (**Fig. 18**).

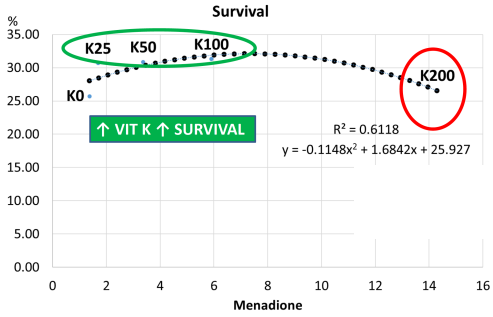


Figure 17. Survival of meagre larvae feeding different levels of vit K in diet.

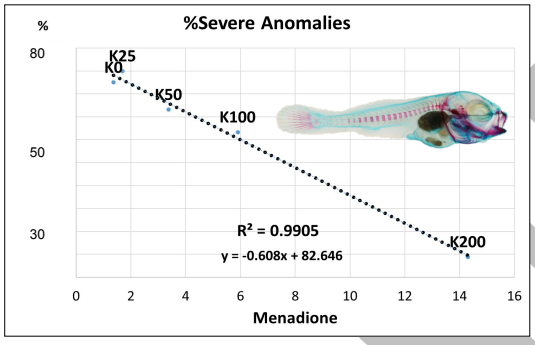


Figure 18. Percentage of severe anomalies in meagre larvae feeding graded levels of vit K.

2.1.4. Highlights to improve larval weaning diets

- ✓ 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.
- ✓ 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).
- ✓ Meagre weaning diets must be supplemented with 2.4 mg/kg vit K, since the absence of this vitamin markedly reduced larval survival.
- ✓ Meagre larvae seem to be very sensitive to hypervitaminosis D and, only mildly to hypervitaminosis A, since supplementation with these vitamins leaded to a growth reduction.
- ✓ 5.90 mg/kg of menadione in diet shows a trend to increase growth and survival of meagre larvae, however, large amount seems to decrease growth and survival, possibly due to its toxic potential.

2.2. The determination of nutritional requirements to promote feed utilization, growth and welfare.

EFA-deficiency is characterised by different symptomatology as reduced growth and survival, swimming disorders, fin erosion, and severe lipid infiltration, particularly in lipid storage tissue, as liver (Izquierdo, 1996). 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.

A feeding trial was conducted with meagre fingerlings with an initial body weight of 2.80±0.23 g (mean±SD) and an initial total length of 6.37±0.20 cm (mean±SD). Triplicate groups of meagre fingerlings, produced at University of Las Palmas de Gran Canaria facilities, were randomly distributed in 15 experimental tanks (200 L fibreglass cylinder tanks with conical bottom and painted with light grey colour) at a density of 45 fish per tank and fed manually one of the experimental diets until visual apparent satiety, three times a day, 6 days per week, during 30 days. Daily feed intake was calculated by recording diet uptake and subtracting uneaten pellets from the tank. The tanks were installed in open system and supplied with filtered seawater (37 mg L^{-1} salinity). Water was continuously aerated and dissolved oxygen was maintained above 6.0± 0.2 mg L^{-1} during the trial. Average water temperature along the trial was 23.0±0.2 ºC. The experiment was run under natural photoperiod. Five isoproteic and isolipidic experimental diets were formulated containing fish oil (FO) and vegetable oils (VO; linseed, palm and rapeseed oils) as lipid sources. Five dietary increasing levels of n-3 LC-PUFAs were defined: 0.8, 1.4, 2.0, 2.6 and 3.6% of dry matter, where DHA and EPA accounted for 93% of total n-3 LC-PUFA. The desired n-3 LC-PUFA content of each experimental diet was achieved by successively replacing VO by FO. Diet composition and proximate analysis are shown in **Table 9**. The experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) with a pellet size of 2 mm.

Table 9. Composition (%) and proximate analysis of the experimental diets for meagre fingerlings

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

1: Skretting, Stavanger, Norway;
2: Cargill Nordic AS, Charlottenlund, Denmark;
3: AAK AB, Karlshamn, Sweden;
4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals; Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

During the feeding trial, no external damage or abnormal behaviour was observed. Survival was high in all treatments and was not affected by diet composition (**Table 10**). After 30 days of feeding, meagre final weight ranged from 9.5 (in fish fed 0.8 n-3 LC-PUFA diet) to 10.7 g (in those fed 2.6 n-3 LC-PUFA diet), achieving a 3-fold increase from initial body weight. Fish fed the 0.8% n-3 LC-PUFA diet showed the lowest values for total length, body weight, WG, SGR and TGC. Increase in dietary n-3 LC-PUFA, significantly ($P<0.05$) increased final total length and body weight. Thus, the highest total length was found in fish fed 2.6% n-3 LC-PUFA diet, being significantly higher than fish fed 0.8%, 1.4% or 3.6% n-3 LC-PUFA. The highest body weight was also found in fish fed 2.6% n-3 LC-PUFA, being significantly higher than those fed 0.8% n-3 LC-PUFA. Dietary n-3 LC-PUFA increase was significantly correlated to final body weight ($P=0.09$, $r^2=0.82$), WG ($P=0.05$, $r^2=0.86$), SGR ($P=0.01$, $r^2=0.94$) or TGC ($P=0.01$, $r^2=0.94$).

Table 10. Growth performance of meagre fingerlings fed the experimental diets for 30 days[1]

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

Feed intake was not affected by the dietary treatment, and fish fed the 2.6% n-3 LC-PUFA diet showed the best K, but not significantly different from fish fed other n-3 LC-PUFA levels. The broken-line linear model fitted best to the dietary n-3 LC-PUFA relation to final length, final weight, WG, SGR or TGC and pointed out a requirement of 2.1% n-3 LC-PUFA for maximum growth of meagre fingerlings (i.e. WG showed in **Fig. 19**).

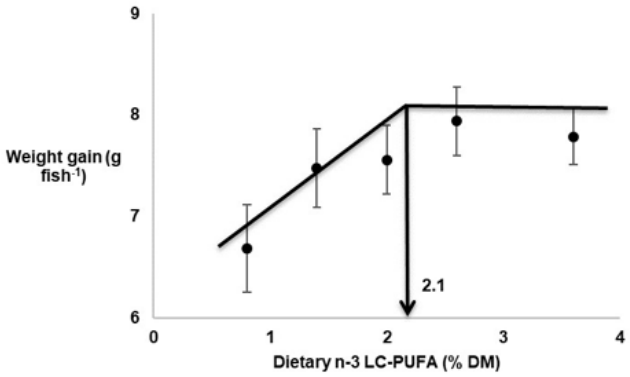


Figure 19. Broken-line linear model fitting dietary n-3 LC-PUFA levels to weight gain of meagre fingerlings fed the experimental diets for 30 days; the arrow indicates the requirement for dietary n-3 LC-PUFA (% DM).

Fatty acid content of juveniles reflected the fatty acid profile of the diet, selectively retaining essential fatty acids in those animals feeding lower levels of essential fatty acids in diet (**Table 11**)

Table 11. Retention efficiency (net accumulation or reduction) of specific fatty acids in whole-body of meagre fingerlings fed the experimental diets for 30 days[1]

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

Histological examination of cross-section of hepatic tissue showed that no necrotic tissue was found in meagre fed different dietary n-3 LC-PUFA levels. However, liver of fish fed 0.8% n-3 LC-PUFA showed a significantly higher degree of steatosis than those fed >2% n-3 LC-PUFA. Additionally, despite gross examination did not revealed the presence of granulomatous lesions in any organ of any fish, those fed the lowest dietary n-3 LC-PUFA level (0.8%) presented higher ($P<0.05$) number of hepatic granulomas than fish fed ≥0.2 %n-3 LC-PUFA (**Table 12**).

Table 12. Histomorphological evaluation of hepatic tissue of meagre fed the experimental diets for 30 days.

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Steatosis[2]	2.6^{a}±0.2	2.4^{ab}±0.2	1.7^{ab}±0.2	1.2^{b}±0.0	1.2^{b}±0.2
Granulomas[3]	5.3^{a}±1.6	2.1^{ab}±1.1	1.4^{b}±1.0	0.1^{b}±0.1	0.7^{b}±0.4

Liver of fish fed 0.8 and 1.4% n-3 LC-PUFA presented a severe steatosis, reflected by the hypertrophy of the hepatocytes (**Fig. 20 A&B**). Furthermore, in the same fish, an extensive infiltration of lipid vacuolization, in hepatocytes was observed and consequently, nuclei were displaced from central position in the cell to the periphery. Hepatic steatotic alterations decreased linearly with the increase of the dietary n-3 LC-PUFA levels (r^2=0.88, P=0.19).

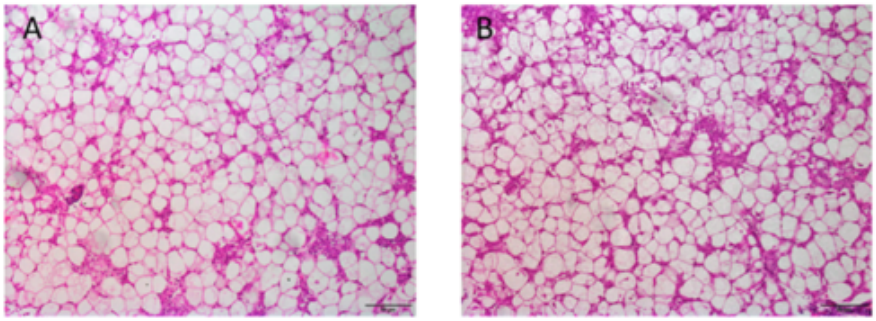


Figure 20. Liver sections from meagre fed different n-3 LC-PUFA levels stained with H&E, Bars 50µm: (A) 0.8% n-3 LC-PUFA; (B) 1.4% n-3 LC-PUFA.

Highlights on the requirements of essential fatty acids by meagre juveniles.

- ✓ Meagre showed the ability to selectively conserve key FA, particularly DHA and ARA over other FA, in response to EFA-deficiency.
- ✓ Meagre fingerlings have n-3 HUFA requirement of 2.1% DM in diets containing 16.5% DM lipids, 0.9 EPA/DHA and 0.4% ARA of total FA contents.
- ✓ EFA deficient meagre showed higher incidence of granulomas than fish fed ≥ adequate levels of n-3 HUFA in diet
- ✓ Hepatic steatotic alterations decreased linearly with the increase of the dietary n-3 LC-PUFA levels.

3. Larval husbandry

Optimum conditions for larval rearing

(Led by IRTA, Alicia Estevez)

Larval rearing of meagre is usually carried out following a protocol based on European sea bass and gilthead sea bream larval rearing. However, different studies have revealed that these protocols need to be adapted to the biological demands of this species, as meagre larvae are quite sensitive to stress produced by high light intensity (more than 500 lux), long photoperiods or high densities of live prey (Roo et al., 2010; Vallés & Estévez, 2013). Although the precise nutritional requirements are under study, larvae show very good growth and survival rates using commercially available enrichment products for live prey (Vallés & Estévez, 2015). Meagre producers do not consider larval rearing to be a major bottleneck for meagre culture (Lazo et al, 2010). On the other hand, cannibalism and variable size distribution in juveniles are considered a main concern, as they reduce production yield and increase the cost of production. Moreover, these problems may be derived from or modulated by the feeding of live food such as *Artemia* nauplii. Therefore, advancing the early weaning of larvae from its dependence on *Artemia* onto a dry feed is a priority and the major focus of the larval work on meagre. Weaning is defined as the switch from live food to inert diet, at a very critical moment during development, which requires gradual and specific dietary protocols for the success of the process (Parma and Bonaldo, 2013). In this sense, a better knowledge of larval digestive physiology under a new feeding protocol will contribute to the optimization of the weaning process and may help to understand functional limitations in the processing capacity of the digestive system to deliver nutrients to the rapidly growing larval tissues under an earlier weaning protocol.

In the project DIVERSIFY we carried out 2 trials using fertilized eggs of meagre obtained in 2014 and 2015 from a wild broodstock maintained in 4000 L circular tanks connected to recirculation units (IRTAMar®) in IRTA Centre of San Carles de la Rápita (Spain) under controlled conditions and after hormonal induction. Floating eggs were stocked in 35 L cylindrical PVC containers provided with airlift systems and high aeration supply. On day 2 post hatching (dph), larvae were stocked into 100 L tanks at a density of 100 larvae L^{-1} and cultured from 2 to 37 dph on different dietary treatments. The 100 L tanks were also connected to IRTAMAR® units. Temperature (18.2 ± 0.5 ºC), salinity (35.4 ± 0.3 g L-1), dissolved oxygen (7.9 ± 0.3 mg L-1) and pH (7.9 ± 0.2) were checked daily, whereas nitrites (<0.25 mg L-1) and ammonia (<0.07 mg L-1) were checked once per week. Light intensity was maintained at 500 lux at the water surface, and the light regime was 12 h light: 12 h dark. Larvae were fed enriched rotifers (Brachionus sp) from 2 dph until 14 dph and *Artemia* metanauplii from 9 dph. Both live preys were enriched using Red Pepper (Bernaqua, Belgium) for 12 h at 28 ºC in the case of rotifers and 6 h at 25 ºC in the case of *Artemia*. Larvae were fed two doses of live prey (morning and evening) every day, whereas dry feed (Gemma Micro, Skretting) was administered by hand every morning at 9h and using automatic feeders every hour, from 9:00 to 20:00h. The amount of feed was adjusted to reach the level of apparent feeding satiation. Every day the bottom of the tanks was siphoned to remove dead fish, uneaten food and faeces. Two experiments were carried out with meagre larvae in 2014 (Trial 1 with 4 different protocols for early weaning, see scheme below) and 2015 (Trial 2 with 2 protocols for early weaning) as described below and shown schematically (**Fig.21)**.

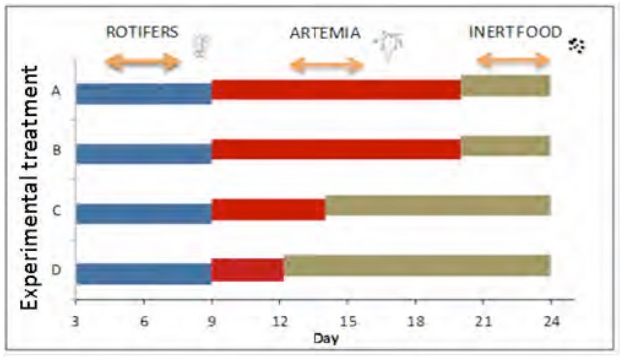


Figure 21. Group A: Weaning on dry feed started from 20 dph and completed on 30 dph, (control group); Group B: Weaning started from 20 dph and completed on 30 dph (same as the control but using half the amount of *Artemia* metanauplii); Group C: Weaning started from 15 and completed on 25 dph; Group D: Weaning started from 12 dph and completed on 23 dph, with three replicates each.

In the experiment carried out in 2014, 10 larvae were randomly collected every week in order to measure growth (SL and DW). Fish were anaesthetized with MS-222. For dry weight, the larvae were placed on glass cover slips, dried in an oven at 60ºC overnight, and weighted in a micro balance (Mettler, MX5, Spain). To examine the development of the digestive system 10 larvae from each of the three replicated tanks were randomly sampled on 10 dph (A,B,C,D groups), 15 dph (D group), 18 dph (C group), 23 dph (A,B,C,D groups) and 35 dph (A,B,C,D groups). Larvae were randomly collected when the weaning started and feed was changed from live to inert diets at 12 dph (D group), 15 dph (C group), 20 dph (A, B groups) and 35 dph, in order to analyse the development of the digestive enzymes, and antioxidant enzyme activities.

Results.- The results in terms of growth performance of the larvae are shown in **Fig. 22**, whereas survival values are shown in **Fig. 23**. Group C, weaned at 15 dph and fed half the amount of Artemia metanauplii showed similar final results in weight than control group (A) ($P > 0.05$), but exhibited longer length than in all the other groups ($P < 0.05$). Although in this group the incidence of cannibalism, and subsequently the growth in length of some individuals, was higher than the rest of the groups, with the exception of group D that showed the highest mortality rate ($P < 0.05$). On the other hand, group B showed a significantly lower growth in weight and length and the highest survival rate with lower incidence of cannibalism (see photographs in **Fig. 24**) ($P < 0.05$) and a similar distribution of size among the larvae.

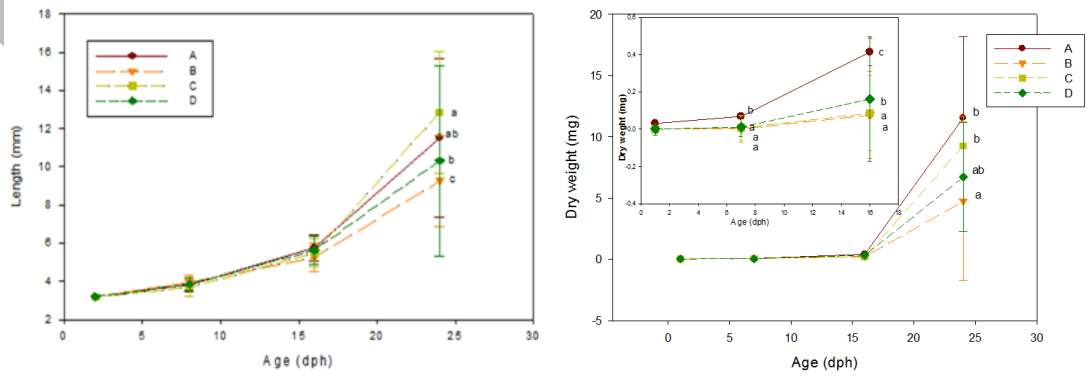
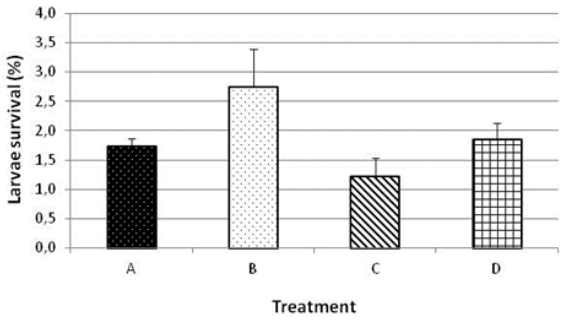


Figure 22. Growth performance of meagre larvae (Mean ±SD), length (left) and dry weight (right). Inserted figure in the right shows the larval growth during the first larval period (1-15 dph). Different lowercase letters show significant differences (ANOVA, $P < 0.05$)

Survival rate was very low in all the groups (1.2 to 2.8%, Fig. 3), and at the end of the experiment big differences in larval growth were detected in each tank as shown in **Fig. 24**, due to the high incidence of cannibalism. Differences in dry weight among small, medium and large larvae are also shown in **Table 13**, with larvae weighing from 1.28 to 38.28 mg of dry weight depending on the group.



T	%
A	$1,7 \pm 0,1^{b}$
B	$2,8 \pm 0,6^{a}$
C	$1,2 \pm 0,3^{b}$
D	$1,8 \pm 0,3^{b}$

ANOVA P=0,005

Figure 23. Survival of meagre larvae from each treatment

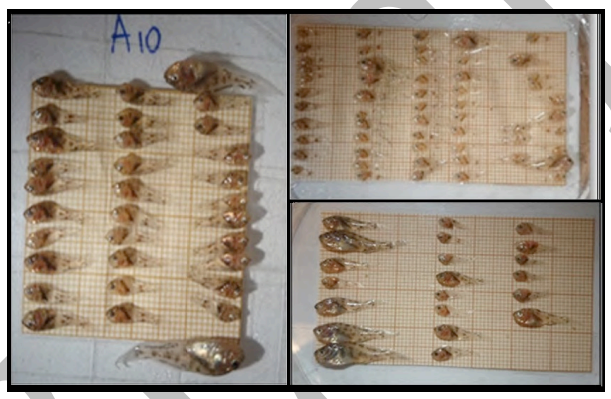


Figure 24. Difference in cannibalism and a similar distribution of size among the larvae.

