



New species for EU aquaculture

Technical Manual – Greater amberjack (*Seriola dumerili*)



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Introduction

Greater amberjack, *Seriola dumerili* (Risso, 1810) is a leading candidate species for enhancing European aquaculture, showing growth rates ten times higher than the European seabass, *Dicentrarchus labrax*. Greater amberjack culture in the Mediterranean region started in the 80s using standard culture conditions, where the feed was first based on fresh fish but quickly progressed to artificial feeds (García-Gómez, 2000). In recent years, interest for this species in the aquaculture industry is expanding, due to its high demand and market price, rapid growth {Thompson, 1999, SERIOLA spp.-yellowtail}, excellent fillet quality, and its capacity to accept inert food (Nakada, 2000) {Nakada, 2000, SERIOLA spp.-yellowtail}. Today, mainly as a result of work undertaken in the DIVERSIFY project, a limited but gradually growing commercial activity with hatchery-produced individuals exists in Greece, and the first market size fish have reached the European market in 2018.



The major bottlenecks for the incorporation of greater amberjack in the EU aquaculture industry at the beginning of the project included lack of (a) reliable reproduction and (b) production of adequate numbers of juveniles. In captivity, reproduction has been problematic (Kozul et al., 2001), but captive-reared broodstock have reproduced after hormonal treatments (Mylonas et al., 2004), and in some cases also spontaneously (Jerez et al., 2006). Also, some knowledge was available on the nutritional requirements of breeders (Rodríguez-Barreto et al., 2012). DIVERSIFY has studied the reproduction in captivity and in the wild, and developed spawning induction methods that are reported here.

Although amberjack larvae were studied before and has been shown that they can readily shift from consuming rotifers to microdiets (Shiozawa *et al.*, 2003), there is still very little information that could lead to an applicable larval feeding protocols and rearing systems of this species in commercial scale. Early trials showed that semi-intensive methods, characterized by stocking density as low as 0.25 eggs l⁻¹, seems to be effective, but the survival rate was rather low, around 3% {Papandroulakis, 2005 #2792}. Therefore, it was necessary to obtain new information on its rearing parameters. The main objectives of our studies were to define some of the parameters related to the larval rearing of the greater amberjack in order to optimize the applied methodologies. The effects of tank type-shape, duration of the photo phase, tank background color and light conditions, stocking density, feeding protocols and nutritional requirements were studied in terms of growth, survival, skeletal deformities, biochemical composition, stress and larval condition.

Another area of concern for the commercial production of greater amberjack is fish health. Since greater amberjack aquaculture is still in its infancy in Europe, there are few if any case reports regarding significant outbreaks due bacterial infections. Epitheliocystis and photobacteriosis have been reported to cause losses in farmed fish, while several known bacterial pathogens have been experimentally demonstrated to be virulent for the species. On the other hand, parasitic diseases have already shown their devastating potential during grow out. Monogenean parasites such as *Neobenedenia* sp. and *Zeuxapta seriolae* have been connected to mass mortalities in farmed fish (Grau et al., 1996), while *Neobenedenia* spp was identified in a major outbreak causing losses in both in juveniles and broodstock. Therefore, DIVERSIFY has studied the potential pathologies that occurred in the course of the project in an effort to develop early diagnosis tools, veterinary solutions and preventive veterinary protocols that are available and support the sustainable rearing of the species.

Although this manual does not include all the possible information that a farmer may need for the successful rearing of this new species, it does provide the majority of information obtained in DIVERSIFY, and we believe it would prove to be a valuable resource for the incorporation of greater amberjack in the Mediterranean aquaculture industry.



Reproduction and Genetics

Development of an optimized spawning induction protocol for captive amberjack in the Mediterranean

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The aim of this study was to examine the two methods of spawning induction using gonadotropin releasing hormone agonist (GnRH_a) either in the form of EVAc implants (sustained release of the substance in the bloodstream) or injections (acute release of the substance in the bloodstream) in terms of spawning kinetics, egg production and quality and deliver a workable protocol to the aquaculture industry.

Amberjack breeders were kept at the facility of Argosaronikos Fish Farm S.A., on the island of Salamina (Greece). The broodstock consisted of 28 fish, captured at the juvenile stage and acquired in May 2014 from the area of Astakos (western Greece), and was maintained in a 1000-m³ sea cage, 300 m offshore from the land-based facility. At the time of the first reproductive season (late spring-summer), the stock consisted of 14 females (mean \pm SD body weight 18.8 \pm 2.1 kg), and 14 males (mean \pm SD body weight 15.1 \pm 3.0 kg). Feed was given to apparent satiation 6 days a week using Skretting Vitalis CAL (22 mm). For the spawning induction trial, fish were transferred to the land-based facility, to 23 m³ flow through round tanks, accordingly to the treatment received, and maintained at a 1:1 sex ratio. Female fish were treated either with GnRH_a injection (20-25 μ g GnRH_a kg⁻¹) or with an EVAc GnRH_a implant (Mylonas and Zohar, 2001), loaded with 750–1000 μ g of GnRH_a, for an effective dose of 49-69 μ g GnRH_a kg⁻¹. Females were treated weekly in injected group, and in both injected and implanted groups on the third week (a total of 3 injections and 2 implants).