Table 13. Differences in dry weight (mg, mean ± SD) among small, medium and large larvae in the four feeding groups (Groups A, B, C and D).

T	Small	Medium	Large
A	4,52 ± 0,97	16,06 ± 9,58	38,28 ± 8,41
B	1,28 ± 0,16	8,00 ± 7,69	18,67 ± 9,75
C	3,73 ± 1,55	12,27 ± 3,79	23,17 ± 5,11
D	3,01 ± 1,29	8,91 ± 3,89	26,41 ± 2,77

The results of the trial carried out in 2015 were different. Growth in weight and length of the larvae is shown in **Fig. 25**. Growth was significantly higher for the larvae of group A (control) compared to early weaned larvae. Survival rates were around 4-5% at the end of the study (**Fig. 26**); these data were within the range of values observed in commercial fish hatcheries. The use of low light intensity (reduced from 500 to 150-200 lux) and the higher number of feed doses supplied had a clear effect on the reduction of cannibalism that at the same time, allowed a higher survival rate.

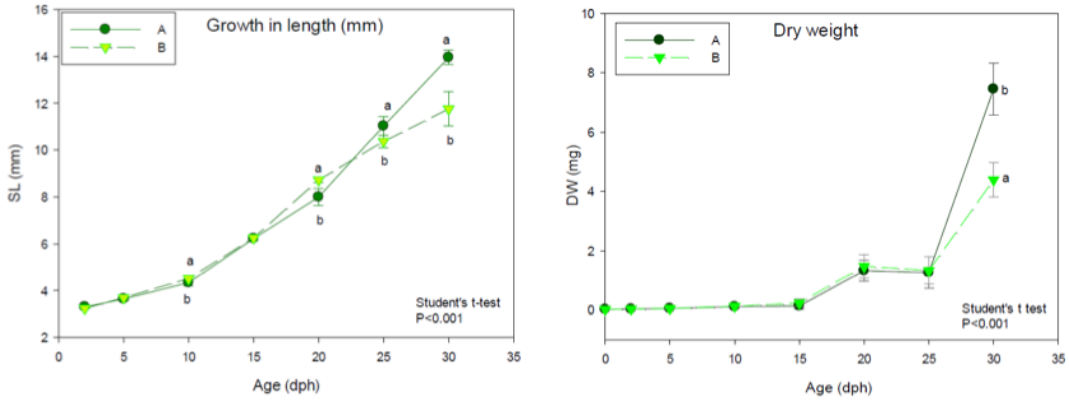
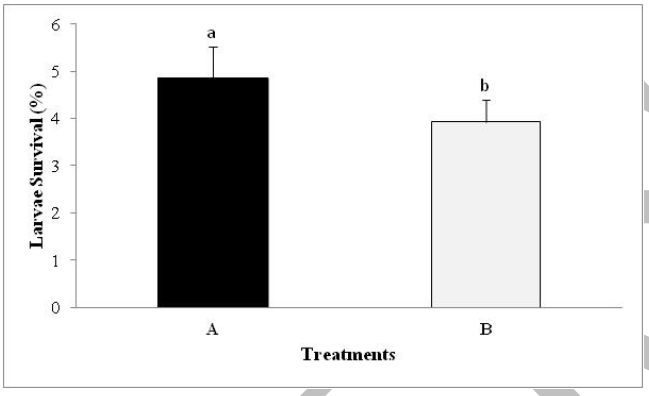


Figure 25. Growth in length (left) and dry weight (right) of meagre larvae. Different lowercase letters indicate significant differences (Student's t test, P < 0.001).



	Survival Rate (%)
A	$4,86 \pm 0,65^{a}$
B	$3,93 \pm 0,47^{b}$

Student's t test P=0,032

Figure 26. Larval survival obtained in the trial carried out in 2015 and Student t-test showing significant differences indicated by lower case letters between the two groups (Groups A and B) (Student´s test P=0.032).

Digestive enzyme activities.- Results of enzymatic activity expressed as specific activity (U mg protein-1) of the larvae from Trial 1, at the beginning of the weaning (12 dph group D, 15 dph group C and 20 dph groups A and B) and at the end of the trial (24 dph) as well as at the end of Trial 2 (37 dph) are shown in Figures 8 and 9. The results clearly show that all the larvae show a similar enzymatic activity when they are fed live prey **(Fig. 27)** and differences could be found only in the alkaline phosphatase production, being higher in the younger larvae (groups C and D). At the end of the weaning phase **(Fig. 28)** pancreatic enzymes tended to be higher in the early weaned larvae, especially in the larvae from Trial 2 and in larvae from the group C larvae Trial 1 with a significantly higher lipase activity compared to the control group (A) (P < 0.05). Alpha-amylase did not show differences between early weaned and control larvae and trypsin also showed higher activity in early weaned larvae (P < 0.05).

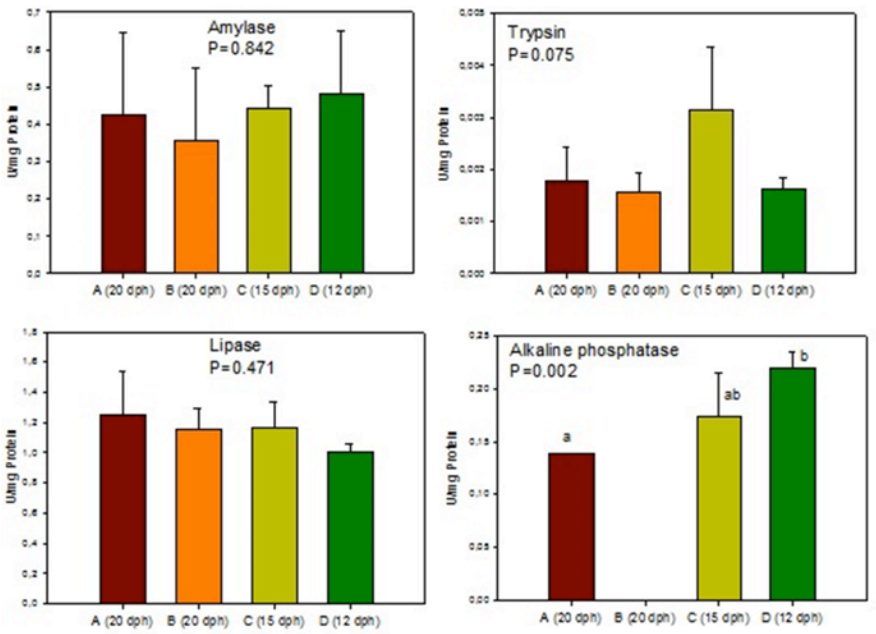


Figure 27. Results of digestive enzyme activity measured in the larvae from 2014 at the end of the live prey feeding period. Different lowercase letters indicate significant differences (ANOVA, $P < 0.05$)

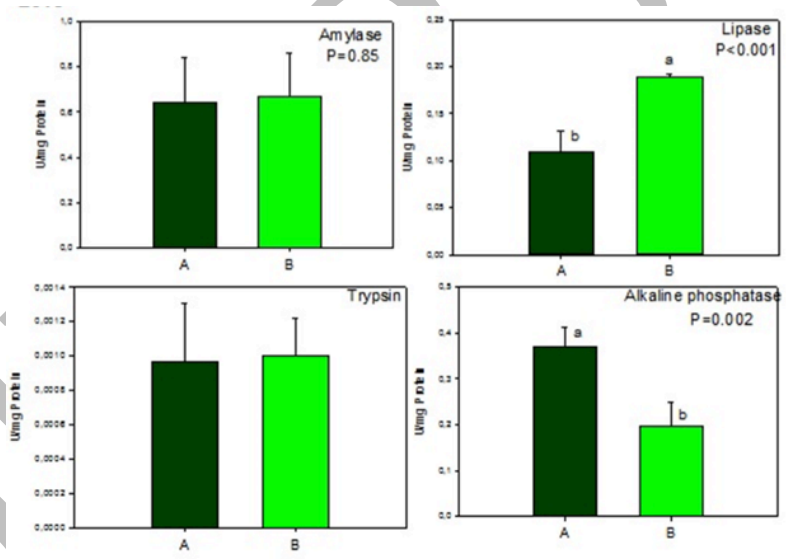


Figure 28. Results of digestive enzyme activity measured in the larvae from 2015 trial at the end of the experiments. Different lowercase letters indicate significant differences (ANOVA, data from 2014 and Student's t test, data from 2015, $P < 0.05$).

DISCUSSION AND CONCLUSIONS

Weaning, the transfer from live food to an artificial diet is successful with most marine fish with a completely developed digestive tract (Person Le Ruyet et al., 1993). In the current study weaning was carried out with a commercial weaning diet (Gemma Micro, Skretting) using a gradual transfer from live prey to thi artificial diet over a minimum period of five days, although in some other

marine species like sea bass ther is an abrupt replacement (Person Le Ruyet, 1990). Duran et al (2009) using a weaning protocol similar to the one used in Trial 2 (2015) obtained similar results in growth and survival. Thus, **early weaning can be carried out with meagre larvae if several measures to reduce cannibalism are in place.**

Cannibalism was controlled in Trial 2 by increasing feeding frequencies, removing dominant individuals, grading regularly and keeping the larvae in the dark when food was unavailable or in short supply. The use of low light intensity before feeding in the morning increased survival of the larvae in the trial carried out in 2015 by reducing cannibalism.

The weaning success of any finfish larvae from live feeds onto a formulated diet is partly dependent on the composition of the diet and the ability of the larvae to select and digest an inert diet. In this sense, the proper development of feeding protocols and diet formulations under fish larval culture conditions requires a deep knowledge and understanding of the digestion processes occurring during early ontogeny in order to synchronize different types of feeds (live prey and microdiets) with production of different digestive enzymes. Therefore, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of fish larvae, food acceptance, digestive capacity, as well as for survival and growth rate predictions. Thus, the synthesis and secretion of digestive enzymes are regarded as indicators for the transition from live feeds to microdiets. The pancreatic secretory process matures during the first three or four weeks after hatching in temperate marine fish larvae. This maturational process can be disrupted when larvae are fed diets that do not meet their specific needs. Proteolytic enzymes from the exocrine pancreas are regarded as being particularly significant in the early life stages of precocious and altricial fish because of the absence of a functional stomach with pepsin. Lipase plays an active role in lipid digestion, especially in the breakdown of triacylglycerol to diacylglycerol and then to monoacylglycerol. In many fish species, including meagre, lipase is active during resorption of the oil globule and the complete transition to exogenous feeding being relevant for the digestion of high levels of triacylglycerols present in the enriched life prey such as *Artemia*. On the contrary, the capacity to digest proteins in the stomach (pepsin activity) was significantly lower in the early-weaned larvae, which coincided with the significantly lower growth rate achieved by this group. Having in mind that pepsin activity is detected after the formation of a functional stomach that in meagre generally occurs between 15 and 20 dph (Papadakis et al., 2013; Suzer et al., 2013), several authors have suggested this age is the best for weaning meagre larvae. This is supported in the present study **that indicated that 10-12 dph (weaning ages used in the experiments) might be premature for larval weaning.** The weak ability of early-weaned larvae for denaturizing and proteolytic cleavage of proteins from the artificial food could be the reason for the poor larval growth achieved at early weaning.

In conclusion, based on these results meagre larvae can be weaned from live feed to an artificial diet at as early as 10 dph, but other important aspects for production success including larval performance and survival should be considered

4. Grow out husbandry

Methodology to avoid size variability in meagre juveniles

(led by IRTA, Alicia Estévez and Neil Duncan)

Size variability in juvenile pre-grow out makes regular grading essential to avoid cannibalism and grades of smaller fish may be related to poor performance when transferred to sea cages. Experiments were carried out with meagre juveniles of a mixture of 4-6 known families (from specific breeding groups), to simulate the commercial hatchery situation and in order to study differences in growth rate. Juvenile fish were stocked in triplicate tanks at the same initial density and fed the same commercial diet (IRTA). At the end of the experiment, fish were genetically characterized for parentage to establish if differences in growth rate was a consequence of genetic origin. Fish with low growth rates were used for compensatory growth studies to determine growth potential of small juveniles and estimate the economic cost of using these fish for production, compared to discarding and using only larger juveniles.

MATERIAL AND METHODS

Two experiments were carried out, one in 2014 keeping the families separated during larval rearing and another in 2015 keeping the families together during larval rearing. In 2014 six different spawnings obtained from hormonal induction of paired fish, which were used for larval rearing. Two families (V8-1 and C2 spawning on April 24th) hatched on April 28th and the other four (V8-1 (2), C1, V6 and V8-2, spawning on May 1st) hatched on May 5th (**Table 14**). Three spawns were from halfsib families (families 1-3) and three spawns were from unrelated breeders (families 4-6). Two cultured females were used as breeders and all other fish were from wild origin.

Table 14. Parents that contributed to each family or half-sib family and spawning date. The number refers to the breeders' unique ID and wild or cultured indicates the origin of the breeder.

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

In 2015 the experimental design was changed according to the suggestions given by the other participants in this task. In this trial four different spawnings obtained from hormonal induction of paired fish (**Table 15**) were used for larval rearing. All the spawnings were obtained on the same day (May 13th 2015) and after incubation the newly hatched larvae (May 15th 2015) were mixed together and distributed in four 1500-l tanks.

Table 15. Parents that contributed to each family and spawning date in 2015. The female and male number refers to the breeders unique ID and wild or cultured indicates the origin of the breeder

Family	Spawning Date (Tank)	Female	Male	Hatched larvae (N)
1	13/05/2015 (V7)	5-wild	19-wild	122617
2	13/05/2015 (V6)	6-wild	23-cultured	141983
3	13/05/2015 (V8-1)	1-wild	20-wild	66500
4	13/05/2015 (V8-2)	8-wild	22-wild	8050

Larvae were reared under intensive conditions following the standard protocol of meagre culture (Vallés & Estévez, 2015). Fifty larvae per litre were used as initial density, a photoperiod of 16L:8D, 500 lux intensity (Vallés & Estévez. 2013), in the water surface, and fed enriched rotifers from 2 to 14 days post hatch (dph), enriched Artemia metanauplii from 8 to 30 dph and weaned a commercial diet (Gemma Micro, Skretting, Norway) from 20 dph onwards. For enrichment, the commercial product Red Pepper was used following the enrichment procedure provided by Bernaqua (Belgium). Every week, 20-30 larvae were sampled and anaesthetized with MS222 to estimate growth in weight and length. Standard length was determined by observation in a stereomicroscope Nikon SMZ800 (Nikon, Japan) equipped with a digital camera Olympus DP25 (Olympus, Germany) and an image analyzer (analysis,SIS GmbH, Germany). The same larvae were used to estimate wet and dry weight, placing the larvae on a pre-weighted coverslip and after drying at 60ºC for 24h in an oven, with a microbalance Mettler Toledo MX5 (Mettler Toledo, Spain).

Juveniles were fed ad libitum with a commercial diet for European sea bass (Mar Perla, Skretting, Norway) in 2014 and using a fixed feeding rate in 2015 (7.5% body weight for fish between 10-30 grams, 5% for fish from 30-60 grams). Growth of juveniles was also registered every 2-3 weeks during ongrowing and specific growth rate calculated following the formula: SGR = (lnWf - lnWi x100) / t, where Wf and Wi are the final and initial weights and t the time (days).

Problems observed during rearing, weaning and ongrowing.- In 2014 larval growth was very different among the six families, thus the larvae from the first week grew faster and bigger than those of the second week. Groups C1 and V6 grew very slowly, larvae were always very small and the weaning became very difficult because the larvae were too small to eat the microdiet. During the weaning phase a high incidence of cannibalism was detected causing very high mortality rates and very high dispersion of sizes was detected. As a consequence on June 19th when the first grading was carried out the survival was very low and a clear high dispersion of weights was detected, with fish of 8 g and of 0.4 g together in the same tank. In 2015 we tried to avoid cannibalism reducing the light intensity of the tanks, increasing the number of feed doses (either Artemia and weaning diets during weaning but also increasing the number of doses of the microdiet after weaning) and separating the floating small weaned larvae from the big ones usually distributed in the lower part of the water column.

RESULTS

In Trial 1 (2014), growth in dry weight was variable between families obtained in the first (spawning of April 24th) and second week (spawning of May 1st)(**Fig. 29**).

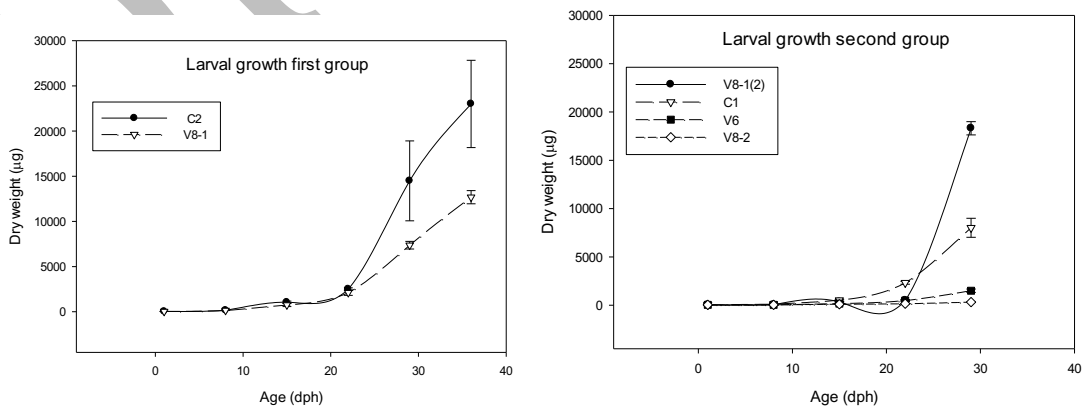


Figure 29. Growth (DW, g) of the six groups of larvae during the first month

On June 19th all the fish were graded according to weight, separated in big (8 g for fish of the first spawning on 24th April and 3 g for fish of the second spawning on 5th May) and small size juveniles (0.6 g from 1st spawn and 0.4 g from 2nd spawn, see **Table 16**). The results of the number of fish graded are shown in Table 3 and the distribution of sizes of the different tanks in Fig 2. After grading the fish were transferred to the nursery. The fish from the different spawning dates (1st date 24th April and 2nd date 1st May) were maintained separate to give a total of four groups in the nursery: big and small fish from the 1st spawns on April 24th and big and small fish from the 2nd spawns on May 1st.

Table 16. Big and small juveniles transferred to the nursery on June 19th

Tank	V8-1	C2	V8-1 (2)	C1	V6	V8-2
Big	5	8	5	14		
Medium	219	141	176		36	89
Total	224	181	181	14	36	89
% Survival	0.64	0.43	0.52	0.04	0.10	0.25

The number and weights of the fish transferred to the nursery (1500-l tanks) is shown in **Table 17.** The fish were stocked into tanks of 1500 l with >400% flow through water supply daily. Water temperature and photoperiod was natural. The fish were fed a commercial meagre diet to satiation, by both hand feeding and automatic feeders.

Table 17. Distribution of fish in nursery on June 19th

	1st spawn April 19th	1st spawn April 19th	2nd spawn May 5th	2nd spawn May 5th
	Big	Small	Big	Small
Nº	13	360	19	212
Avg Weight (g)	8.83	0.64	2.82	0.35
SD	2.24	0.30	0.8	0.16
Biomass (g)	114.73	230.4	53.66	73.78

A new classification of all the fish (big and small, see **Table 18**) was carried out on July 24th to separate the fish in 3 sizes: big, medium and small fish (**Table 18**). In the new classification the small grades from June 19th were separated into medium and small grades for each spawning date to give a total 6 groups: big medium and small for each spawning date: 1st spawns on April 24th and 2nd spawns on May 1st (**Table 18, Fig. 30**).