Implanted fish spawned for 9-10 times after the 1st treatment, while only 4 times after the 2nd treatment (Fig. 1). On the contrary, injected fish spawned 7 times after the 1st treatment, 3-5 times after 2nd treatment and 1-3 times after the 3rd treatment, respectively. Mean daily relative fecundity was higher in implanted fish (15,170 \pm 2,738 eggs kg⁻¹day⁻¹) compared to the injected fish (6,119 \pm 2,790 eggs kg⁻¹day⁻¹)(Fig. 5). Total relative fecundity was also higher in implanted fish (102,402 \pm 20,337 eggs kg⁻¹tank⁻¹) compared to the injected (26,517 \pm 9,938 eggs kg⁻¹tank⁻¹), respectively. Total egg production was decreasing in injected fish after consecutive GnRH_a treatments, while in implanted fish no statistical differences were observed among treatment number (P=0.17).

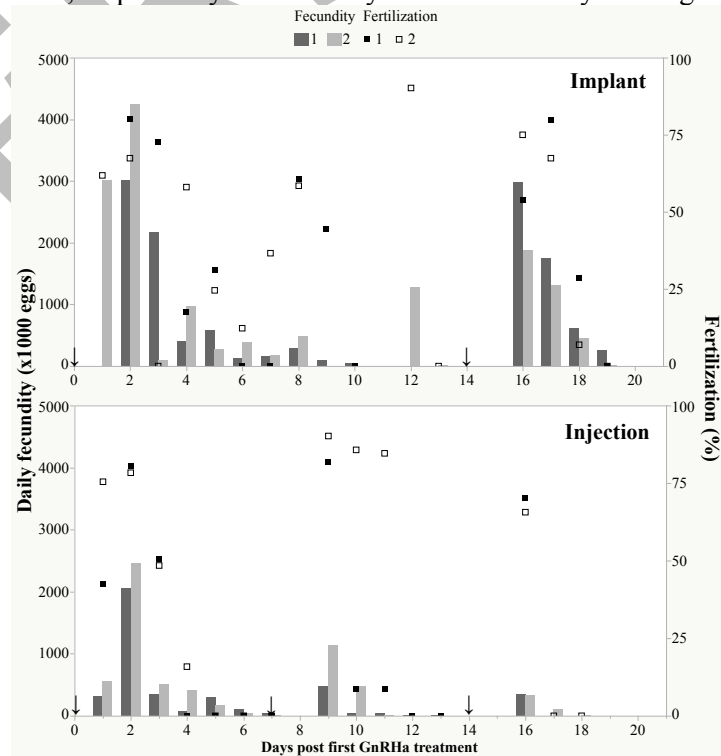


Figure 1. Daily fecundity (bars, x1000 eggs) and fertilization success (marks, %) of GnRH_a implanted or injected greater amberjack. Arrows (n=2 for GnRH_a implanted and n=3 for injected, respectively) indicate the time of treatment. The first application was done on 7 June 2016.



Fertilization success, 24 h embryo survival, hatching and 5d larval survival was similar among treatment methods, while no statistical differences were observed among different treatment number (**Fig. 2**).

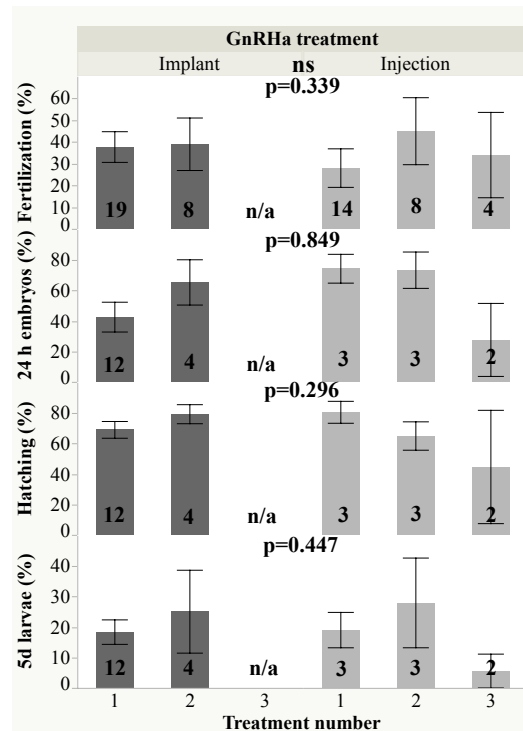


Figure 2. Mean (\pm SEM) fertilization, 24h embryo survival, hatching and 5d larval survival after consecutive treatments with GnRH α implants or injections. The numbers in bars indicate the spawns constituting each mean. No statistical differences were observed ($P < 0.05$).

Greater amberjack were induced successfully to spawn using either GnRH α implants or GnRH α injections, producing fertilized eggs. GnRH α can reliably induce multiple spawning of viable eggs, overcoming the observed reproductive dysfunctions of the species in captivity. This is important, because in the Mediterranean no reliable spontaneous reproduction of the species has been reported, when fish are maintained in land-based tanks. Two treatments with GnRH α implants compared to three treatments with GnRH α injections, produced more eggs in terms of fecundity without altering the quality of eggs in terms of fertilization, 24h embryo survival, hatching and 5d larval survival. This means that less handling is necessary using the first treatment protocol producing significantly higher amount of eggs for rearing.

Development of an optimized spawning induction protocol for captive amberjack in the eastern Atlantic

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The main objective of the present study was to examine the potential of captive-reared wild greater amberjack to produce natural spawns, and compare the quality of the produced eggs to eggs produced from induced spawns with GnRH α , applied with either injections or implants. In January 2013, the twenty-two greater amberjack of a mean weight of 8.27 ± 1.11 kg for females and 8.12 ± 1.82 kg for males, were transferred to the new broodstock station of the PCTM, and kept in three circular tanks of 40 m³ (5 m x 2.35 m). The selected fish were distributed in three circular tanks of 40 m³ in volume, as follows. In tank 1 (2 ♀ and 5 ♂), the fish were not induced hormonally and were left as controls. In tank 2 (3 ♀ and 3 ♂), the fish were injected intramuscularly with gonadotropin releasing hormone analogue (GnRH α) at a dose of 20 μ g kg⁻¹ body weight. These hormonal treatments were applied twice a week



alternating the broodstock (1 ♀ and 1 ♂). The breeders of tank 3 (3 ♀ and 3 ♂), were induced using 500 µg GnRH_a implants (Mylonas and Zohar, 2001) (**Fig. 3**).