Table 18. Results of the second grading on July 24th.

	Week 1			Week 2		
	Big	**Medium**	**Small**	**Big**	**Medium**	**Small**
Nº	12	46	168	19	49	122
Av Weight	114.54	14.14	9.22	59.59	16.60	11.01
SD	8.71	3.73	1.48	5.84	3.58	3.80
Min	50.60	11.00	5.20	23.26	12.97	5.65
Max	78.50	30.3	11.80	41.23	23.70	22.70

On the 21st August all the fish were weighed, measured (length), photographed and fin clips were taken for genetic analysis, and in addition to fin clips 16 fish were sacrificed and samples of liver and muscle stored in RNA-Later for transcriptome analysis (see WP2, Task 2.5). During the first two months (June 19th to August 21st) in the nursery the fish exhibited good growth, the largest group from the 1st spawning (April 24th) grew from 8.8±2.2 g to 101.8±22.3 g and the largest fish from 2nd spawning (May 1st) grew from 2.8±0.8 g to 56.7±13.6 g (Fig 3). The small and medium grades of fish grew from 0.6±0.3 g to 21.8±5.0 g (small grade) and to 35.2±4.3 g (medium grade) for the first spawning and from 0.3±0.2 g to 26.0±4.1 g (small grade) and to 50.7±12.7 g (medium grade) for the second spawning. The large fish exhibited a SGR of 5.6 and 6.9% day-1 for the first period (June to July) and 1.8 and 2.1% day-1 for the second period (July to August). The medium and small grades exhibited a SGR from 8.0 to 10.1% day-1 for the first period (June to July) and a range from 3.1 to 4.0% day-1 for the second period (July to August). The proportion or percentage of the population attaining larger weights was low indicating a few fish grow faster than the majority of the population. The large grades of fish represented 3 and 8% of the population, the medium grades 21 and 26% and the small grades 76 and 65% of the population (**Fig. 30**).

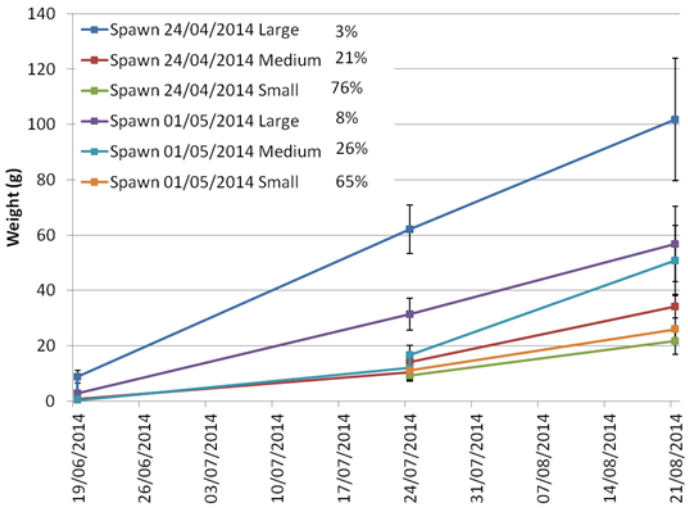


Figure 30. Growth, mean wet weight (g) with 1 standard deviation of the juveniles from different spawning dates and graded by weight. Initially the population was divided into large and medium fish and then the medium fish were divided into medium and small. The percentages in the legend refer to the percentage of the population in each grade**.**

The fish were stocked into tanks of 1500-l with >400% flow through water supply daily. Water temperature and photoperiod was natural. The fish were fed a commercial sea bass diet (Perla Skretting, Norway) to satiation, by both hand feeding and automatic feeders. A random sample of 50 fish from each group was weighed and measured (length) on the 18th Sept., 8th Oct., 29th Oct., and 19th Nov. The growth performance of these three groups was similar and the SGR in the first period (August to Sept.) was 2 % day-1 in all groups, 1.6-1.8 % day-1 in all groups in the second period (Sept. to Oct.), 1.4-1.7 % day-1 in all groups in the third period (Oct.) and 0.8-0.9 % day-1 in all groups in the fourth period (Oct. To Nov.). The large fish have grown from 27.2±1.5g to 113.9±21.0g, medium fish have grown from 22.7±12.2g to 94.2±19.8g and small fish have grown from 17.9±1.8g to 71.6±21.3g **(Fig. 31)**. On all sample dates there have been significant differences ($P<0.05$) between the grades and the fish in each group have grown significantly ($P<0.05$). The different size grades appear to have very similar growth potential, but the large and medium grades always presented the same of slightly higher growth than the small grade. This trail finished in November 2014.

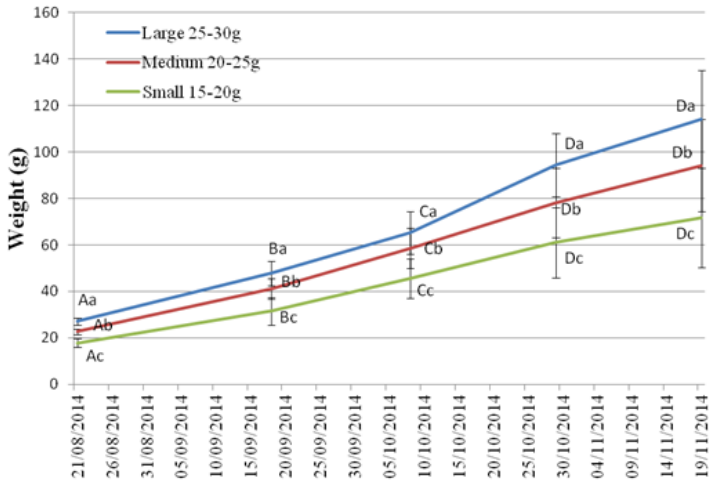


Figure 31. Growth, mean wet weight (g) with 1 standard deviation of the juveniles classified to three grades large (initially 25-30g), medium (initially 20-25g) and small (initially 15-20g). These fish represented 70% of the population from five spawns on two different dates. Capital letters represent significant differences (P<0.05) between sample dates for the same size grade. Lower case letters represent significant differences (P<0.05) between size grades on the same sample date.

The results obtained in 2014 are also summarized in **Table 19** and **Fig. 32**, including the dates of grading, age of the fish (days post hatch), weight and growth rate (SGR).

Table 19. Summary of results obtained in the growth in weight of 2014 juveniles

	2014																							
	53 dph		88 dph			116 dph			144 dph			164 dph			185 dph			205 dph			227 dph		
	19/06/2014		24/07/2014		SGR	21/08/2014		SGR	18/09/2014		SGR	08/10/2014		SGR	29/10/2014		SGR	19/11/2014		SGR	11/12/2014		SGR
S	0,43	0,37	9,42	1,82	2,27	17,855	1,786	2,80	31,488	6,052	3,35	45,55	8,57	3,65	61,01	15,14	4,04	71,58	21,31	4,07	79,47	24,40	4,18
M			15,47	3,91		22,682	1,288	3,02	41,214	4,488	3,61	58,59	8,55	3,88	78,13	14,81	4,28	94,18	19,79	4,33	101,33	23,48	4,41
L	5,26	3,36	43,23	16,7	3,72	27,184	1,544	3,17	47,768	5,311	3,75	65,26	9,06	3,99	94,39	13,46	4,47	113,93	21,00	4,51	125,55	26,49	4,62

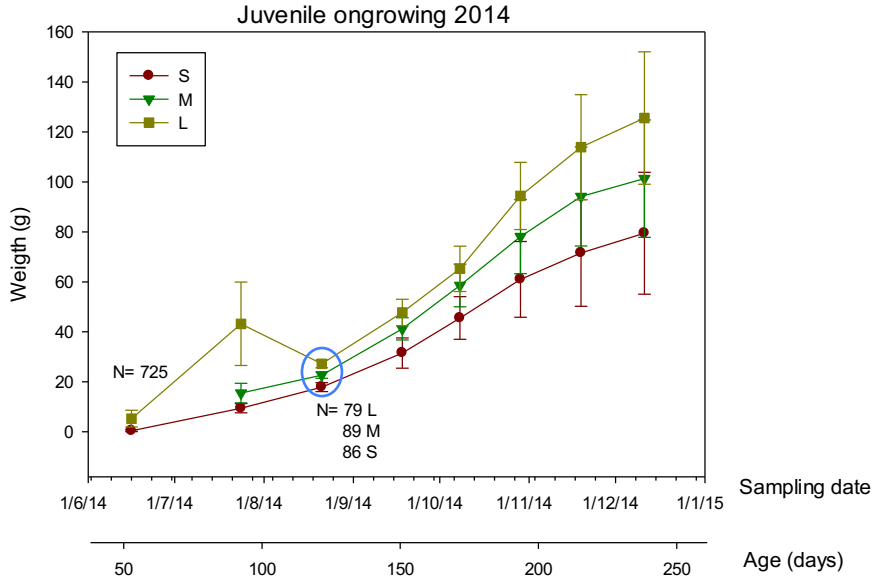


Figure 32. Growth in weight of juveniles obtained in 2014 after grading in small (S), medium (M) and large (L)

Trial 2 (2015).- All the larvae obtained from the 4 spawns were reared mixed together in 4 tanks. On July 2nd 150 fish were individually weighed to check the size distribution before separating the fish in small, medium and large individuals. On July 17th all the fish were counted and graded into large, medium and small fish and distributed in two RAS modules according to **Table 20**.

Table 20. Distribution of fish in ongrowing tanks on july 17th 2015

14/07/2015									
Mod 5						Mod 3			
Tank 1		Tank 2		Tank 3		Tank 1		Tank 2	
N	Average WW (g)	N	Average WW (g)	N	Average WW (g)	N	Average WW (g)	N	Average WW (g)
551	0,26	802	0,43	361	1,20	660	0,44	650	0,43
Biomass (g)	145,00		343,72		433,94		291,90		278,57
7,5% feeding rate (g)	10,88		25,78		32,55		21,89		20,89

The fish were kept in these tanks (an additional movement was carried out in August to redistribute M size fish and reduce the biomass in tank 2 Mod 5 and tanks 1 and 2 Mod 3) for 2 months and fed using automatic feeders at feeding rate of 7.5% from July 17th until September 3rd when the fish were graded again in large (L 28-32 g), medium (M, 19-24 g) and small (S, 12-16 g) fish and fin clips taken for parental assignment. Fish were graded in L, M and S, and distributed in triplicate tanks each with 100 fish that were fed also using automatic feeders at a feeding rate of 7.5% for fish between 12 and 30 grams and 5% for fish weighing more than 30 g. Every 2-3 weeks until November 5th 2015, the fish were weighed to build the growth curve and calculate the standard growth rate as in 2014. Results are summarized in **Table 21** and **Fig. 33**.

Table 21. Summary of results obtained in the growth in weight of 2015 juveniles

2015																				
	49 dph		83 dph			110 dph			112 dph			134 dph			155 dph			190 dph		
	02/07/2015		05/08/2015		SGR	01/09/2015		SGR	03/09/2015		SGR	25/09/2015		SGR	16/10/2015		SGR	05/11/2015		SGR
S	0,263	0,030	4,806	1,20	1,61	17,841	5,646	2,82	13,96	1,39	1,20	19,07	2,79	2,83	26,13	4,04	3,12	29,89	5,31	3,30
M	0,434	0,093	7,030	1,83	1,97	22,171	5,776	3,03	21,50	1,47	1,52	30,43	3,53	3,28	38,61	6,13	3,49	45,83	8,79	3,72
L	1,202	0,494	12,359	5,69	2,51	37,950	14,961	3,54	29,18	1,56	1,56	39,76	5,40	3,53	55,12	8,40	3,83	66,62	11,88	4,08

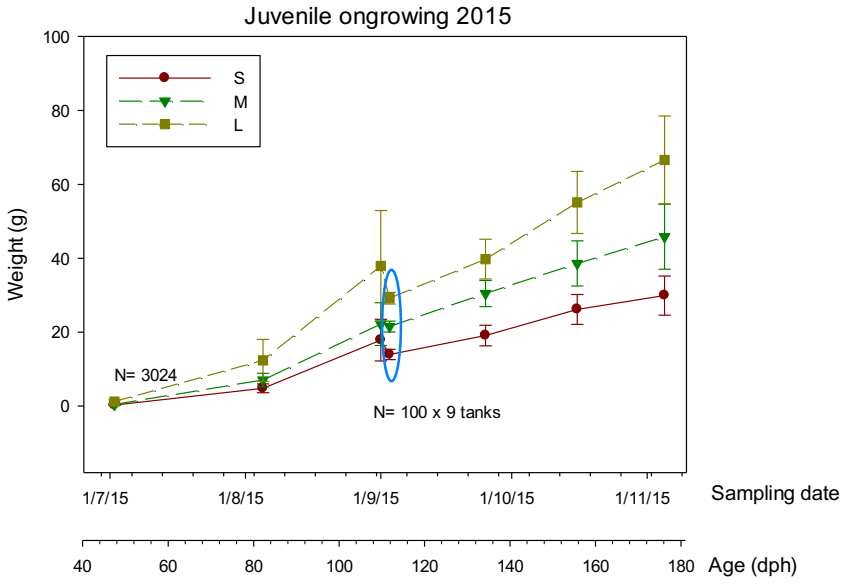


Figure 33. Growth in weight of juveniles obtained in 2014 after grading in small (S), medium (M) and large (L)

With the results obtained we have calculated the growth curves for 2014 and 2015 for S, M and L fish and calculated the differences in growth for the different groups of fish. The results are presented in **Table 22** and clearly show that S fish always grow more slowly than M and L fish and there is no compensatory growth when the fish are graded in different sizes. Thus, if S fish are kept in the fish farm they will have a delay of around 6 months to attain the same size of L fish. Although the growth of the fish was different between the two years (2014 and 2015) the differences in growth of S versus L fish were almost the same.

Table 22. Theoretical growth of S, M and L fish in 2014 and 2015

Growth of fish in 2014			
	S	M	L
100 d	7,82	10,84	15,67
200 d	66,67	85,94	104,18
360 d	160,83	206,10	261,79
540 d	266,76	341,28	439,11
Growth of fish in 2015			
	S	M	L
100 d	12,27	19,26	24,24
200 d	33,17	50,33	73,33
360 d	66,61	100,04	151,87
540 d	104,23	155,97	240,24

We have observed that in meagre there is no compensatory growth when the fish are graded in sizes during ongrowing. Slow growing fish (S) always show a lower growth rate than medium or fast (L) growing fish that have as a consequence a delay of around 6 months to get commercial size with clear economic consequences for producers (**Table 23**). The prices used were obtained from a feed producer (Skretting) and the central market of Madrid, Spain.

Table 23. Production cost of L- and S- growing fish.

PRODUCTION COST OF L- AND S- GROWING FISH (1000 juveniles)		
	L- fish	S- fish
Juveniles (0.6€/unit)	600	600
Food 10-30 gr (2.4€/Kg)	90	136,8
30-250 gr (2.04€/Kg)	1526	2557,7
250-500 gr	1943,1	3243,6
Total	**4159,1**	**6538,1**
Market price (9,3€/Kg)	4650	4650

CONCLUSIONS

1.- Size variability and different growth rate in meagre juveniles exist and seems to have a genetic origin (parental assignment still in progress).

2.- There is no compensatory growth of the small size juveniles when graded and transferred to new tanks and offered enough food.

3.- After grading, large fish always show a higher growth rate that it is maintained along the ongrowing period. On the contrary, slow growing fish always show a lower growth rate that is maintained along the whole ongrowing period causing a delay of approx. 6 months in attaining commercial size.

4.- Our recommendation for hatchery producers is to cull and eliminate slow growing fish from the production chain before the fish are transferred to ongrowing facilities.

The effect of cage depth and light intensity on growth

(led by HCMR, Nikos Papandroulakis)

The objectives of this work were the adaptations in the existing methodologies for grow out in cages related to the rearing environment (depth and light conditions). Two trials were implemented in order to study the effect of cage depth and the effect of light intensity in the cages.

For the cage depth, the trials were performed in 180 (6x6x5) and 290 (6x6x8) m^3 cages at the HCMR pilot farm using fish belonging to two size-groups of (200-600 g) and (800- 1.5 kg). For the first trial, four groups were created, two of ~5,150 for the 180 m^3 cages and two of ~8,240 for the 290 m^3 cages in order to keep similar stocking densities for the two conditions. The wet weight at the beginning of the trial was **200 ± 20 g**. For the second, 4 groups were created, two of ~2,000 individuals for the 180 m^3 cages and two with ~3,200 for the 290 m^3 ones. The initial weight at the beginning of the trial was **867 ± 18 g**. The duration of each trial was 8 months. Feeding was performed with automatic feeds and the growth performance was estimated with monthly samples. Every second month hematological (hematocrit, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid, free fatty acids), immunological (lysozyme, myeloperoxidase serum), hormonal (cortisol) evaluation was performed. Also, the vertical distribution in cages was monitored using an echo integrator.

For studying the effect of light intensity in the cage, rearing was performed with and without shading at an SME farm (ARGO) applying standard commercial procedures. The rearing was for 2 rearing periods (each with 2 cages). 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 (**Fig. 34**) while the second was covered only with a bird protecting net.

(a)  (b) 

Figure 34. Experimental cages at ARGOSARONIKOS SA. Shaded (a) and not shaded (b).

The first trial started with groups of 11.000 individuals each with an individual weight of 135±25 g. 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. The duration of each trial was minimum 8 months. Groups were fed manually, 3 times per day, with standard commercial diets. Samples to estimate growth rate were regularly taken, while the vertical distribution in cages was monitored.

Effect of cage depth. The growth performance **(Fig. 35)** observed in the two experimental phases (200 g and 800 g fish) was without differences between the tested conditions. During the 1st phase the growth rate was ~2 g d^{-1} while for the second phase it was increased to 3.5 g d^{-1}.

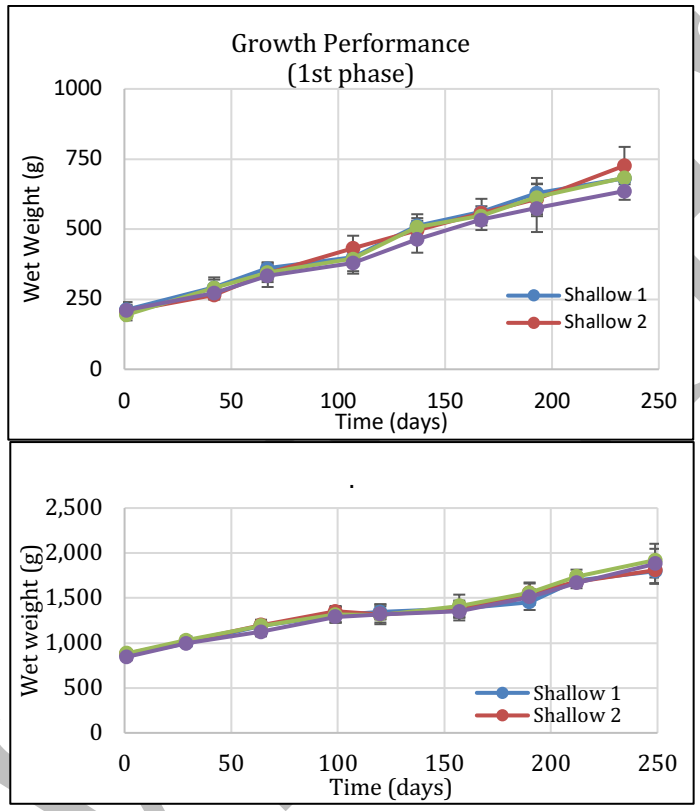


Figure 35. Growth performance in terms of averageweight, of meagre. Vertical bars showthe standard deviation (n=10)

Regarding other performance indicators, **Table 24** shows the mortality (%) and the food conversion ratio (FCR). Significant differences were obtained only during the first period with the groups reared in the deep cages (D1 and D2) cage showing almost half the mortality rate and also ~25% lower FCR compared to the shallow groups (S1 and S2).

Table 24. Performance indicators during the two experimental phases

		S1	S2	D1	D2
1st phase	**Mortality (%)**	23,5	24,2	12,1	13,9
	FCR	1,92	1,92	1,58	1,60
2nd phase	**Mortality (%)**	10.8	9.7	7.9	8.1
	FCR	1.67	1.70	1.50	1.47

Regarding the physiological status, during the 1st Phase, glucose and lactate (**Fig. 36**) showed differences between the two depths but statistical interactions make interpretation difficult

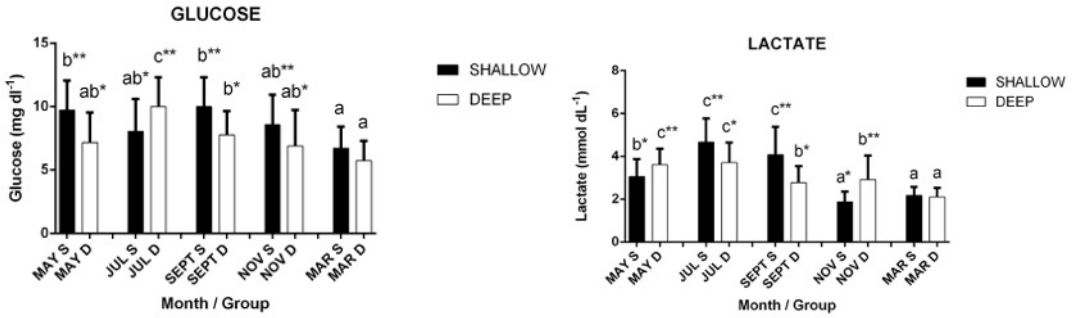


Figure 36. Plasma glucose and lactate levels during the period from May 2014 to March 2015. Letters indicate differences between the different samplings (months), while asterisks differences between the different cages, $P < 0.05$.

Cortisol levels in plasma (**Fig. 37**) presented only seasonal fluctuation. Observed higher levels in March may reflect stress due to crowding or lower temperature.

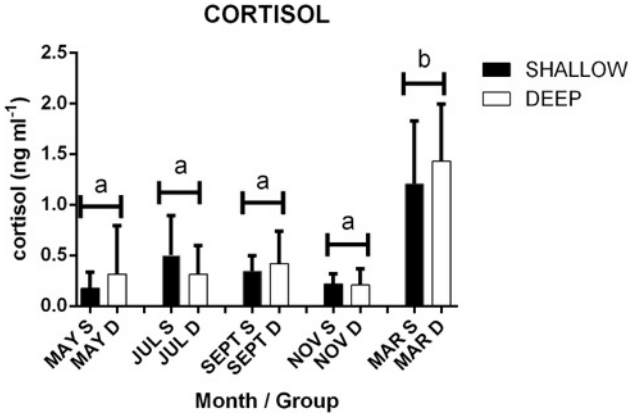


Figure 37. Plasma cortisol levels during the period from May 2014 to March 2015. Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), while asterisks differences between the different cages, $P < 0.05$.

During the 2nd Phase, plasma cortisol and lactate levels (**Fig.38**) presented statistically significant higher values in fish reared in the SHALLOW net than in the DEEP net at the end of the trial.

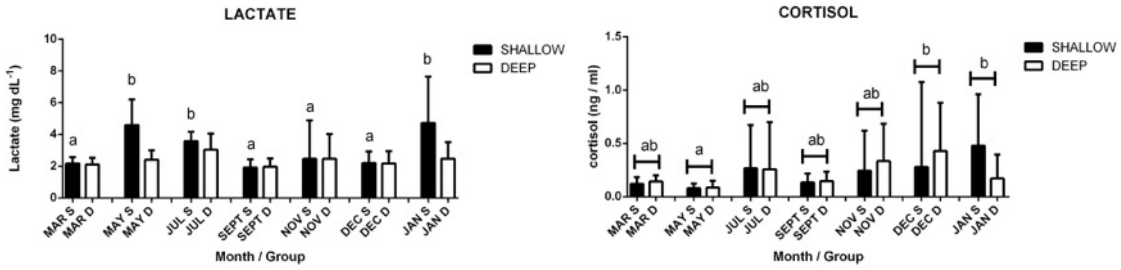


Figure 38. Lactate and plasma cortisol levels during the period from March 2015 to January 2016. Values are given as mean ± S.D. (n = 10 per group and sampling month). Letters indicate differences between the different samplings (months), $P < 0.05$.

From the immunological parameters measured, a first estimation of the innate immune status of meagre is available. Compared to other Mediterranean species, lysozyme activity (**Fig. 39)** was double in meagre than in European sea bass and 6 times stronger than in gilthead seabream but 5 times lower than in shi drum.

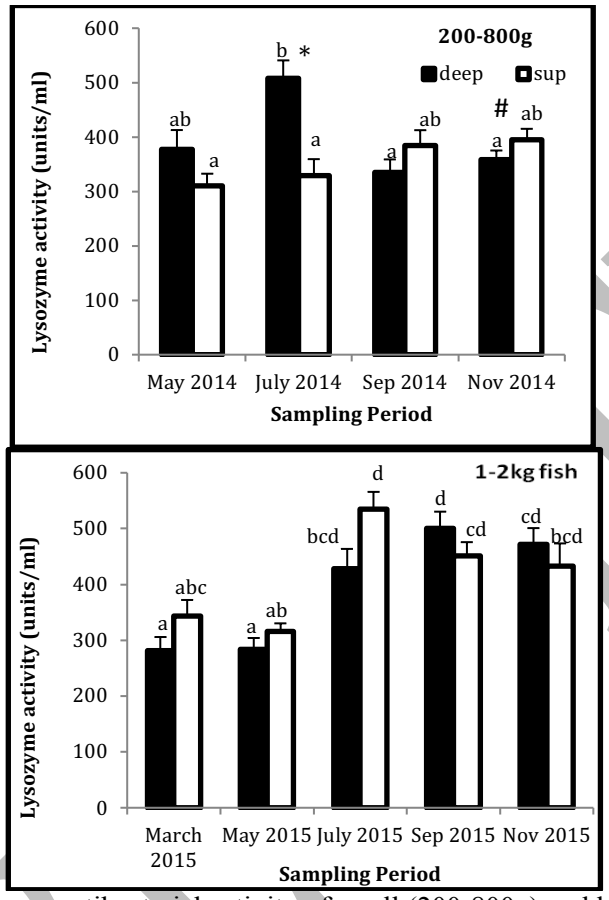


Figure 39. Serum lysozyme antibacterial activity of small (200-800g) and large (1-2kg) fish kept in cages at different depths (deep or superficial). Asterisks * show significant differences between different sampling times. Hashtag # shows a significant difference between the 2 depths. Different letters show significant differences between depths at different sampling times (One-Way ANOVA, $P<0.05$, Student-Newman-Keuls't-test). n=10.

Myeloperoxidase activity (**Fig. 40**) was also very strong in meagre.

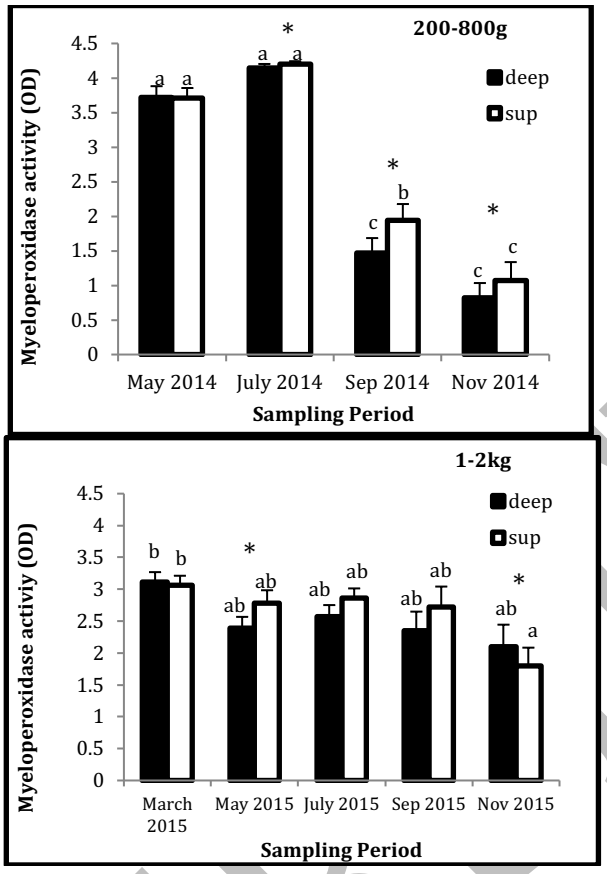


Figure 40. Serum myeloperoxidase activity of small (200-800g) and large (1-2kg) fish kept in cages at different depths (deep or shallow). Asterisks * show significant differences between different sampling times. Hashtag # shows a significant difference between the 2 depths. Different letters show significant differences between depths at different sampling times (One-Way ANOVA, $P<0.05$, Student-Newman-Keuls't-test). n=10.

Comparison between small and big fish showed that small fish have a strong arsenal against bacterial infections (both Gram positive and negative). The cage depth affected significantly the lysozyme and complement antibacterial activity (**Fig. 41**) of small fish in an opposite manner making impossible to give a recommendation about optimal cage depth. Cage depth does is not significantly involved in the health status of large fish. Seasonal variations (water temperature and photoperiod) had a stronger effect on the fish immune parameters tested than the depth of the cages

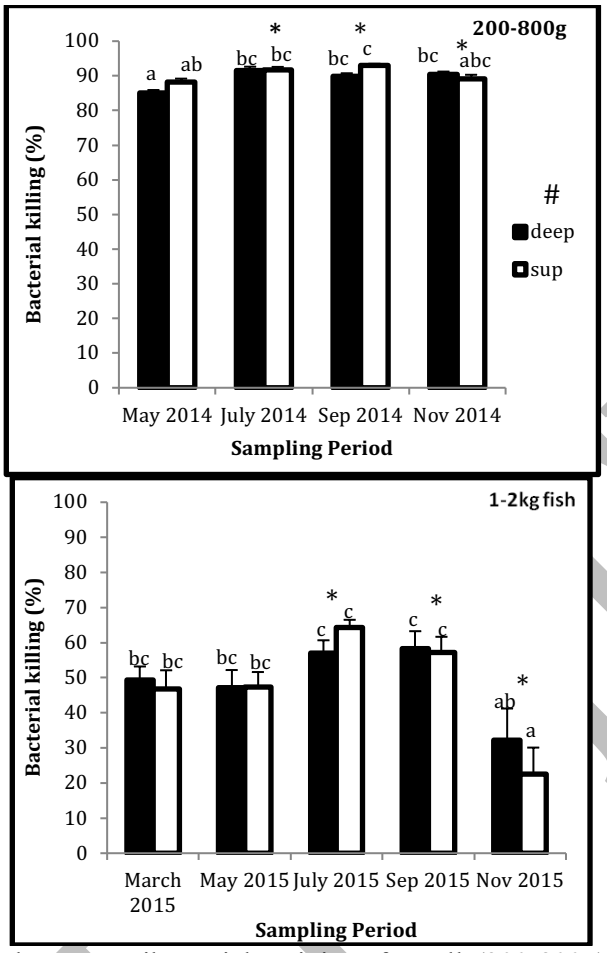


Figure 41. Serum complement antibacterial activity of small (200-800g) and large (1-2kg) fish kept in cages at different depths (deep or superficial). Asterisks * show significant differences between different sampling times. Hashtag # shows a significant difference between the 2 depths. Different letters show significant differences between depths at different sampling times (One-Way ANOVA, $P<0.05$, Student-Newman-Keuls't-test). n=10.

Regarding the behavioral monitoring, all data collected were analyzed and no significant alteration was observed either within or between the experimental groups. The expected behavior during the day light was apparent in all the cases with the majority of the individuals concentrated at the lower layers of the cage and with observed movements towards the surface when feeding occurred.
Hence, the vertical distribution of meagre was mostly in the lower half of the cage for a period of approximately 12 hours while the rest of the period the meagre were distributed almost homogeneous in the whole available volume of the cage (**Fig. 42**).

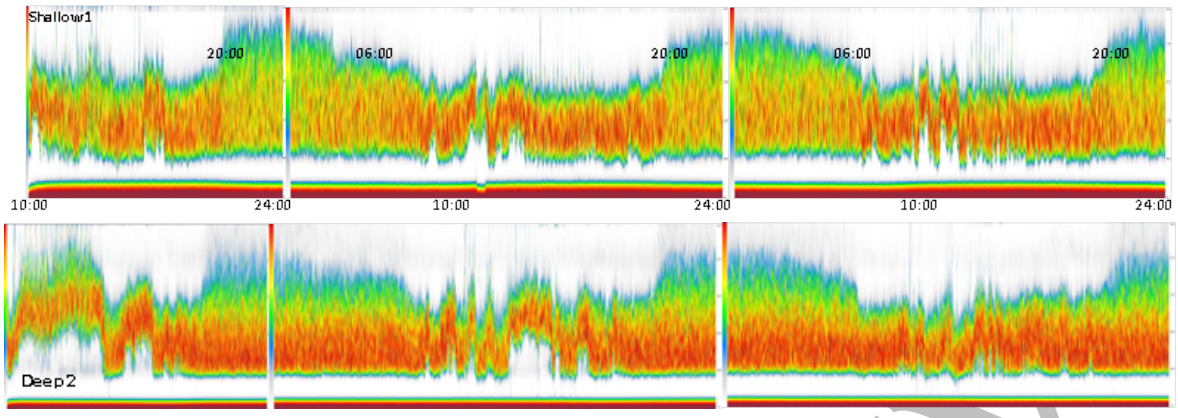


Figure 42. Vertical distribution of meagre in the experimental cages for a period of 3 days.

The only period that the fish appeared to be in stress was during the high temperature period of late August – September when the individuals with body weight ~1,5 Kg were sluggish and with limited appetite. This was not the case for the younger groups (weight of appx. 500g).
This behavior is in general different from what was observed in salmon or European seabass reared in cages. Meagre in general appears as a species with high tolerance to variable conditions and with a very conservative behavioral pattern. The observed nocturnal behavior that, to our knowledge, was observed for first time, may represent a potential alternative husbandry period for the species, i.e. a period that the species can be fed a hypothesis tested and the results are presented in the next section.

Effect of light intensity in the cage.

The growth performance presented no difference between the tested conditions (**Fig. 43**) During the first trial, growth rate was 1.2 g d^{-1} for the fish in the shaded cage while it was 1.3 for the second group. During the second trial, both groups performed significantly better than the first trial 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. Some additional indices for the biological performance of the groups are presented in **Table 25.** The size variation was higher in the first trial while there was an almost similar variability between the experimental conditions.

The results showed that in the first experiment, and only for the 1st phase of the rearing, the performance of the fish in deep cages was better, mostly based on the results of mortality and the Food Conversion Ratio (FCR), while for the other parameters tested, no statistically significant difference between the depths was observed, and only seasonal differences were detected. Specifically, both the FCR and the mortality rate had better values in cages with deep net. For the 2nd phase of the rearing no difference was observed between the tested conditions. The same holds also for the shading experiment.

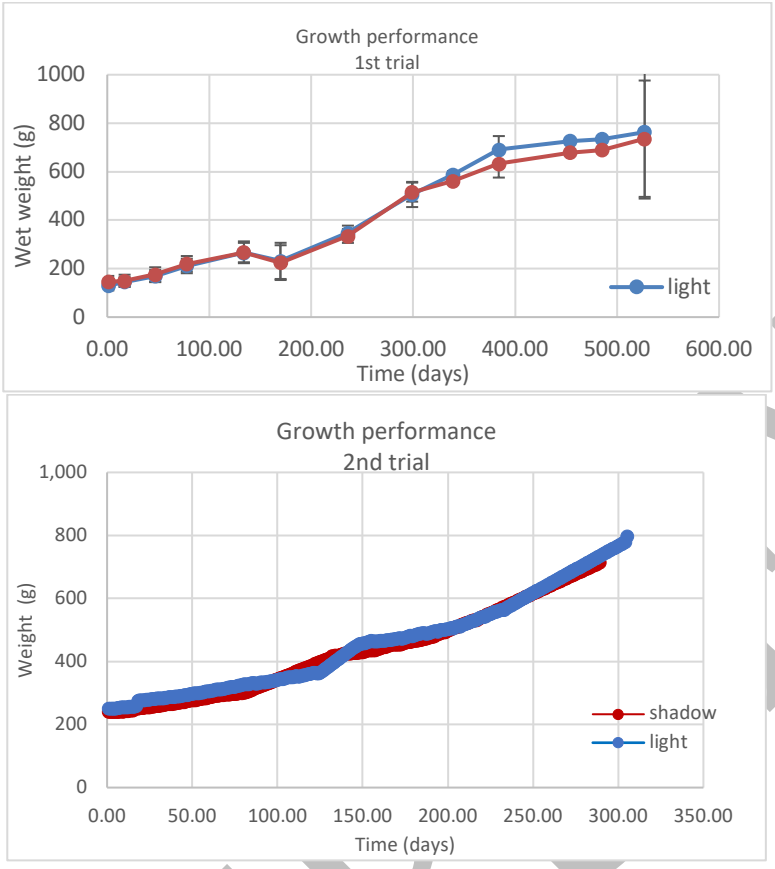


Figure 43. Growth performance of the experimental groups. Vertical bars show the standard deviation of the mean

Table 25. Performance parameters of the experimental groups

	Trial 1		**Trial 2**	
	Shadow	Light	Shadow	Light
FCR_{econ}	3.0	2.9	2.0	1.8
Survival (%)	91.4	92.7	98.3	93.3
Final Body Weight (g)	825.8±240.1	910.6±274.0	714.3±169.8	796.5±181.9
Variation Coef. (%)	29.1	30.1	23.8	22.8

Development of feeding methodology

(led by HCMR, Nikos Papandroulakis)

For the development of an appropriate feeding methodology the following steps were implemented. The **first step** was to test **in tanks** whether meagre responds to **different feeding stimuli** (mechanical, optical, etc). For this, groups of two different individual sizes (50-100 and 700-900 g) at different tank sizes (500 and 5000 l respectively) were used for testing mechanical and optical feeding stimuli for a period of 4 months (each group). Monitoring with video recordings allowed the definition of the optimal feeding stimuli.

The **second step** was to test **in cages different feeding periods** related to the presence of light (day-night) and also **different feed distribution methods**. For this an experiment was implemented to test the performance of meagre in cages when fed exclusively either during the day or during the night. In a second experiment the feed distribution from the surface and from the bottom were compared. The tests were performed during two duplicated trials in the HCMR pilot farm in (6x6x8 m3) cages. One size group was used for a period of 8 months for each trial. Growth performance was estimated with monthly samples while every second month hematological, biochemical, immunological and hormonal evaluation was performed. Also, the vertical distribution in cages will be monitored using an echo integrator.

The **third step** was the comparison of **automatic and demand type feeding in tanks** for a production cycle. Comparison in each season of the year of (a) demand feeding and (b) feeding with automatic feeders programmed to follow the feeding routines that are used customarily in meagre cage farms (feeding in farms is now based mainly of automated feeding). Three replicate control tanks (automated feeding) will be compared to three experimental demand-feeding tanks. Video cameras and sensors will be installed to register the activity of the fish and behaviors related to feeding and aggression. Experimental conditions will be natural photoperiod and simulated natural temperature controlled to be similar to sea cage growing areas for the specific season. The parameters to be evaluated were feeding time, feed delivered, growth, size variation in the population, FCR, pattern of fish activity, level of aggressive behaviors and fin condition.