Under the environmental conditions of photoperiod and water temperature in the Canary Islands, it is possible to obtain natural spawns from a number of wild-caught great amberjack broodstock, as shown earlier by Jerez et al. (Jerez et al., 2006), after maintenance of the fish without much manipulation for at least 2 years in tanks of 40-m³ in volume. The reproductive performance, and quality of the eggs and larvae produced from these spontaneously spawning females were the best, compared to GnRH_a induced individuals. In addition, GnRH_a therapy in the form of multiple injections or implants of controlled release was shown to be very effective in inducing multiple spawns. GnRH_a injections given on a weekly basis were more successful in inducing spawns of high fertilization success than fish given GnRH_a implants, and are thus recommended for this species under the conditions in the Canary Islands.

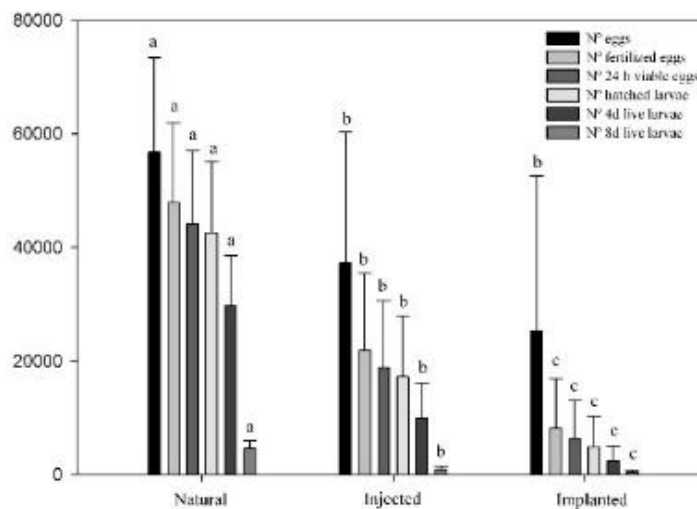


Figure 3. Production of eggs, embryos and larvae of greater amberjack (per kg female and spawn) females spawned spontaneously (Natural) or induced with GnRH_a treatment (injections or implants). For each examined parameter bars with a different letter superscript differ significantly ($P < 0.01$).

Development of an optimized spawning induction protocol for F1 hatchery produced amberjack in the eastern Atlantic

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The objectives of the present study were to examine the reproductive development of hatchery produced F1 generation greater amberjack, and to evaluate the potential of controlled-release GnRH_a delivery systems (implants) to induce oocyte maturation, spermiation and spawning of fertilized eggs, and to monitor spawning kinetics and gamete quality. The developed method shows great potential for the development of the aquaculture industry for greater amberjack, by enabling the use of hatchery produced broodstock for further breeding selection.

Rearing was undertaken in the facilities of the Centro Oceanográfico de Canarias, Tenerife, Spain. The broodstock consisted of 14 hatchery-produced F1 fish, from eggs obtained from wild-caught breeders between 2005 and 2009. Fish were maintained during the year in two outdoor covered 50-m³ tanks, supplied with well-water (10 renewals day⁻¹) at ambient water temperature and photoperiod until the beginning of the experiments on 13 May 2015. After the 1st GnRH_a treatment and for the duration of the study, the selected fish were placed in a single outdoor covered raceway tank of 500 m³ with continuous water supply (6 renewals day⁻¹) under natural photoperiod. Fish were treated with an Ethylene-Vinyl acetate (EVAc) GnRH_a implant (Mylonas and Zohar, 2001) loaded with GnRH_a at the sampling times of May, June and July (**Fig. 4**).

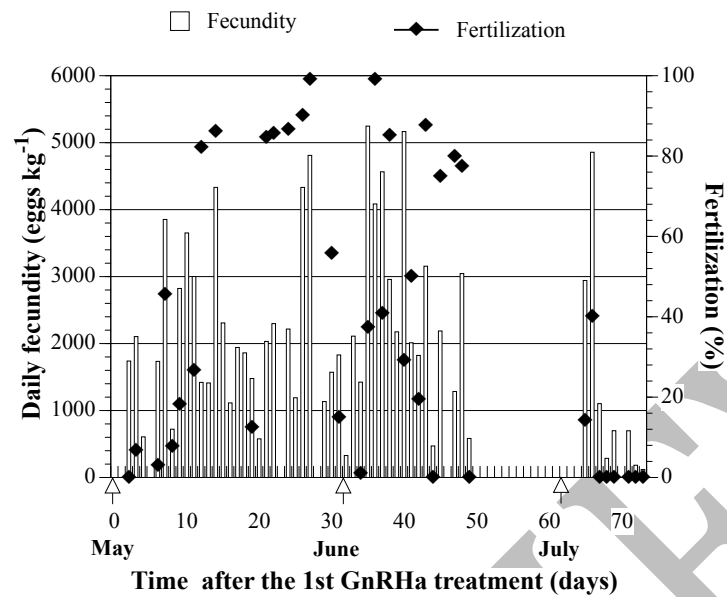


Figure 4. Daily fecundity and fertilization of F1 greater amberjack in response to three treatments with GnRH α implants. The white arrows on the X-axis indicate the times of the GnRH α treatments.