All these steps provided sufficient information to propose a Methodology for meagre feeding.

The effect of various stimuli on feeding behavior

(led by HCMR, Ioannis Papadakis)

Three consecutive experiments took place at the facilities of HCMR, in Crete, Greece. The first two were performed indoor with individuals ranging between 50-100 g in body weight while the third was performed outdoors with individuals ranging between 600 to 900 g.

For the small fish, four experimental groups were used related to the stimulus used. For the first group, called ‘’Light’’, the stimulus was a fading light, coming from waterproof LED lamps that were placed in the water column 10 cm below the water surface under the feeder used. For the second group, called “Air”, the stimulus was air bubbles released from a 5-mm plastic tube that was placed at the same position as before under the feeder. For the third group, called “Air and Light”, a combination of the previous stimuli was used, ‘but the “Light” stimuli were performed during morning and afternoon while the “Air” stimuli during noon. The fourth experimental group was the control group with no stimuli before feeding. In order to monitor the behavior of the fish a recording camera was placed above each tank (**Fig. 44**).

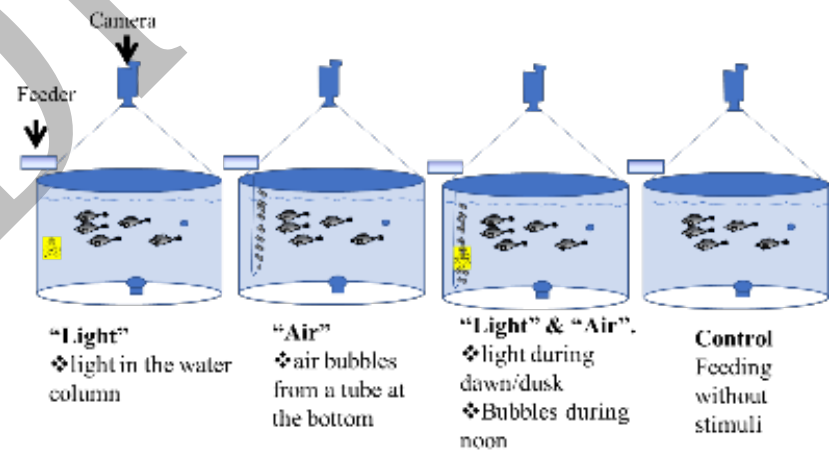


Figure 44. A schematic representation of the experimental tanks with the different types of stimuli.

Based on the results of the experiments with the small fish, three experimental groups of large fish (600-900 g) were created. For the first, called ''Light'', the stimulus was a fading light, from a waterproof ribbon of LEDs, placed in the water column 10 cm below the water surface under the feeder used. The maximum light intensity of the lamps, as it was measured from a distance of 40 cm, ranged between 350-400 lx. The intensity of the natural light at noon with sunshine had a value from 24,000 lx to 34,000 lx. For the second group, called "Air", the stimulus was air bubbles released from a plastic 5 mm tube placed at the same position in the water column as the light stimulus. The third experimental group was the control group with no stimuli before feeding.

From the total 60 experimental days, 14 days were selected for the analysis, one every 4 days. Following similar procedures as presented for the previous experiments, from each 5-min video, 70 pictures were extracted and analyzed and the coordinates of each fish (the head) in the tank was determined. In particular, each tank was divided into six equal square areas (**Fig. 45**).

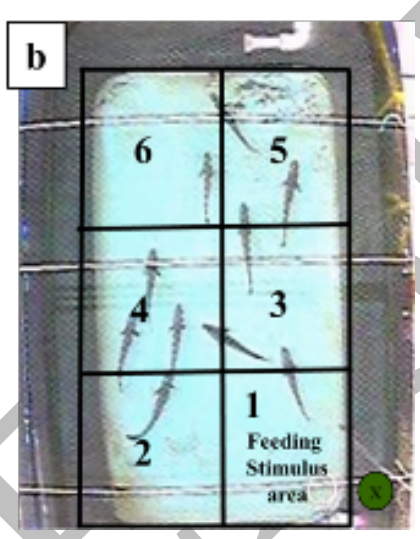


Figure 45. Representation of the different areas that tanks were divided. Area 1 included both the stimulus and feeding area, whilst the green circle represents the position of the feeder**.**

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 considered 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.

The effect of feed distribution methods

(led by HCMR, Nikos Papandroulakis)

The objective of the trial was to test two feeding distribution methods that was from the surface of the cage and using a submerged distribution device. The trial was performed at the pilot scale

installations of HCMR in duplicates using cages of 290 (6x6x8) m^3 indicated as Normal and Submerged. The initial wet body weight at the beginning of the trial was 290 ±20 g. The duration of the trial was planned to be 8 months. It started in May 2017 and was completed on December 2017. Feeding was similar in all cases (proportional to biomass of each group) but the distribution was different according to the condition tested. The standard feeding with feeders located on the surface of the cage was compared with feed distributed submergible. The submerged feeding was performed by transferring feed together with sea water through a flexible tube from the surface to a predefined depth approximately the medium depth of the cage (i.e. 4m). An electric pump located on the platform pumped water into the cage while an electric dosing mechanism delivered the required feed quantity (**Fig. 46**). During the experimental period, growth performance was estimated with monthly samples. Every second month blood samples were taken for haematological (hematocrite, hemoglobin), biochemical (glucose, lactate), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. The vertical distribution of the populations in cages has been monitored using an echo integrator (CageEye 1.3, Lindem Data Acquisition AS, Norway).

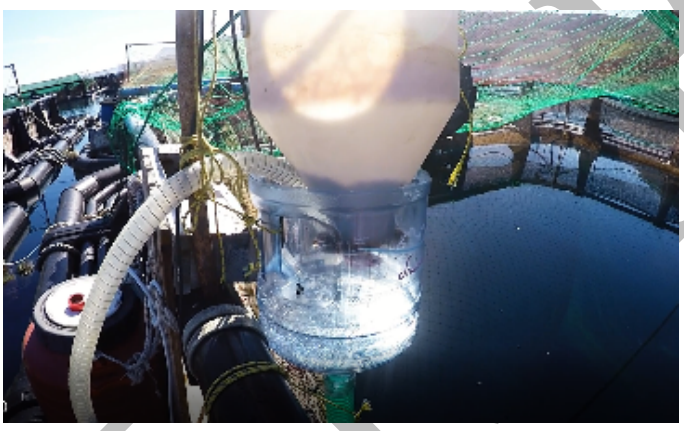


Figure 46. Feeder installed during the trial for the submerged feeding

The feeding pipe was installed at the center of the cage. A rotating S-form ending ensured an even distribution of the pellets (**Fig. 47**).

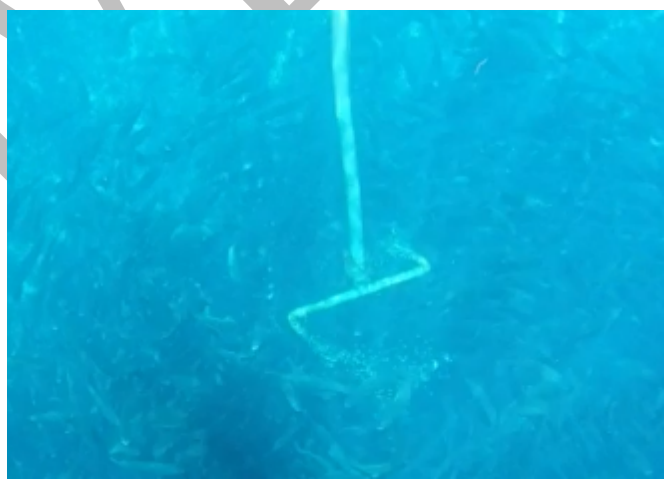


Figure 47. The S-form ending of the submerged feeding system

During the experimental period, rearing was implemented without any particular problem such as pathologies that could have resulted in significant changes in the experimental conditions. The growth rate was ~3.8 g d^{-1} for all conditions tested with no significant difference observed between

or within the tested conditions (**Fig. 48)**. The final weight presented a coefficient of variation for all groups between 20.6 and 25.7% without differences between the tested conditions. The size distribution at the end of the trial was normal for all groups, although a tendency towards bigger sizes was observed for the submerged fed groups.

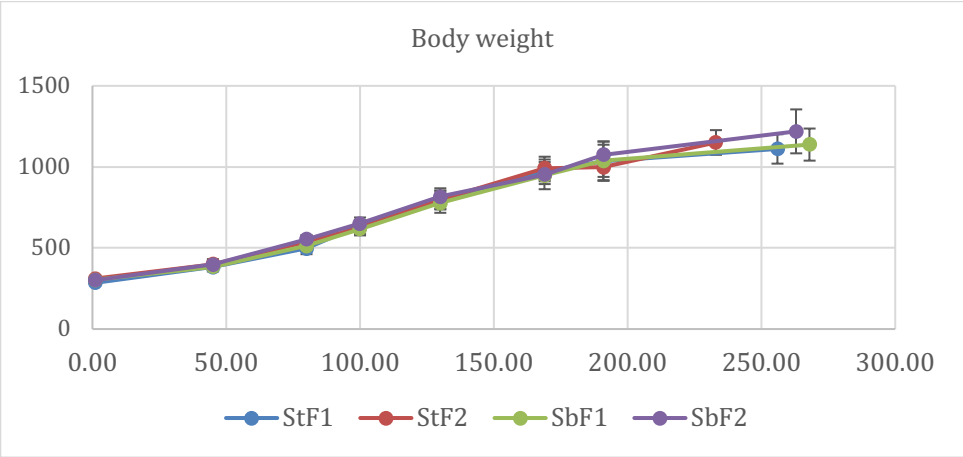
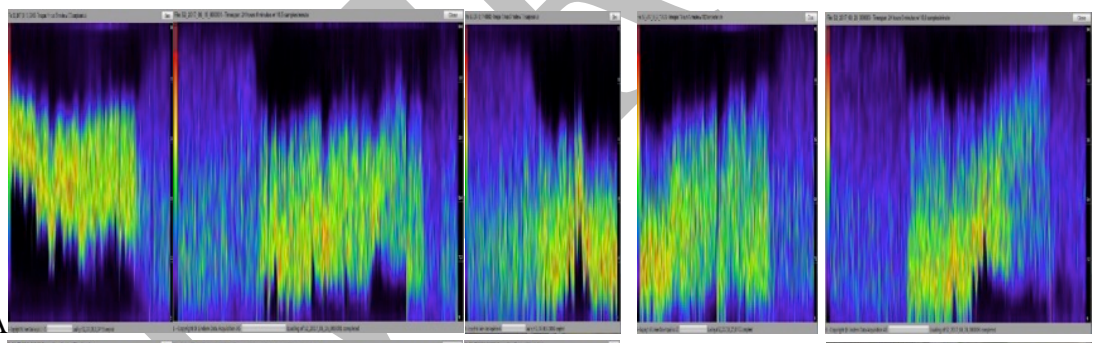


Figure 48. Growth performance, mean weight, of meagre. Error bars are the standard deviation (n=10).

Regarding the behaviour of the fish in the different groups, in terms of their vertical distribution in the cage the pattern was similar to the one observed during previous experiments in Souda cage farm (**Fig. 49**). The fish were mostly located in the bottom of the cage during the daylight hours of the day and spread in the whole available volume during dark.

A



B

Figure 49. Echograms with the vertical distribution of the reared groups in (a) Standard feeding and (b) Submerged feeding. Horizontal Axis is the time while Vertical is the cage depth (from bottom to top). Different colors (from violet to red) represent different densities.

Comparison of automatic and demand type feeding in tanks

(led by IRTA, Neil Duncan and Alicia Estevez)

The experiment used 1200 trained fish randomly distributed into six 1500 L tanks, 200 fish per tank. The tanks were connected in pairs to a recirculation system (IRTAmar®) (**Fig. 50)**. As the fish grew, biomass was removed from the tanks by reducing the number of fish per tank. All tanks were fitted with a net to avoid fish jumping from the tank. The photoperiod was natural and the temperature regimen simulated the temperature of the seawater on the Mediterranean coast of Spain where meagre are grown out in sea cages. The photoperiod was maintained with a clock that switched the lights on and off in relation to the natural photoperiod for the region (latitude: 40.628º longitude: 0.665º). Fluorescent daytime lights that provided 120 ± 12 lux at the water surface provided daytime illumination. A low light illumination from a white LED covered with a perforated aluminium foil was used at night to simulate moon or star light illumination. The illumination was enough to visually see the outline of the shapes of the fish, but did not register intensity on the lux meter (0 lux).

Figure 50. Experimental set up with six tanks connected in pairs to RAS, IRTAmar. Each pair of tanks was a programmed feeding regimen (Prog) and a auto-demand feeding system (AD).

Growth between the two treatments was similar (**Fig. 51**). Fish in the two feeding systems, programmed and auto-demand, grow significantly ($P<0.05$) respectively from 58.0 ± 0.9 g and 58.5 ± 1.0 g to 340.5 ± 9.5 g and 322.1 ± 7.6 g. There were no significant differences in weigh of the groups from the two treatments during 10 months. However, on two months the programmed feeding groups were significantly ($P<0.05$) larger than the auto-demand groups. In March 2017, the mean weight of the fish with the programmed feeding system was 188.2 ± 3.5 g compared to 171.4 ± 3.4 g with the auto-demand feeding system and in May the programmed feeding system was 268.1 ± 5.4 g compared to 251.2 ± 5.9 g with the auto-demand feeding system. The different replicas of the same treatments were held in different recirculation systems and there was no significant interaction between the effect of the treatment and recirculation system on growth. However, the recirculation system did have a significant effect on growth and in the months February, March, May and June the fish in recirculation system 2 were significantly ($P<0.05$) larger than the other recirculation systems. No differences amongst recirculation systems were observed in any other month.

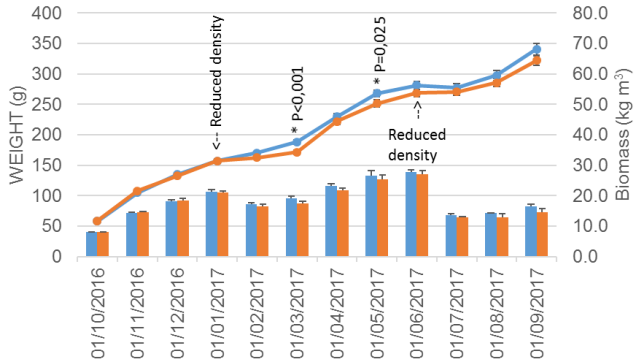


Figure 51. Growth in mean wet weight (lines left axis) and tank biomass (columns right axis) of meagre (*Argyrosomus regius*) fed with two feeding systems, programmed (blue) and auto-demand (brown) (n=180, 60 x 3 replica tanks). Arrows indicate when density number of fish was reduced. An asterisk indicates significant differences between feeding treatments with the actual P value.

The growth of the fish in the two feeding systems exhibited a similar pattern over the year. There were no significant differences in specific growth rate (SGR) between fish fed with the two feeding systems or between fish held in different recirculation systems. The highest growth and SGR was observed at the beginning of the experiment. As the temperatures decreased the SGR decreased to a low during February and March. The SGR increased during the spring with rising temperature before decreasing in May, June and July due to high biomass, disturbance of reducing numbers and / or high summer temperatures. The SGR increased towards the end of the trial in August and September.

There were no differences in FCR between fish feed with the two feeding systems, programmed and auto-demand. There was no difference over the entire experiment and the fish with programmed feeding had a mean FCR of 1.50 ± 0.02 and fish with auto-demand feeding had 1.42 ± 0.01. There were also no differences within each month and fish feed with the two feeding systems exhibited similar FCRs ranging from less than 1 during October and March to a high of more than 3 in August (**Fig. 52)**. The FCR above 1.5 coincided with the extremes in temperature (15ºC and 25ºC) and/or the highest stocking densities >20 kg/m^3 (**Fig. 52**). The reductions in numbers to reduce stocking density also appeared to impact negatively on feeding and growth particularly in June.

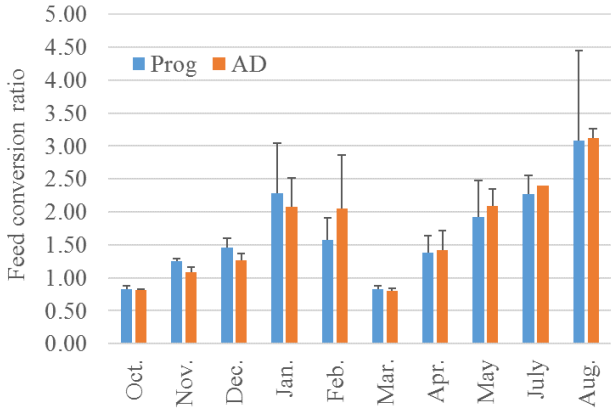


Figure 52. Mean feed conversion ratio (FCR) of meagre fed with two feeding systems, programmed (blue) and auto-demand (brown) (n=3 replica tanks). There were no significant differences between feeding system (P=0.252).

The cumulative number of demands over the entire experiment was significantly higher during the night compared to the day (**Fig. 53**). During the night (19:00 to 06:00) there was a mean of 720 ± 22 demands / hour compared to during the day (07:00 to 18:00) with a mean of 439 ± 22 demands / hour. The pattern of feeding during the night appeared to change from the first part of the experiment to the second part of the experiment. During the period Oct. 2016 to April 2017, more feed was demand in the first part of the night from 19:00 to 22:00 (**Fig. 47 - middle**), whilst during the period from April 2017 to Sept. 2017 the higher demand was the entire night period 20:00 to 08:00 (**Fig. 53 - right**).

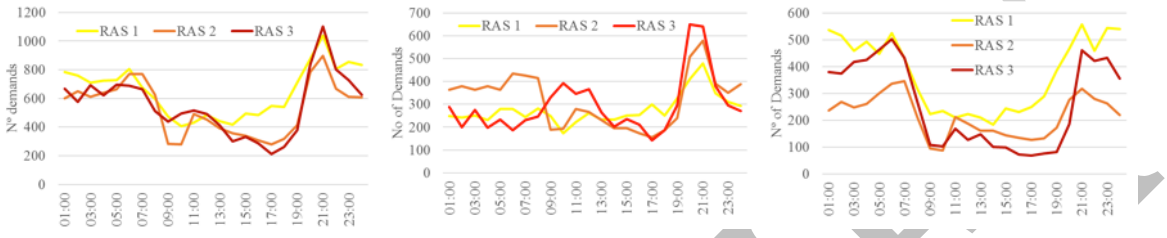


Figure 53. Cumulative number of demands during each hour of meagre (*Argyrosomus regius*) in three replicas in different recirculation systems, RAS 1 (yellow), RAS 2 (orange) and RAS 3 (red). The three figures represent demands during the entire experiment (Oct. 2016 to Sept. 2017) (left), during the period Oct. 2016 to April 2017 (middle) and the period April 2017 to Sept. 2017 (right).

Meagre exhibited a social non-aggressive behaviour towards conspecifics and an opportunistic feeding behaviour over the entire 24-hour day. The fish clearly presented nocturnal behaviour, both feeding and using the entire tank from the bottom to the surface during the night, compared to the day, when the fish remained low in the water column. However, the behaviour was not exclusively nocturnal and the fish were active and fed during the day. The feeding system, programmed or auto-demand did not have a significant effect on growth, size variation or feed conversion ratio (FCR). The aim of the trial was to determine feeding patterns that may improve growth, FCR and reduce size variation. Other species feed with auto-demand systems have exhibited improved growth, improved FCR, less size variation and less aggressive behaviours compared to systems with specific feeding times. However, meagre did not present a clearly defined feeding pattern. Over the entire year the cumulative amount of feed eaten each hour did show a pattern with elevated feeding during the night compared to the day. A mean of 720 ± 22 demands / hour were made during the night (19:00 to 06:00) compared to 439 ± 22 demands / hour during the day (07:00 to 18:00), which indicated that over the entire year 62% of the feed was demand during the night and 38% during the day. The observed feeding over the entire 24 hours of the day compared to programmed feeds at 09:00 and 17:00, did not have an effect on the growth, size variation or FCR.

There were no differences in growth rate (SGR), size distribution or FCR within any month during the experiment. However, there was considerable variation in both SGR and FCR between months, indicating that factors other than the feeding system influenced the SGR and FCR in both feeding systems. The most obvious factor was the temperature and the winter low of 15ºC clearly supressed appetite and growth. Optimal growing conditions were in October and March, with an SGR above 1.5 g/day in October and 0.75 g/day in March and FCR below 1 in both months. October had temperatures of 23ºC and stocking densities of less than 10 kg/m^3 and March had temperatures of 16ºC and stocking densities of 20 kg/m^3. However, it would appear that during the summer months growth and FCR were negatively influenced. Amongst possible negative factors were the high temperatures (25ºC from July to Sept.), stocking densities (>25 kg/m^3 during May), size of fish in relation to the size of the tanks and the reduction of numbers (January and June). It appeared that stocking density and the reduction in number impacted negatively during May and June and fish

size or temperatures impacted negatively in July and August. Although at the end of the experiment mean weights were 322 - 340 g the highly positive skewed populations contained fish as large as 700 – 800 g. This size variation is similar to the situation in cage culture and Deliverable 20.1 demonstrated that faster growing juvenile fish obtain and maintain a growth advantage. The size variation was not related to feeding system.

Generally, the behaviour of the meagre was not affected by the feeding system. Meagre were non-aggressive towards conspecifics, observations of the general behaviour and in videos did not identify aggressive behaviours such as chasing or biting and there were no differences in fin condition between the fish in the two feeding systems. The pectoral, dorsal and tail fins showed light fin damage that was normal for the holding condition. There was no difference in the upper and lower sensor in the tanks with different feeding systems. All fish were observed to stay low in the tank during the day and rise to fill the whole tank during the night. The only difference observed in behaviour between the feeding systems was in the swimming velocity. The fish in the auto-demand feeding system had lower swimming velocity than fish in the programmed feeding system. This appeared to be related to feeding and availability of feed. The fish in the auto-demand feeding system had feed availability at all times and did not increase swimming speed in response to hunger and/or feed delivery. However, the fish in the programmed feeding system had higher swimming speeds in response to hunger and periodic feed delivery. This was also highlighted, as the fish in the programmed feeding system had a changing swimming velocity in response to hunger and food delivery during the day. The swimming velocity of fish in the programmed feeding system was highest in the morning (10:30) immediately after feeding (09:00-10:00) and decreased to the lowest level at 18:30 immediately after the afternoon feed (17:00-18:00) that completed the ration for the day to meet the daily feed requirement of the fish.

5. Fish health

Systemic Granulomatosis

(Led by HCMR, Pantelis Katharios)

Systemic Granulomatosis (SG) is a pathological condition affecting the majority of farmed populations of meagre. SG is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic (Ghittino et al., 2004; P Katharios et al., 2011). SG is not associated with high mortalities; however, it may lead to reduced growth and physiological performance during grow-out and, in addition, it affects the final product, making it unacceptable to the consumer. The aetiology of the disease is unknown; however, two hypotheses have been raised. The first is that it is caused by bacterial pathogens most likely *Nocardia* spp. (Elkesh et al., 2013) and the second that it may be a metabolic disorder (Ghittino et al., 2004; P Katharios et al., 2011) similar to systemic granulomas observed in other cultured fish species.

Externally, heavily affected fish can be emaciated, with fin erosion, exophthalmia and in several cases unilateral blindness. Visible granulomas of various sizes are usually scattered in the internal organs. Liver, kidney and spleen are the organs affected more by SG. In heavily affected fish with many visible granulomas, large part of the liver and the kidney can be necrotic and calcified, while the heart can be completely covered by white to cream colored nodules (**Fig. 54, 55**). At stereoscopic level, fresh squash preparations of affected tissues reveal the presence of granulomas encapsulated by several layers of fibrous tissue with an "onion-like" appearance (**Fig. 56**). This characteristic "onion-like" appearance was also evident in photos from scanning electron microscopy (**Fig. 57**).

Figure 54. A,B. External lesions on the eye and the tail fin of meagre related to SG. C,D. Dystrophic calcification of the liver and the kidney.

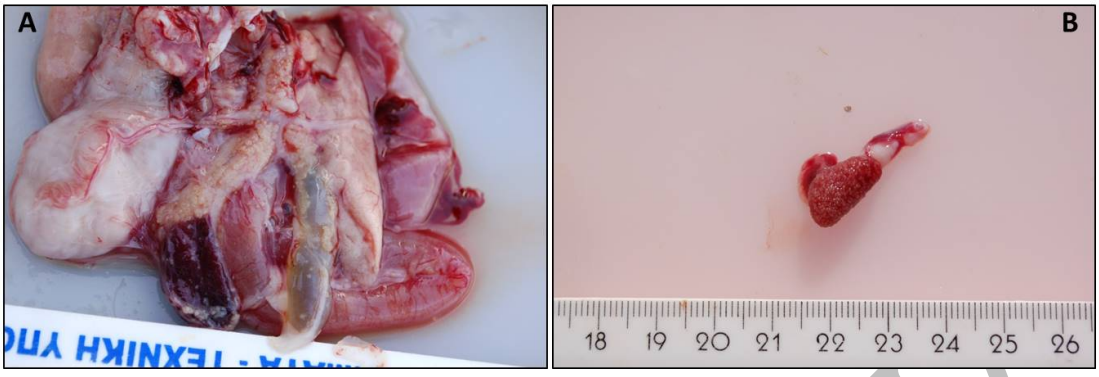


Figure 55. A. Multiple granulomas in the soft tissues of meagre. B. Heart of meagre fully covered by granulomas.

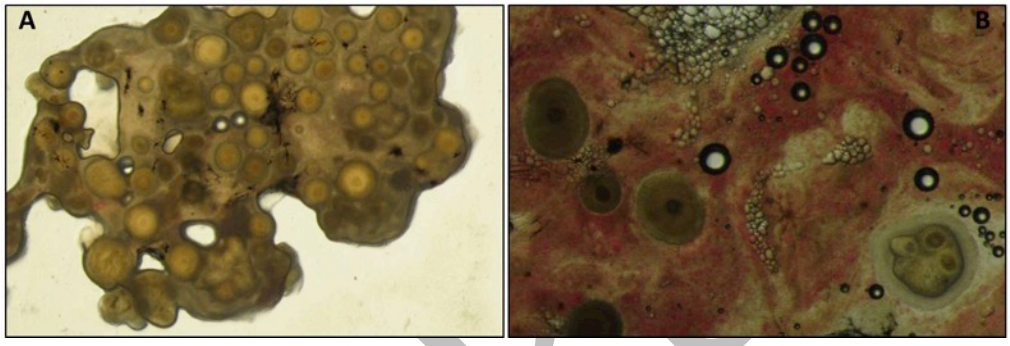


Figure 56. Fresh squash preparation from liver (A) and kidney (B) with granulomas (A:stereoscope x1, B:stereoscope x2.5).

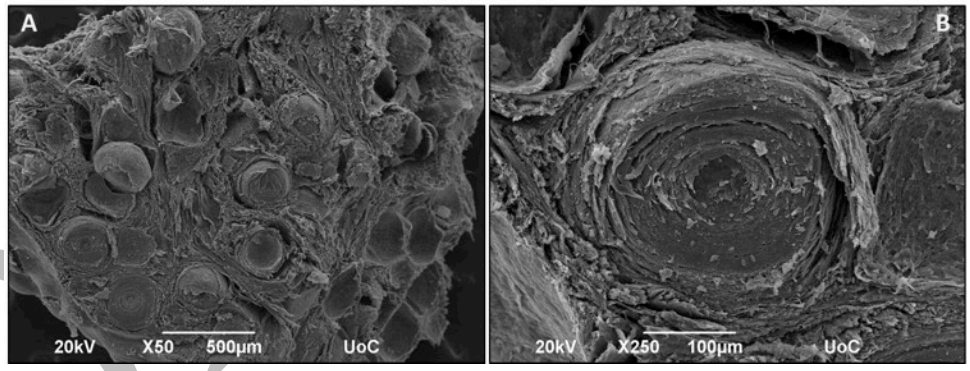


Figure 57. A. SEM microphotograph of the heart covered by granulomas. B. SEM microphotograph of a heart granuloma with "onion-like" appearance.

Histologically, the morphology of the granulomas consists of a central necrotic amorphous area surrounded by a multilamellar layer of epithelioid cells and fibrous tissue. Several stages of the granuloma formation with the characteristic epithelioid cells can be identified in the examined tissues ranging from immature granulomas, multilayer mature granulomas to big nodules, possibly a result of the merging of several adjacent granulomas that had big areas of central necrosis with dystrophic calcification (**Fig. 58, 59**).

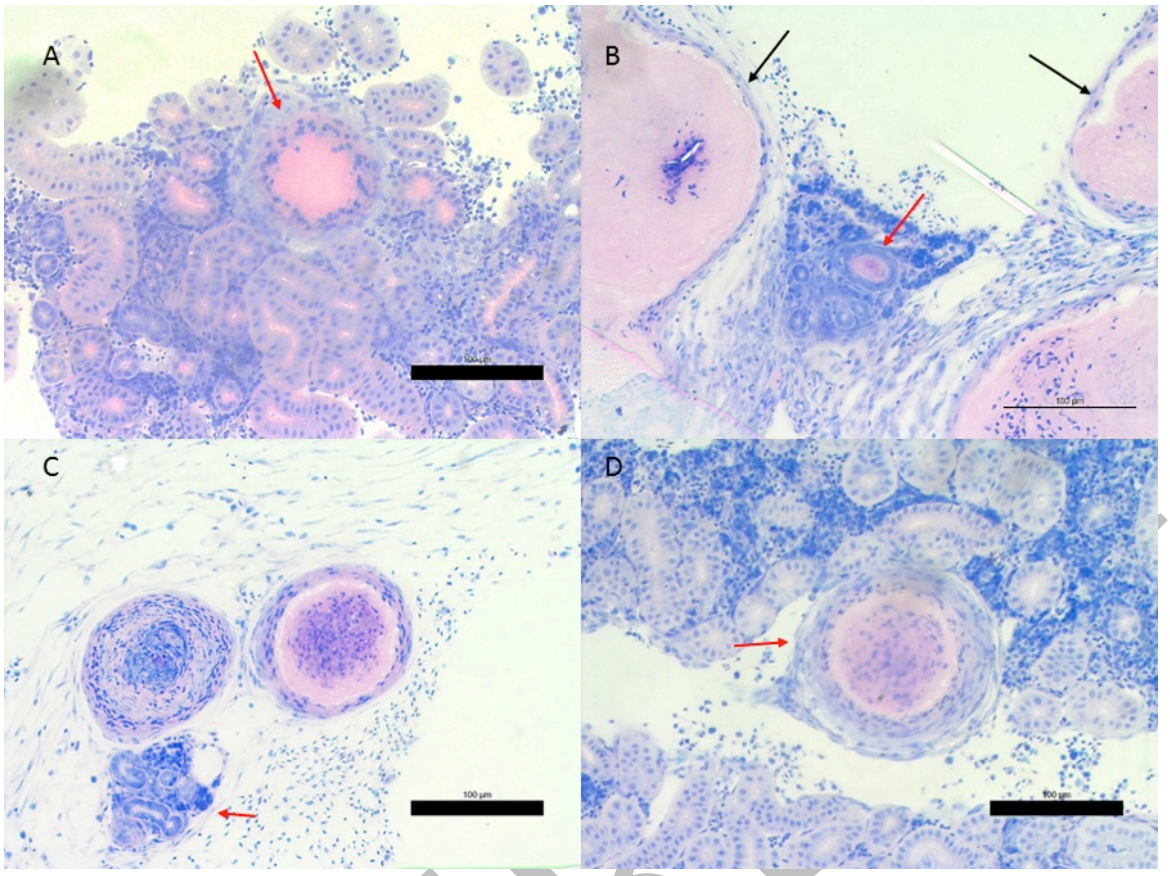


Figure 58. A. Immature granuloma in the kidney of meagre. There is an amorphous, acellular area, which is surrounded by inflammatory cells. B. An immature granuloma in the kidney (red arrow) in a small area of a kidney with normal appearance. In this particular fish there was extensive caseous necrosis in this organ, a small part of which is indicated with black arrows. C. Two adjacent granulomas sectioned at different levels over a small part of renal tissue (red arrow). D. Typical appearance of a "young" granuloma in kidney.

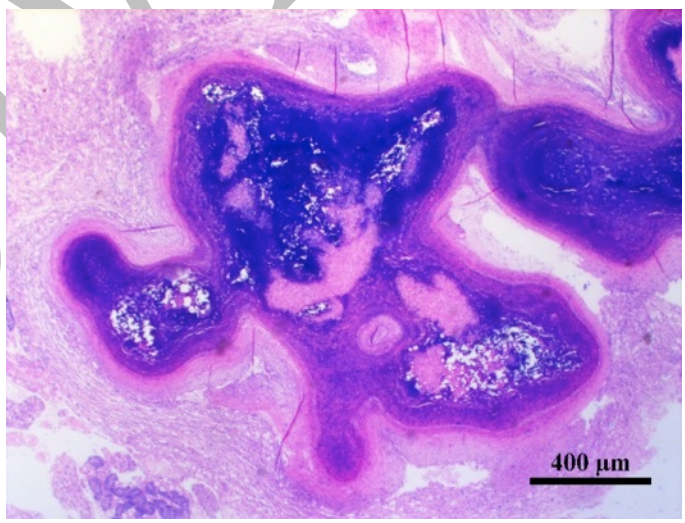


Figure 59. Dystrophic calcification in the kidney of meagre.

In some cases, mainly in livers, the initial stages of the granulomas are located at the blood vesicles resembling vasculitis (**Fig. 60**). In these cases, there is also an involvement of rodlet cells. Rodlet cells are present in large numbers in all tissues. Rodlets are aligned like epithelial cells in the peritoneal membranes but they are also found in livers, pancreas and intestine (**Fig. 61**). The distinctive characteristics of these pear-shaped cells are the collection of the rodlets (linear crystal structures) within their cytoplasm and the thick surrounding membrane.

Under specific conditions rodlet cells expel their rodlets into the extracellular environment. The composition of the rodlets is not known, however it has been shown that they contain the antimicrobial peptide piscidin. Thus, their secretory nature might be connected to the defense mechanism of meagre against infection. However, this cannot be fully supported since there is no data on the presence of these cells in normal or wild specimens.

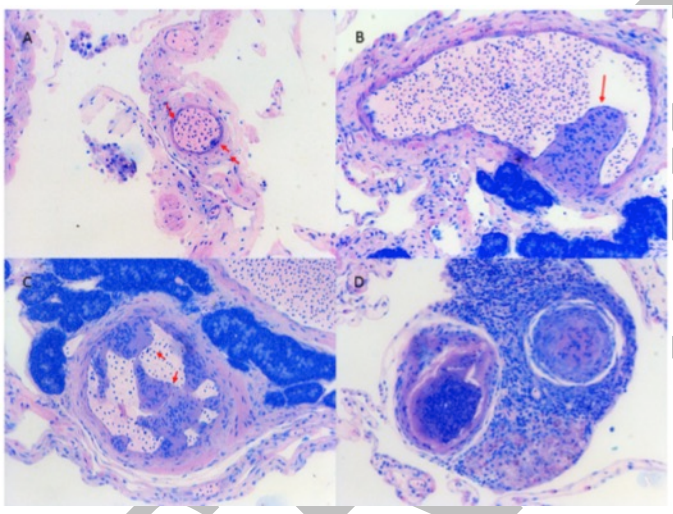


Figure 60. Blood vessel implication is evident in the manifestation of the disease. Various sections of blood vessels from the peritoneal membranes and the liver of affected fish are shown. There are specific growths composed of inflammatory cells at the endothelium of the vessels, which are indicated with red arrows. In more progressed stages (C, D) these growths seem to block the lumen of the vessel.

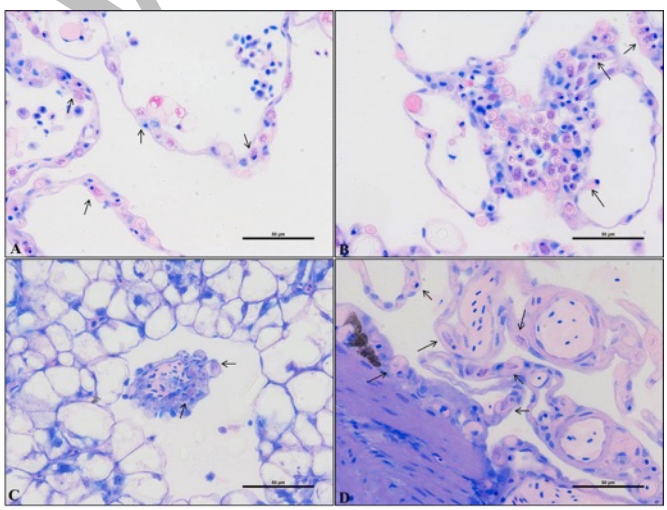


Figure 61. Rodlet cells (black arrows) aligned in the peritoneal membranes (A, B) and surrounding blood vessel walls (C,D).

Since the infectious agent hypothesis was also investigated in the project, we screened a large number of fish for the isolation and characterization of *Nocardia* spp. which has been proposed as the aetiological factor of SG. In order to demonstrate the presence of infectious agents, we have applied special stains in many different meagre samples with granulomas. The results of these specific stains (Ziel-Neelsen, FiteFaraco and Gram stain) were negative. Following extensive sampling we have identified only one case of nocardiosis in meagre, originating from the same geographical are where it was first reported. Histological analysis of the Nocardia-positive fish revealed the presence of filamentous, beaded and branching bacteria, morphology consistent with the description of *Nocardia* spp. in meagre (Elkesh et al., 2013). Ziehl-Neelsen stain was weakly positive in the colonies located in the skin lesions. The bacterial colonies were not demarcated by a granulomatous formation (**Fig. 62 A, B, C**). Typical granulomas were also present in all tissues examined. In these granulomas, no bacteria could be seen (**Fig. 62D**).