The present study showed that hatchery-produced greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning after multiple administrations of gonadotropin releasing hormone agonist (GnRH α) in a controlled-release delivery system, over an extended spawning period lasting from May to September in the Canary Islands, Spain. The use of GnRH α -delivery systems resulted in multiple spawns of fertilized and viable eggs. Egg production was high and egg quality was adequate for the implementation of larval rearing for commercial purposes. The repetitive handling required to administer the GnRH α implants during the prolonged spawning season did not result in any negative effect on the welfare and reproductive performance of the fish based on evaluation of a number of biochemical parameters.

Sperm quality parameters that were evaluated included (a) sperm density (number of spermatozoa ml⁻¹ of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (sperm motility, %), (c) duration of forward sperm motility of $\geq 5\%$ of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4°C (sperm survival, days). In males, there were in general no differences in sperm quality parameters of the males during the present study (**Fig. 5**), showing similar values to wild-caught GnRH α -treated greater amberjack reported earlier (Mylonas et al., 2004). A gradual reduction in the sperm motility duration during the reproductive season was observed in another study with wild-caught breeders in the eastern Mediterranean Sea (Zupa et al., 2017). However, as water temperatures do not rise to the same levels in the summer in the Canary Islands, greater amberjack apparently remain in spermiating condition for a much longer period of time, reported from May to October (Fernández-Palacios et al., 2015; Jerez et al., 2006). The sperm density in GnRH α -treated F1 males showed an increasing trend over the course of the spawning period, although it was always lower than the one obtained in wild-caught males treated with GnRH α implants, but it was in the same range of a study using untreated males.

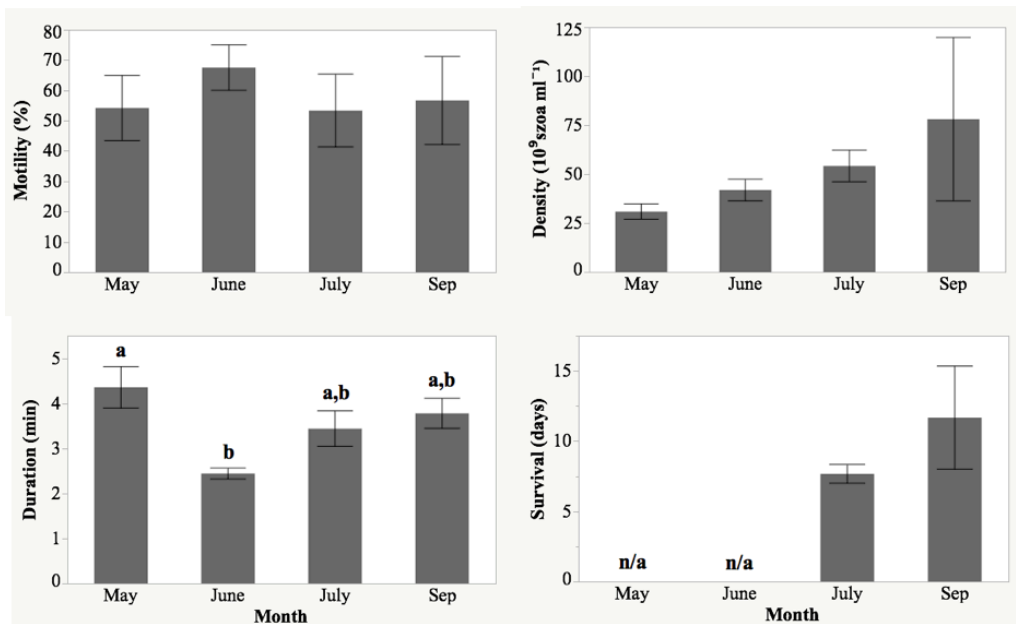


Figure 5. Mean (\pm SEM) sperm quality parameters of F1 greater amberjack at different times during the reproductive season (spermatozoa forward motility, density, duration of motility and maximum survival during storage at 4°C). Statistically significant differences among sampling times are indicated by different lower-case letters ($P \leq 0.05$). na = not available.

The developed reproduction control method shows great potential for the development of the aquaculture industry for greater amberjack, by enabling the use of hatchery produced broodstock for further breeding selection (**Fig.6**). For the time being, there is a need for maintaining the breeders in sea cages during the year, until such time new large broodstock tanks (>70 m³) supplied with filtered seawater of the appropriate quality are constructed by the aquaculture industry.



Figure 6. Greater amberjack broodstock (left) and incorporation of hormonal implants in a specimen of greater amberjack (right).



Nutrition

Hatchery and grow out nutrition for greater amberjack

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The overall objective of the present task was to determine the nutritional requirements and optimum levels of DHA, EPA, and combined PUFA-carotenoids in greater amberjack enrichment products at the time of both rotifer and *Artemia* stages, evaluating their effects on survival, growth, welfare and stress resistance/tolerance, bone development and tissue composition.

1. Optimum docosahexaenoic acid (DHA) in enrichment products for live prey for greater amberjack

Greater amberjack of 17 dph, at a total density of 1000 larvae per tank (mean total length 6.39 ± 0.44 mm; mean fresh weight 2.94 ± 0.57 mg), were randomly distributed in 15 experimental tanks of 200 l capacity at facilities of Las Palmas de Gran Canaria University. From 17 to 22 dph, there was an overlap between rotifers (unenriched) and *Artemia* with a gradual reduction in the amounts of rotifers from 5000–0 individual's l⁻¹ and a progressive increase of enriched *Artemia* from 125–500 individuals l⁻¹. From 23 to 35 dph, greater amberjack larvae were fed exclusively with enriched *Artemia* from one of the five dietary treatments.