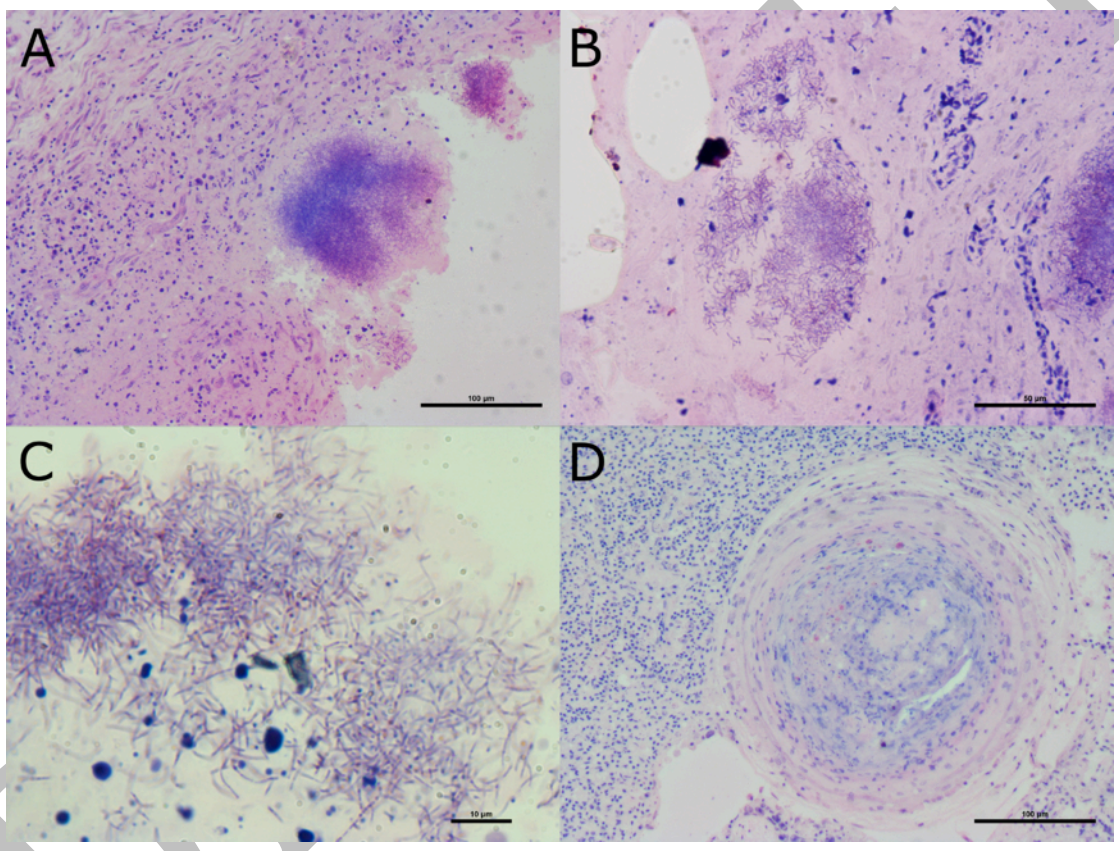


Figure 62. A, B. Histological section of a dermal lesion of a Nocardia-positive meagre (methylene blue/azure II/basic fuchsin stain). There are several bacterial colonies, which have elicited a moderate host response. C. Higher magnification of the bacterial colonies from dermal lesions. Note the filamentous branching morphology of the bacteria which are consistent with the descriptions of Nocardia spp. in other fish species. Ziehl Neelsen stain. D. A non-bacterial granuloma in the spleen with the typical morphology observed in SG (methylene blue/azure II/basic fuchsin stain).

Biochemical analysis

The analyses of serum liver enzymes such as ALT, AST and ALP have been proposed to be the main biomarkers for liver diseases. In general, the elevation of ALT and AST concentrations may indicate hepato-cellular diseases, while the elevation in ALP may indicate cholestatic diseases of

the liver. Analysis of these serum enzymes showed that, regardless of the diets, ALP, ALT and AST activity increased in fish with granulomas or tissue calcification compared with fish without (**Fig.63**). Increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation, while elevated plasma ALP activity corresponds to an inflammatory reaction of the bile ducts. In damaged tissues, cell membranes become more permeable, releasing some enzymes into the blood and thus modifying normal plasma values. In fish, elevated plasma ALP and AST have been associated with liver or bone disorders, so those results may be associated with SG, but further investigation needs to be done.

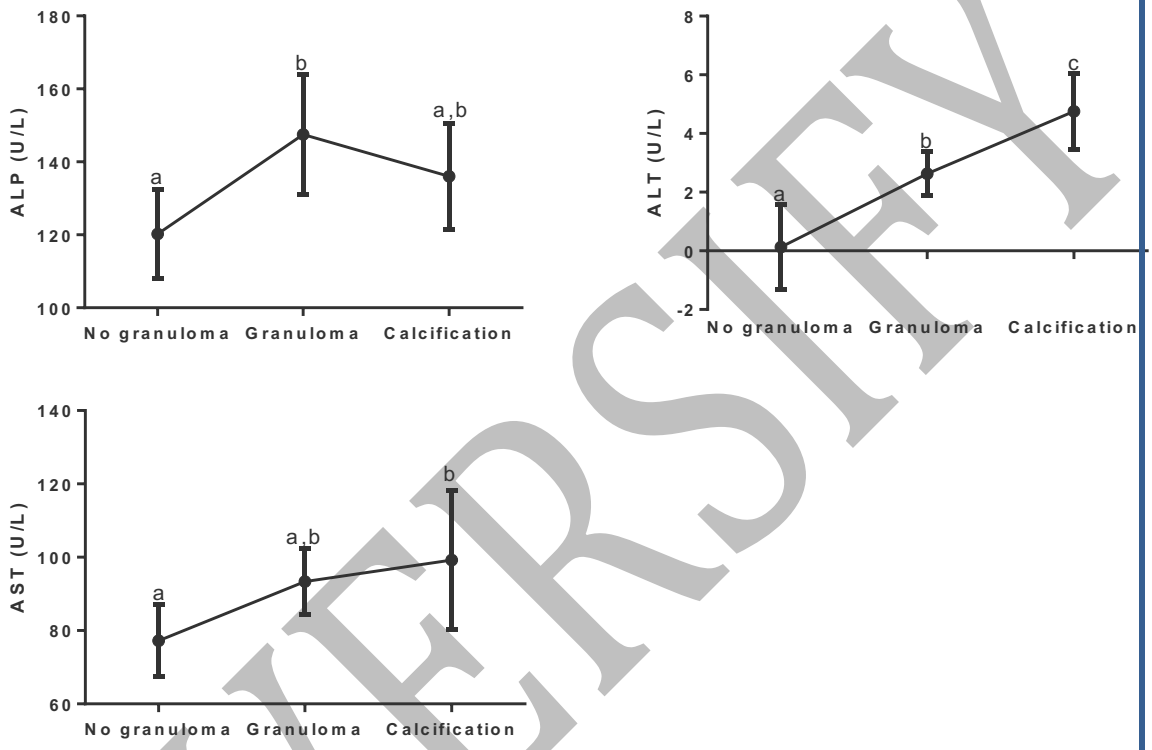


Figure 63. Mean concentrations (±SD) of ALP, ALT and AST in meagre with no granulomas, granulomas and calcification of even one tissue at the end of Vitamin D3 experiment (Task 24.1). Different letters (a, b) show statistically significant differences between the three conditions ($p<0.005$).

Several nutritional trials were performed in the DIVERSIFY project in order to investigate the metabolic disorder hypothesis. The metabolic disorder hypothesis (Ghittino et al., 2004; P Katharios et al., 2011) have been raised due to the similar systemic granulomas observed in other cultured fish species such as the gilthead sea bream (*Sparus aurata*), the rainbow trout (*Salmo gairdneri*) and the turbot (*Scophthalmus maximus*). In all cases, the development of the disease has been associated with nutritional imbalance in minerals and vitamins or inadequacy due to the use of plant protein sources or long-term stored formulated feeds or frozen fish.

We ran several feeding trials to assess the effect of vitamin D and Ca/P levels in feeds (HCMR), the effect of plant ingredients in the feeds (HCMR), as well as the effect of minerals and vitamins levels (FCPCT)

Trial 1. The effect of vitamin D_3 inclusions in diets in the development of SG in meagre (HCMR)

For vitamin D_3 trial four experimental diets with increasing levels of vitamin D_3 were prepared at HCMR (Athens, Greece). Meagre juveniles of 4 g average weight (n=600) were used for the feeding trial. Three replicates were used for each diet. The feeding trial lasted 93 days. At the end of the feeding trial samples were taken for granuloma evaluation and histology, estimation of specific biomarkers (CYP27, CYP24 enzymes) and antioxidant enzymes activity and plasma analysis.

To assess fish status regarding the presence of granulomas, a semi-quantitative method was developed based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. Each fish was dissected and internal organs were examined macroscopically. Fresh squash preparations of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney were assessed under a stereoscope. For the general state of each individual, the sum of the scores from the various tissues was calculated. The assessment scale used was according to the following scoring system shown in **Table 26**.

Table 26. The assessment scale used for the evaluation of granulomas**.**

Score 0	No granulomas
Score 1	Granulomas visible only with microscopy
Score 2	Granulomas visible macroscopically
Score 5	Tissue calcification

Main result: Supplementation with vitamin D_3 has no effect on the development of SG.

Trial 2. The effect of Ca:P ratio in the diet on the development of SG (HCMR)

For these trial nine experimental diets with different levels of Ca and P were formulated at the SKRETTING Aquaculture Research Centre (SARC), Norway. The basal diet was formulated to contain about 53% crude protein and 15% crude lipid. P was supplemented separately to the basal diet of the mixture to obtain various concentrations of P, while the amount of Ca that was supplemented in the basal diet was calculated to be either equal or double the amount of P. Meagre juveniles of 1 g average weight (n=1350 were used for the feeding trial. Three replicates were allocated to each diet. The feeding trial lasted 4 months. At the end of the feeding trial samples were taken for granuloma evaluation and histology, body and mineral composition, estimation of specific biomarkers (CYP27, CYP24 enzymes) and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in Table 1.

Main result: The high P content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis.

Trial 3. The effect of high plant protein diets in the development of SG (HCMR)

The purpose of this third trial was to examine whether FM replacement by PP sources affects the development of SG. Furthermore, due to the results obtained in previous trial we also investigated whether P supplementation in PP diets has any effect on SG. Four experimental diets were formulated at SARC with 60% (FM) and 14% fishmeal (PP) and increasing levels of P in the diets with 14% fishmeal (PP+medium P, PP+high P). Meagre juveniles of 2 g average weight (n=600) were used for the feeding trial. The feeding trial lasted 3 months (August- November 2016). At the end of the feeding trial samples were taken for granuloma evaluation, histology and plasma analysis. Granulomatosis was assessed using the semi-quantitative ordinal-scale scoring system described in **Table 26**.

Main result: Plant proteins in the diets of meagre were found to negatively affect 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.

Trial 4. The combined effect of vitamins E, C and carotenoids in the development of SG (FCPCT)

Six experimental diets were prepared by adding different levels of vitamin E, C and astaxanthin. Meagre juveniles of 79 g average weight (n= 900) were obtained by broodstock induced spawning at the ECOAQUA facilities (FCPCT, University of Las Palmas de Gran Canaria, Taliarte, Canary Island, Spain). The feeding trial lasted for 135 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in **Table 27**. The score was organ dependent, because the number of granulomas in each organ was variable.

Table 27. Severity score of granulomas in liver, kidney and heart

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Main result: The combination of a high dietary content of the antioxidants vitamin E and C increased the incidence and number of fish with lower severity of SG.

Trial 5. The effect of Se, Mn and Se in the development of SG. (FCPCT)

Five isolipidic and isoproteic fish meal and fish oil-based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se. Meagre juveniles of 15 g average weight (n= 2100) were obtained by broodstock induced for spawning. The feeding trial lasted for 90 days. Samples were taken for macroscopic evaluation of granulomas, histology, biochemical analysis and gene expression of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). The severity of granulomatosis was scored in each organ using a quantitative method that was developed according to the following criteria shown in Table 2.

Main result: The addition of target minerals did not ameliorate the granuloma incidence or severity, but recommended levels of minerals are: 40 $mg \cdot kg^{-1}$ of Mn, 200 $mg \cdot kg^{-1}$ of Zn, and 1.5 $mg \cdot kg^{-1}$ of Se.

General conclusions and recommendations for SG in meagre

- Nocardiosis is present in Greece, most probably in a confined geographical region; however it is not the cause of SG.
- Vitamin D_3 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 increased the incidence and number of fish with lower severity of SG
- The addition of Se, Mn and Se did not ameliorate the granuloma incidence or severity.

Taken together the improvement of SG by change in the diet with the absence of pathogens in SG-affected population we believe 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.

Considering all the above results, our recommendations for prevention of SG in meagre are:

- A combined diet with high percentage of fishmeal (60%) and high dietary content of P ($15 gkg^{-1}$) and 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.

Chronic Ulcerative Dermatopathy (CUD) in meagre

(Led by HCMR, Pantelis Katharios)

The lateral line is a mechanosensory system found in all fishes and in the larvae of aquatic amphibians, which is used for the detection of water movements and/or pressure fluctuations (Bleckmann and Zelick, 2009; Webb and Shirey, 2003). The receptors of the lateral line that detect water flow are called neuromasts and they are distributed on the head, the trunk and the tail of the fish. Neuromasts can be either superficial in the skin or enclosed in the fluid-filled canals of the lateral line that open to the environment through a series of pores (Bleckmann and Zelick, 2009; Webb, 1989). A schematic appearance of the lateral line system, the canals and the neuromasts is presented in **Fig. 64**. It has been demonstrated that the lateral line canals develop through a bone remodeling process with the implication of both osteoblasts for bone apposition and osteoclasts for bone resorption (Wada et al., 2014).

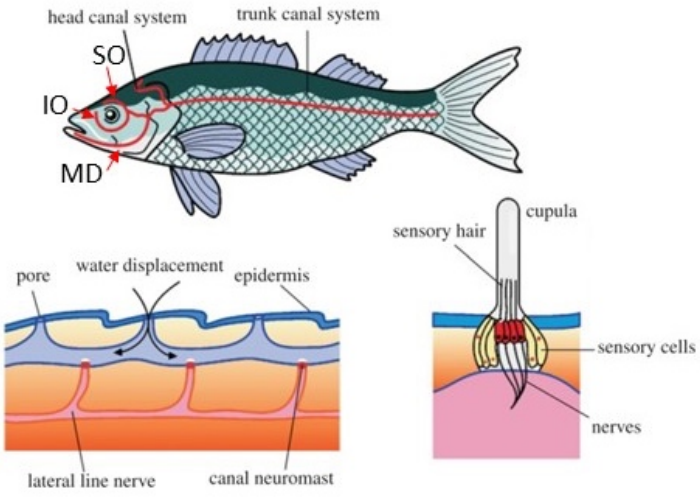


Figure 64. Lateral line system in fish. Structure of lateral line canal and of a neuromast. SO: supraorbital canal, IO: infraorbital canal, MD; mandibular canal (from Dagamseh et al., 2013 with modifications).

Chronic Ulcerative Dermatopathy (CUD) is a newly described condition affecting the lateral line canals of many cultured fishes both freshwater and marine. It has been described in the Australian

freshwater fish Murray cod, *Maccullochella peelii peelii* in sites supplied by groundwater (Baily et al., 2005; Schultz et al., 2011, 2008). The disease results in focal erosion, ulceration and loss of epidermis around the lateral line canals of the head and the trunk, and fin erosion. It has been associated with reduced growth rates, increased mortalities and significant reduction of marketability due to the severe disfigurement of the affected fish (Baily et al., 2005; Schultz et al., 2008). The same condition was also reported for goldfish *Carassius auratus* after exposure to freshwater groundwater (Baily et al., 2005). Concerning marine species, CUD was reported to affect the sharpsnout sea bream, *Diplodus puntazzo*, after culture in saline groundwater (Katharios et al., 2011). For the sharpsnout seabream, the authors suggested that there is an indication of osteoclastic enzymatic activity in the affected fish. The enzymes implicated in bone remodeling of the lateral line canals are the tartrate resistance acid phosphatase (TRAP) and cathepsin K for bone resorption, and vATPase for bone apposition. Both for Murray cod and sharpsnout seabream, the authors reported that the lesions resolve if fish are transferred to natural freshwater and seawater respectively and they could not associate the disease with any infectious agent. The final conclusion of both studies was that the development of the disease is correlated with the use of groundwater sources. However, the aetiology is still unknown since they could not establish the exact component of the water which results to the development of the disease (Baily et al., 2005; Katharios et al., 2011; Schultz et al., 2011, 2008). A similar condition under the term 'lateral line depigmentation' has been reported in channel catfish. In this case the authors concluded that the causative agent for development of the disease was the exposure of fish to chronic nutritional stress by 12 months of fasting (Corrales et al., 2009).

Meagre is one of the sensitive CUD fish species. The disease affects 100% of the population and results in ulceration of the skin overlying the lateral line canals, however is not associated with mortalities (Rigos and Katharios, 2010). The aim of this study was to describe the disease in meagre using histology and SEM and to investigate osteoclast activity using molecular markers. Through this study, the final goal was to investigate the aetiology of the disease and suggest preventive measures.

Two parallel rearing trials of meagre in borehole and natural seawater were conducted in order to study the development of CUD. Eggs produced in the facilities HCMR, Crete, Greece were used for the rearing trial, which was performed in duplicate 40-m^3 tanks. The rearing trial lasted from 1-56 days post hatching (dph). Every day, measurements of pH, CO_2, O_2 and T were made in the two water sources.

At the end of the rearing trial all the fish reared in borehole water had visible lesions associated with CUD in comparison with the fish reared in natural seawater (**Fig. 65).**

Figure 65. Meagre reared in natural seawater (left) and borehole water (right). All fish reared in borehole water had visible lesions on the head associated with CUD.

The average length and weight of the fish of the different water sources at the end of the rearing trial (56 dph) are presented in **Fig. 66**. The growth performance of the fish was not affected by the different source of water (p>0.05)

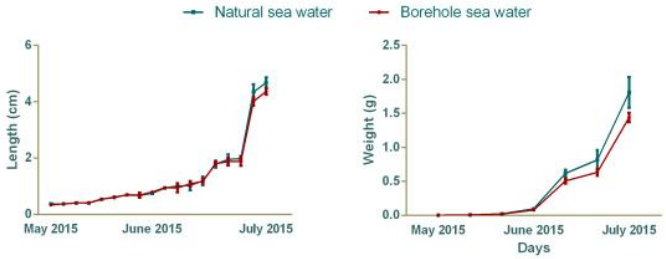


Figure 66. Average length and weight of meagre reared in borehole and natural seawater. The values are mean±SD.

The transfer from borehole water to natural sea water of CUD affected meagre led to almost full recovery of the lesions within 5 months (**Fig. 67)**.

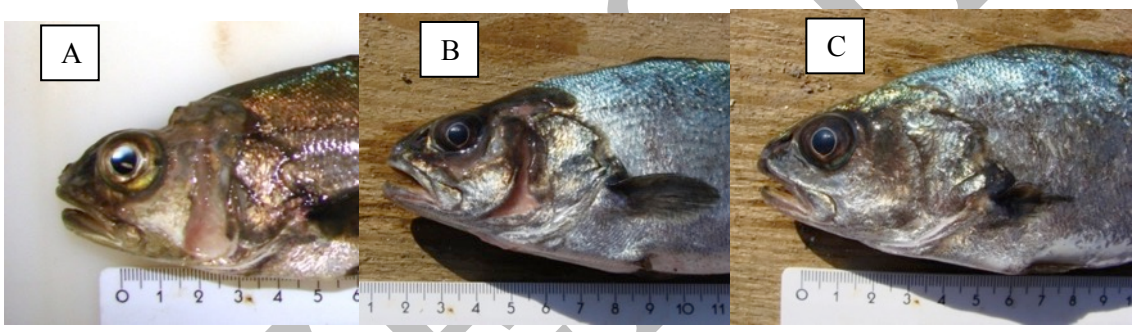


Figure 67. A. Nine-month-old meagre reared solely in borehole seawater. B. Nine-month-old meagre transferred to natural seawater for 5 months, with partial resolution of the lesions. C. Nine-month-old meagre transferred to natural seawater for 5 months, with complete resolution of the lesions.