Five experimental emulsions, which varied in the DHA contents (0-50%) were formulated. Experimental emulsions were prepared, mixing increasing amounts of high DHA content commercial methyl ester oil (DHA-70, Maruha Nichiro Foods, Tokyo, Japan) containing 70% of Total Fatty Acid (TFA) as DHA, 12% as EPA and 2% as ARA; Oleic Acid oil (Sigma-Aldrich; Madrid, Spain) including 77% of TFA as oleic acid and soya lecithin (SL, Korot SL, Alcoy, Spain) containing mainly 54% of TFA as linoleic acid (18:2n-6, LA) and trace amounts of EPA and DHA. In addition, to prevent the oxidation of high DHA levels, experimental emulsions were fortified with 3000 mg kg⁻¹ vitamin E (DL- α -tocopherol acetate, Sigma-Aldrich, Madrid, Spain) and 2500 mg kg⁻¹ vitamin C (L-ascorbic acid, Asc, Sigma-Aldrich, Madrid, Spain,) (Table 1). Larval survival was calculated by daily counting of dead larvae from 17 dph and by counting all the remaining alive larvae at the end of the experiment. Thirty larvae per tank at 35 dph were submitted to acute stress, handling them out of the water for 30 and 60 seconds and returning them to a bucket with aerated seawater. Survival rate was determined 24 hours later, counting all the surviving larvae. To determine the skeletal anomalies incidence, 100 larvae were collected per tank at 35 dph.

Table 1. Emulsion ingredients, proximate and fatty acid composition of the resultant enriched *Artemia* containing increasing levels of DHA.

Experimental Emulsion	DHA-0	DHA-1	DHA-2	DHA-3	DHA-4
<i>Ingredients (g kg⁻¹ diet)</i>					
DHA-70 ^a	0	300	450	600	900
Oleic acid	900	600	450	300	0
Soy bean lecithin	100	100	100	100	100

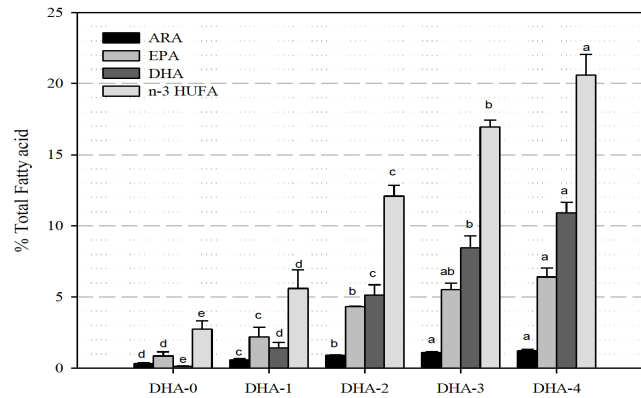


Figure 7. Fatty acids content in *Artemia*

The lowest level of DHA in experimental emulsions was present in DHA-0 with 0.5% in TFA, while the DHA contents in DHA 1 to 4 increased from 10.5% to 52.5 % in TFA.

Results

Larval performance

The relationship between final total length and *Artemia* DHA content was described in Fig. 3. The growth models suggest that maximum growth was achieved in the range of dietary DHA concentrations tested, between 5-10 %TFA with a maximum around 6.5-7% DHA content in *Artemia* (**Fig. 7**). Larval survival was significantly ($P \leq 0.05$) affected by dietary DHA at 35 dph, the lowest survival was recorded in those larvae receiving the lowest DHA in the *Artemia* (DHA-0) (**Fig. 8**)

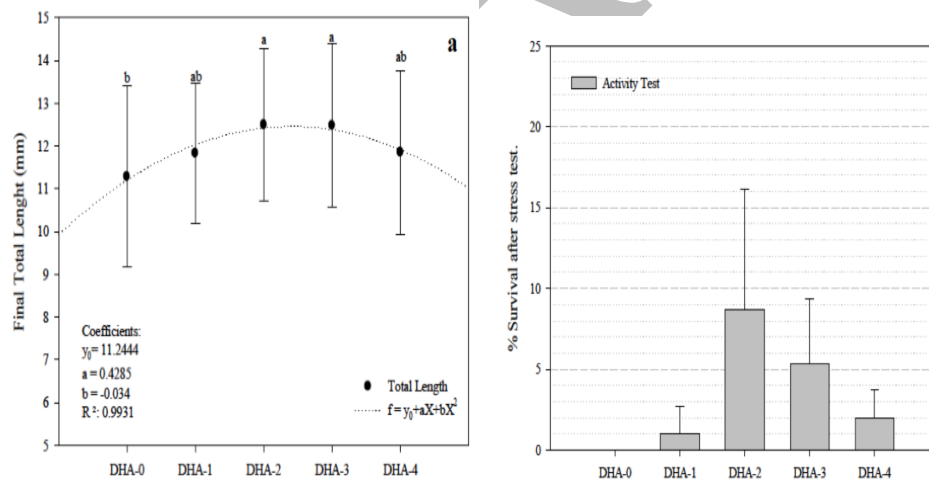


Figure 8. Relation between (a) total length (mm) and dietary *Artemia* DHA content in larvae 35 dph. Survival after activity test

Welfare

The incidence of total acute skeletal deformities measured as sum of lordosis, kyphosis, scoliosis, vertebral anomalies and cranial anomalies were relatively low for all the dietary DHA levels assayed. This parameter was not significantly affected by the dietary DHA with an average value for all the dietary treatments of $5.01 \pm 1.09\%$ (**Fig. 9**).

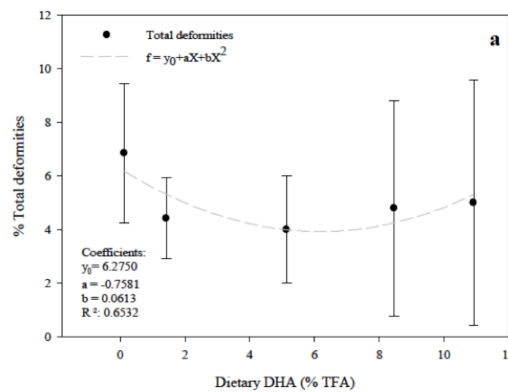


Figure 9. Relationship between total acute deformities (%) to dietary *Artemia* DHA content (%TFA) in larval at 35 days post-hatch

2. Optimum Eicosapentaenoic acid (EPA) in enrichment products for live preys for greater amberjack

Methodology. Larval performance

With same protocol than previously described, in the facilities of Las Palmas de Gran Canaria University, five experimental emulsions, which varied in the EPA contents (0-60%) were formulated. Experimental emulsions were prepared, mixing increasing amounts of high EPA content commercial triglycerides oil (Incromege EPA 500 TG, Croda, Barcelona, Spain) containing 63% of Total Fatty Acid (TFA) as EPA, 8% as DHA and 3% as ARA ; Oleic Acid oil (Sigma-Aldrich; Madrid, Spain) including 77% of TFA as oleic acid and soya lecithin (SL, Korot SL, Alcoy, Spain) containing mainly 54% of TFA as linoleic acid (18:2n-6, LA) and trace amounts of EPA and DHA. In addition, to prevent the oxidation of high DHA levels, experimental emulsions were fortified with 3000 mg kg⁻¹ vitamin E (DL- α -tocopherol acetate, Sigma-Aldrich, Madrid, Spain) and 2500 mg kg⁻¹ vitamin C (L-ascorbic acid, Asc, Sigma-Aldrich, Madrid, Spain,) (**Table 2**).

Table 2. Emulsion ingredients, proximate and fatty acid composition of the resultant enriched *Artemia* containing increasing levels of EPA.

Experimental Emulsion	EPA-0	EPA-1	EPA-2	EPA-3	EPA-4
	<i>Ingredients (g kg⁻¹ diet)</i>				
EPA 500TG ^a	0	300	450	600	900
Oleic acid	900	600	450	300	0
Soy bean lecithin	100	100	100	100	100

Results.

Larval performance

Artemia enriched with different experimental emulsions resulted in five enriched *Artemia* treatments (**Fig. 10**). The EPA content in *Artemia* was directly correlated to EPA content in the experimental emulsion ranging from 1.08 to 22.9 %; (**Table 2**), other fatty acids such as DHA (range 0.14-3.01 % TFA), ARA (range 0.39-1.72 % TFA) and total n-3 HUFA (range 3.24-29.07 % TFA) were also directly correlated with their emulsion content.

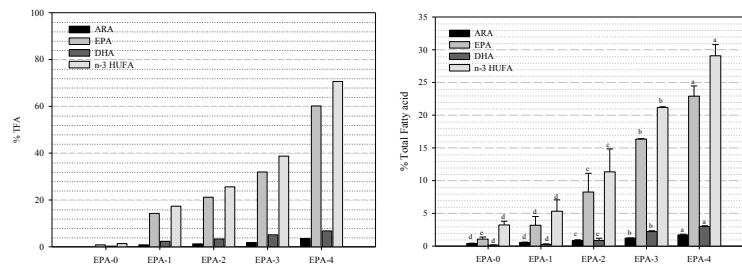


Figure 10. Fatty acids content (percentage of total fatty acids) in the experimental emulsions and in *Artemia*.

Larval growth was significantly affected by the dietary EPA levels along the feeding trial (Fig. 11). Growth models suggest that maximum growth was achieved in the range of dietary EPA between 8-16 %TFA with a maximum around 13-14% DHA content in *Artemia*, when low dietary DHA were provided (0.8-2.2% TFA). Larval survival was significantly ($P < 0.05$) affected by dietary EPA at 35 dph, the lowest survival was recorded in those larvae receiving the lowest EPA in the *Artemia* (EPA-0)

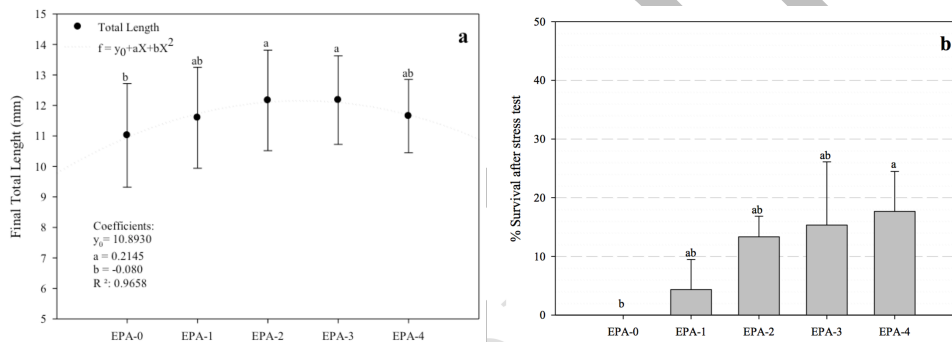


Figure 11. Relationship between (a) total length (mm) and dietary *Artemia* DHA (22:6n-3) content in greater amberjack larvae 35 dph. (b). Survival after activity test

The incidence of total acute skeletal deformities measured as sum of lordosis, kyphosis, scoliosis, vertebral anomalies and cranial anomalies were relatively low for all the dietary EPA levels assayed.