The results of the studies in DIVERSIFY indicated that CUD in meagre is induced by the use of borehole water, which is in agreement with the conclusions of Baily et al. (2005) and Schultz et al. (2008) for Murray cod and of Katharios et al. (2011) for sharpsnout seabream. Furthermore, another similarity with Murray cod and sharpsnout seabream is that the lesions resolve if fish are transferred to natural seawater. The results from histology and SEM confirmed that the lesions were limited to the lateral line organ in the head.

From the physicochemical analysis of the two water sources it is noteworthy that the pH was lower and CO_2 higher in borehole water in comparison with natural seawater. Katharios et al. (2011) hypothesized that borehole water which is rich in CO_2, as indicated also by the lower pH compared to the pH of natural seawater, increases the enzymatic activity of the osteoclasts. The CO_2 activates the osteoclasts, which are in close proximity with the environment, such as the osteoclasts of the lateral line canals. In this scenario there would be an environmentally induced imbalance between osteoclasts (bone resorbing cells) and osteoblasts (bone depositing cells) that would cause the lesions seen in the fish, located exclusively in the lateral line canals. Based on these results, we performed a second rearing trial in order to investigate whether CO_2 in borehole water is the aetiological agent that causes the development of CUD lesions. In this trial, we used 2 parallel rearing tanks supplied with natural sea water. In one of these tanks we adjusted the pH to 7.4 by

infusing CO_2. We cultured meagre from eggs to 60 dph. The lack of lesions in the head and the trunk of the fish following visual examination in this study, suggests that neither pH nor CO_2 are the factors affecting the development of CUD lesions.

Eisler and Gardner (1973) found that copper alone or in combination with zinc or cadmium damages the epithelium of canals in the head of mummichog (*Fundulus heteroclitus*). The facilities and the water sources we used for this trial were the same that Katharios et al. (2011) used for the study of CUD in sharpsnout sea bream. From the heavy metal analysis of water samples, they found that borehole water had higher concentrations of copper, lead, nickel and zinc than natural seawater, however these levels were within the acceptable limits for marine aquaculture and much lower than the toxic limits. Our results from the metal analysis of the head of meagre reared in the two different water sources showed that the concentration of copper was significantly higher in the head of meagre reared in borehole water than in the head of meagre reared in natural sea water. However, concentrations of all metals where comparable to published data from other farmed and wild fish species where lesions are absent (Alasalvar et al., 2002; Kalantzi et al., 2016, 2013; Zotos and Vouzanidou, 2012). Nevertheless, metal toxicity as a causative factor for the development of CUD cannot be ruled out because of the lower pH of the borehole water and the longer exposure times of the fish.

Furthermore, another interesting similarity between CUD-affected meagre and CUD-affected Murray cod is the presence of the enigmatic rodlet cells. Schultz et al. (2014) found a significantly greater number of rodlet cells in the gills, kidneys and intestines of CUD-affected Murray cod and assumed that it was a response to a toxicant in the groundwater. In this task we didn't examine the soft tissues of meagre. However, in trials to investigate the causes of systemic granulomatosis we used meagre reared in borehole water with visible lesions associated with CUD. As we have described in deliverables 24.1, 24.2 and 24.5, rodlet cells in meagre are present in large numbers, aligned like epithelial cells in the peritoneal membranes, liver, pancreas, intestine and kidney. In both meagre and Murray cod, no pathogens were identified in any tissue, so the secretory nature or rodlet cells might be connected to defense mechanisms of fish against a toxicant in the water. However, this hypothesis cannot be fully supported since no data exist on the presence of these cells in normal (not affected by either systemic granulomatosis or CUD) or wild meagre.

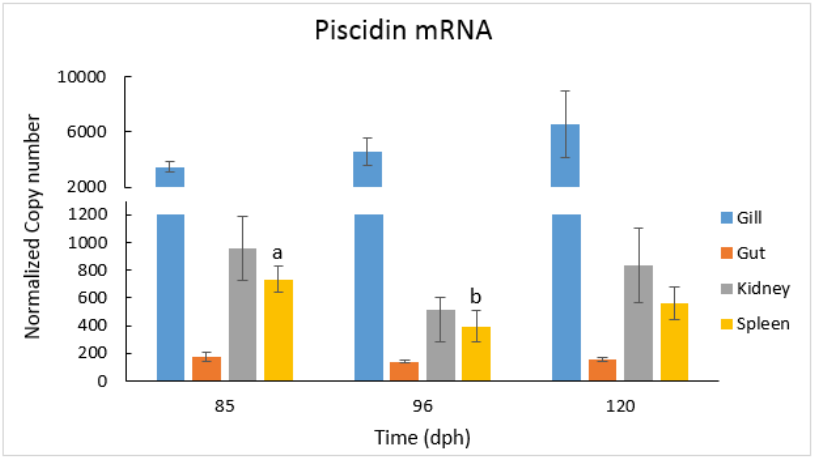
Although the disease is directly associated with the use of borehole water, the causative agent is still unknown for meagre, as well as for Murray cod and sharpsnout seabream. For all species the lesions resolve when the fish are transferred to natural freshwater or seawater (Baily et al., 2005; Katharios et al., 2011). Furthermore for Murray cod, Schultz et al. (2011) found that the retention of groundwater into a vegetated earthen pond or in a tank containing biofilms growing on an artificial macrophyte for 72 h prevents the development of CUD. Thus, it is recommended to avoid borehole seawater for the rearing of meagre if natural sea water sources are available and to pay careful attention to the source of the water used. Alternatively, the residence time of meagre in borehole water should be reduced to the minimum necessary, and fish should be moved to natural seawater (*e.g.* in sea cages) as soon as possible once the nursery phase is completed, in order to allow the tissue regeneration process to complete before marketing the fish.

Fish health issues and antiparasitic treatments

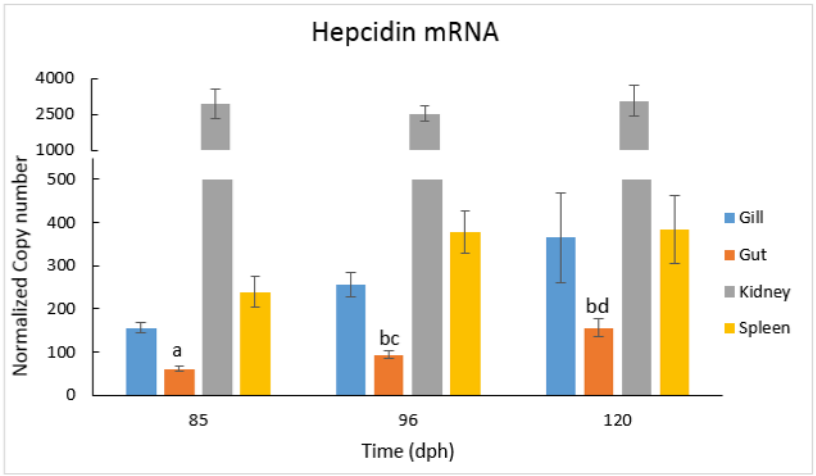
(Led by IRTA, Andre Karl and Ana Roque)

In all sectors of aquaculture the health of the animals is a limiting factor for sustainable economic development. To gain some lead in this topic for the benefit of industry the project has undertaken studies of various known health related issues, as seen above in the sections regarding systemic granulomatosis and chronic ulcerative dermatitis. Studies of the immune system of meagre have identified various genes invoked during an immune response. Among other genes examined are innate response effectors (C3 complement, metallothionein, lysozyme, MX protein, cyclooxygenase 2), antimicrobial peptides (hepcidin, piscidin, defensin) (Campoverde et al. 2017),

some cytokines (interleukins and interferons) and immunoglobulins (immunoglobulin M and immunoglobulin T). The ontogeny of expression was examined for the purpose of understanding the rate of maturation of the immune system and gain a better understanding of the time at which the immune system is fully functional. Future vaccine development will rely on the capacity of specific immune memory, and this hinges on the full functioning of all these various genes and their protein products. Much of this immune response is carried out within lymphoid tissues and specific cells of the peripheral blood. In this work we focused on four tissues: mucosal immune tissues of the intestine and gills and lymphoid tissues of the kidney and spleen. Two important points that were noted were the significant changes in expression that occur at the time of weaning, and the expression of the full suite of genes examined was not accomplished until 86 days post-hatch. Therefore, 86 days post-hatch would be the earliest date at which the immune system would be responsive to vaccination. Weaning is a time of physiological stress for the fish larvae. The change of feed means the immature immune system is being exposed to new antigen sources for the first time. This is seen clearly with the expression of antimicrobial peptides whose expression is triggered by exposure to bacterial proteins and their component antigenic peptides. Each of these antimicrobial effectors is differentially expressed in particular tissues. Piscidin is preferentially expressed in the gills, a primary site of exposure to antigens from the environment, while hepcidin is more highly expressed in the kidney where the blood is filtered and many foreign particles are phagocytized by the white blood cells produced in the pronephron kidney. (**Fig. 68**).



A)



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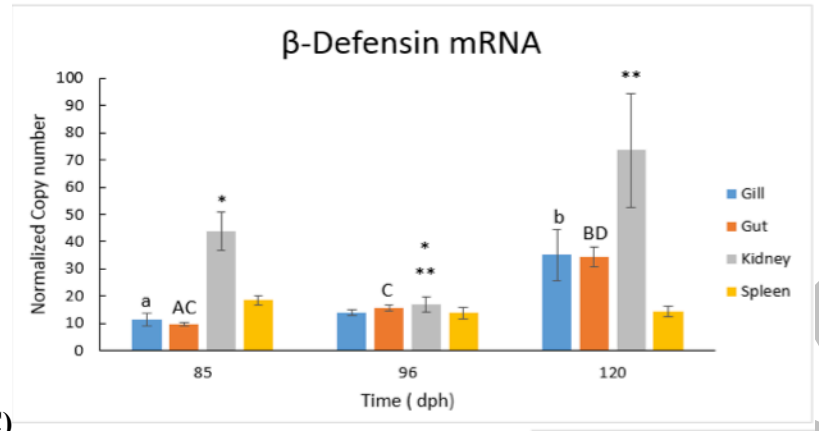

Figure 68. A). Piscidin expression at three different timepoints in four distinct tissues. B. Hepcidin expression at three different timepoints in four distinct tissues. C. Defensin expression at three different timepoints in four distinct tissues. Expression of each of the identified antimicrobial peptides are time and tissue specific.

Proinflammatory responses are controlled by multiple signaling pathways that use cytokines as messengers. During the investigations, there were encountered multiple isoforms of tumor necrosis factor and interferon (Milne et al 2017; Milne et al. 2018). The multiple isoforms of interferon (IFNc, IFNd, and IFNh) are interesting in that it suggests a better antiviral response of the immune system since this cytokine is one of the main signaling molecules for eliciting an antiviral immune response. As seen in **Fig. 69** the different isoforms are expressed in a distinctly different way in each of the tissues analyzed (Milne et al. 2018).

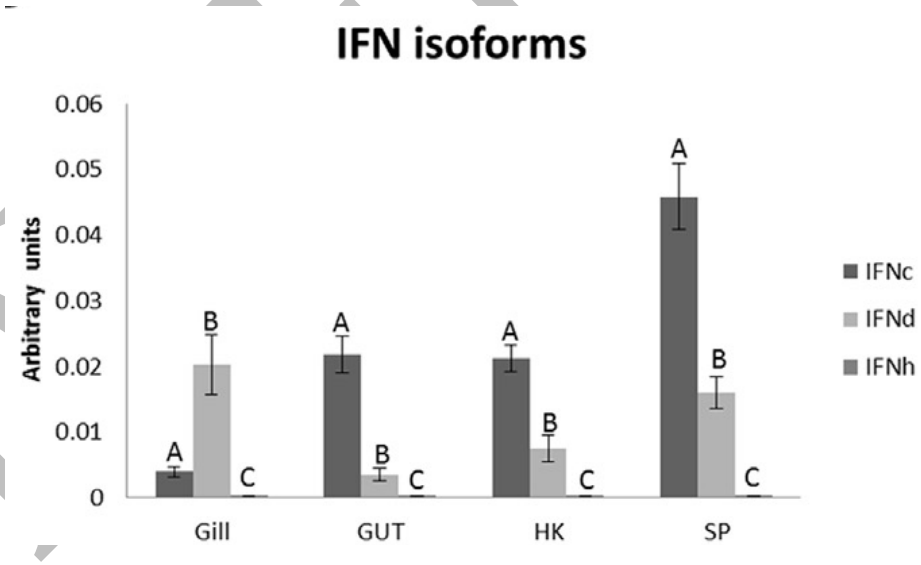

Figure 69. Constitutive expression of meagre IFN isoforms in four immune tissues. Total RNA was extracted from the tissues and IFN transcripts detected by qPCR and normalised to GAPDH. Graph shows how each subgroup is expressed relative to each other within a tissue. HK = head kidney, SP = spleen. Bars are mean arbitrary units ± SEM, N = 10. Letters denote significant differences ($P < 0.05$) between subgroups within a tissue.

As shown above, the innate antibacterial and antiviral repertoire of responses of the immune system of meagre are not insignificant. The response to parasitic pathogens that lead to pathology is somewhat less-well documented. A review of the pathology affecting meagre shows there are some reports of bacterial diseases (Vibriosis, Nocardiosis), as well as ciliates (*Amioloodinium sp.*) and several trematodes (Merella et al., 2009; Terengo et al. 2010; Elkesh et al. 2012; Soares et al. 2018). Parasites such as *Sciaenacotyle panceri,* found on the gills of meagre (Merella et al. 2009), are known to cause mortality in farms in the Mediterranean and require development of appropriate treatments.

Towards this objective, research focused on the use of essential oils (EO) with vermicide properties which had the potential to be mixed in the feed, so they could be delivered orally. 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 analyzed 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 analyzed for levels of cortisol, glucose and lactate which are general stress indicators. Treatments that demonstrated potential efficacy were repeated. Four trials were run. The first one to choose oils accepted by fish, the second and third evaluated the toxicity of cinnamon and mint oil over 6 weeks. Although the digestive tract was not altered, fish did not feed well and grew less. However they had lower levels of glucose in the blood, a possible indicator of reduced stress or related to the reduced feeding behavior. A challenge model was also developed wherein fish were infected using eggs of the parasite, *Sciaenacotyle panceri,* in order to test the therapeutic properties of the selected oils.

A final experiment was set up to test the efficiency of cinnamon as a parasiticide for meagre infested with *S. panceri*. Fish were confirmed to have parasites before the experiment started. For the treatment experiment three diets were prepared, untreated diet, diet with EO of cinnamon and a new diet prepared with an aqueous extract of Echinacea. Echinacea was selected since recently several studies have demonstrated its immunostimulant properties in fish (Bulfon et al, 2017; Guz et al 2014; Oskoi et al 2012), and it was decided to investigate whether an immunostimulant would benefit the fish with parasites. After three weeks prevalence in each of the treatment groups is shown in **Table 28**:

Table 28. Results summary.

Treatment	Prevalence (%)	Intensity (>5 ind/ gill)	Intensity (>8 ind/gill)
Control	100	15	2
Echinacea	100	15	10
Cinnamon	60	0	0

Stress and immune defense parameters were evaluated, but no significant differences were detected among treatments. Cinnamon oil was also tested in vitro and at a concentration of 0.2% where it killed 100% of the *S. pancerii* individuals in 4’21”. Cinnamon oil also showed immunostimulant properties. Overall, cinnamon showed a clear potential to treat a parasitosis with *S. pancerii* when administered orally to juvenile meagre.

6. Market, consumer perception, new products and business model

(led by Gemma Tacken, Wageningen University and Research, The Netherlands)

The socio-economic research in DIVERSIFY includes applied market development approach, clarifications on perception of aquaculture products, market demand evaluation, consumer preferences, new product development (**Fig. 70**), value adding and market development. The studies have been performed across five European fish markets: France, Germany, Italy, Spain and the United Kingdom.

Market analysis

Machiel Reinders, Wageningen University and Research, The Netherlands

The market analysis demonstrated that important buyers (*i.e.* retailers) in the five countries find it very difficult to position the 6 new species (*e.g.* meagre) in relation to the current species in the market.
Species such as meagre is sometimes known as wild catch but less as aquaculture products. However, industrial buyers do not easily position this fish in relation to other fish species.
Buyers are open to welcome new species under the following conditions:

- The product must be cultured in a sustainable way,
- The product should be available as a fresh product (especially southern-Europe),
- The product must be easy to prepare and/or ready to eat (Germany and United Kingdom),
- The product must be priced competitively.

New Product Development

Marija Banovic, MAPP Centre, Department of Management, Aarhus University, Denmark; Rocio Robles, Ctaqua, Spain.

Co-creation with consumers identified very promising product ideas for new fish products per investigated country. The most important drivers and barriers for the choice of the new product ideas have also been identified as well as recommendations for new product development of selected fish species.
Twelve product ideas have been evaluated for production technical feasibility and shelf-life, three of them from meagre: ready to eat salad with fish (meagre), frozen meagre fillets divided in double portions and fish hamburger with the shape of a fish **(Fig. 70).**

Figure 70. Meagre new products developed in Diversify: ready to eat salad with fish (meagre) (top left); fish hamburger with the shape of a fish (top right) and frozen meagre fillets divided in double portions (bottom).

Sensory characterization of new fish species and consumer acceptance of the new developed products

Luis Guerrero, IRTA, Spain.

New fish species need to be properly introduced to create a diversification in the current market.

Sensory, compositional, instrumental texture parameters and somatic properties of DIVERSIFY five emerging fish species, namely wreckfish, greater amberjack, grey mullet, meagre, and pikeperch, were examined for characterization purposes. Regarding the compositional parameters, fat content was among the most relevant discriminating aspect between species, while hardness was among the most differentiating ones when dealing with texture. Greater amberjack was described with sour flavor, pikeperch was described as a crumbly, pasty white fish and grey mullet was characterized by bitter flavor. Sensory firmness was clearly distinctive for wreckfish, while meagre related to juicy texture. The species in this study exhibited a wide range of physicochemical and sensory characteristics that show their potential for being further exploited when designing new products.

In a **consumer acceptance** test, it was demonstrated the influence of providing the product information on the consumer acceptance degree **(Fig. 71**). In the case of meagre, it was presented as a ready to eat fish salad an d fish burger with the shape of a fish. In general, products with a lower degree of processing were those who generated higher expected and actual acceptance.

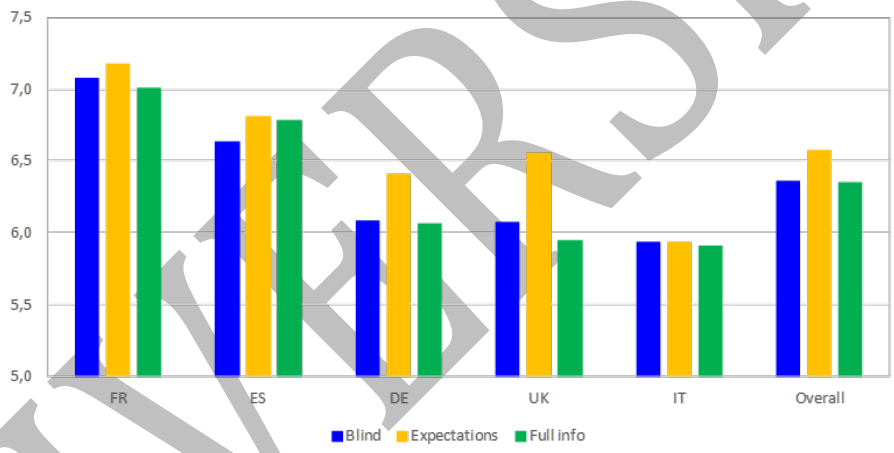

Figure 71. Results of the consumers' acceptance tests for new developed products performed in 5 European countries. Consumers were not informed about the product (blue bar), then knowing the product to be tested, they were asked about their expectation (orange bar) and finally they had the full information before tasting the product (green bar).

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