3. Combined effect of PUFA-rich lipids and carotenoids in enrichment products for live prey (rotifers) for greater amberjack

According to the carotenoid and lipid and fatty acid composition of wild greater amberjack female gonads and eggs, some preliminary studies were conducted at University of La Laguna (ULL) and Instituto Español de Oceanografía (IEO) in Tenerife Island, in which three different emulsions were assayed. Those emulsions were designed with different lipid sources varying in their chemical structure and combined to supply high LC-PUFA levels and DHA/EPA/ARA ratios resembling those of wild amberjack eggs total and polar lipids. **E1** was based on a polar rich (PL-rich) emulsion containing a marine natural lecithin LC60 (PhosphoTech Laboratories, France) with up to 60 % phospholipids (40% PC + 20 PE) rich in DHA, and a DHA/EPA ratio of 2.5/1. **E3** was based on a mixture of different TAG sources (Incromega DHA500 TAG and cod liver oil) although a slight supplementation with soybean lecithin was performed to help emulsification and absorption of lipids. Finally, **E2** emulsion was formulated on a blend of these three lipid sources. The three experimental emulsions were additionally supplemented with free arachidonic acid (AA) (Sigma Aldrich, Madrid, Spain) and emulsified with 0.5 g of egg yolk. A commercial booster rich in TAG was also used as a control (C).



it was concluded that rotifers enriched for short periods (3-6h) with 6% of the marine lecithin with a slight supplementation of arachidonic acid in combination with a range of carotenoids (50, 100 or 150 ppm Naturose ~2% astaxanthin), well below 50 ppm, might improve larval performance at early life stages.

Larval rearing

Newly hatched larvae of greater amberjack, at a total density of 5000 larvae per tank (mean total length 3.14 ± 0.08 mm), were randomly distributed in 12 experimental tanks of 100 l capacity. From 3 to 11 dph, rotifers in the tanks were adjusted to 5 individuals ml^{-1} and increased to 10 individuals ml^{-1} until the end of the trial. Rotifers were enriched with one of four treatments: the rotifer enrichment commercial protocol (C) plus three experimental emulsions (E1, E1,10 and E3,10) added at a 6% concentration for 3h to the rotifer enrichment tanks. For welfare indicator, cortisol level in whole larvae was determined.

Results

Rotifer and larval performance

The different treatments assayed showed significant differences at 14 dph. Fish total length (TL) in Control group was significantly lower. The lowest survival was recorded in those larvae receiving the treatment based on triacylglycerols (TAG) (E3), plus Naturose (Fig. 12) and it was significantly different to treatment based in lipid sources rich in polar lipids (E1), plus Naturose.

Welfare

Elevated and significantly higher ($P < 0.05$) whole body cortisol levels were observed in larvae fed treatment E1 at 14 dph. On the contrary the cortisol level of larvae from treatment E1,10 was the lowest one.

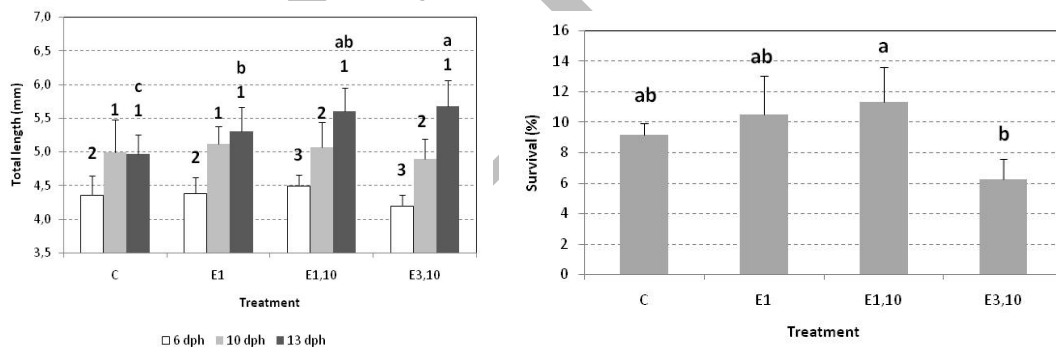


Figure 12. Total length (mm) and survival of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental emulsions at 6, 10 and 14 dph.

The list of optimum levels and ratios of EFA and carotenoids in enrichment products after the studies conducted is:

- ✓ DHA in enrichment products for *Artemia* 10-17% TFA
- ✓ EPA in enrichment products for *Artemia* 14-20% TFA
- ✓ DHA/EPA in enrichment products for *Artemia* 1-5
- ✓ DHA in enrichment products for rotifers 14% TFA
- ✓ EPA in enrichment products for rotifers 6% TFA
- ✓ DHA/EPA in enrichment products for rotifers 2.3
- ✓ Carotenoids levels in enrichment products 10 ppm



At on-growing level, an experiment with different levels of Lysine was conducted at Institute of Marine Biology, Biotechnology and Aquaculture, HCMR, in Crete island, Grece. (By Kotzamanis, Y. (HCMR) and Fontanillas, R. (Skretting ARC). Six different levels of added lysine were formulated (**Table 3**). Growth and antioxidant enzymes were studied.

Table 3. Diet formulation and analyzed chemical composition of the experimental diets based mainly on plant ingredients and supplied with different levels of lysine

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

The antioxidant capacity was investigated through determining the activity of catalase (CAT) in the liver and in the intestine. The specific activity of CAT was significantly diminished in the liver and the intestine of fish fed the L3 diet, indicating a possible protecting mechanism of lysine substitution in this dose.

The results from the present study indicated that the dietary lysine requirements, based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, was 2.11% of diet. No significant effect of lysine levels on the expression of HSP in liver or intestine was found. Lysine supplementation found to affect the specific activity of CAT in liver and intestine of greater amberjack fed the diet containing 2.11% lysine (**Fig. 13**)

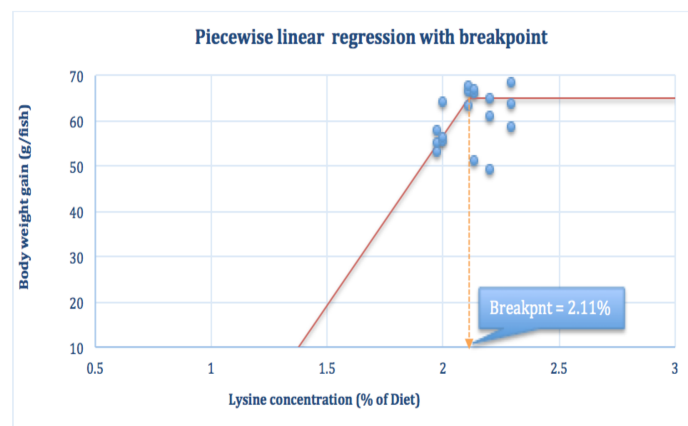


Figure 13. Broken line analysis of weight gain (g/fish) in greater amberjack fed graded levels of dietary lysine. Values of the X-axis are the lysine levels in the experimental diets, while each Y-axis values represent the body weight gain values of each tank. $Y = (1.215 + 0.0135 * X) * (Y \leq 2.11) + Y = 60.58 * (Y > 2.11)$, $R^2 = 0.91$



Feeding regimes for broodstock to optimize reproduction



Figure 14. Greater amberjack embryo (insert) and anaesthetised broodstock in the tank ready for manipulation.

Three different dietary experiments were conducted in the facilities located at Canary Islands (Spain) (**Fig.14**). The first experiment was designed to determine the effect of increased protein, histidine and taurine dietary levels on egg quality, the second one to determine the optimum ARA, DHA and EPA levels as essential fatty acids for reproductive success, and the third one to examine the effects of an experimental diet with a potentially improved formula of lipids, on reproductive development of hatchery produced greater amberjack.

Effects of increased protein, histidine and taurine dietary levels on egg quality

Hipólito Fernandez-Palacios, Marisol Izquierdo, Samira Sarih (University of Las Palmas de Gran Canaria), Ramon Fontanillas, Grethe Rosenlund (Skretting ARC).

Greater amberjack broodfish (12.19 ± 1.35 kg and 11.79 ± 2.05 kg females and males body weight, respectively) were distributed in three 40 m³ (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) in order to achieve a similar initial biomass in all tanks (1.29 kg/m³, 1.29 kg/m³ and 1.24 kg/m³) at Facilities of Parque Científico Tecnológico of University of Las Palmas de Gran Canaria (Canary Islands). From June 29th to October 31st, broodfish were fed with three different diets. Diets were formulated and produced in Norway by Skretting ARC. Three diets were formulated to be higher in either histidine, taurine or protein (**Table 4**).

Fish were hand feed twice a day and 5 days a week (1% of biomass day⁻¹). After 24 days of feeding each experimental diet, spawning quality was separately studied for each of the 2 couples for each diet during 10 consecutive spawns. Spawning quality was determined as: fertilization rate (%), egg viability rate (%), hatching and larval survival at 1 and 3 days post-hatching (dph), using two replicates of 96-well microtiter plates. With these percentages, the total numbers of fertilized, 24h viable and hatched eggs and larvae produced at 1 and 3 dph were calculated. Also, for each spawn the female fecundity (egg/female), the number of eggs per spawn and the relative fecundity (eggs/female kg) were determined.

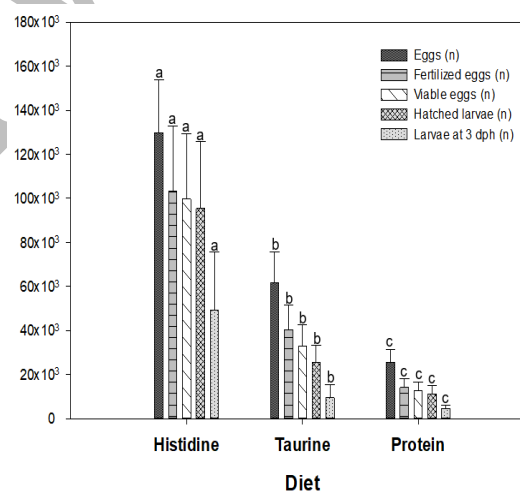
**Table 4.** Raw material composition and proximate analysis of the diets experimental diets

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

Fish were hand feed twice a day and 5 days a week (1% of biomass day⁻¹). After 24 days of feeding each experimental diet, spawning quality was separately studied for each of the 2 couples for each diet during 10 consecutive spawns. Spawning quality was determined as: fertilization rate (%), egg viability rate (%), hatching and larval survival at 1 and 3 days post-hatching (dph), using two replicates of 96-well microtiter plates. With these percentages, the total numbers of fertilized, 24h viable and hatched eggs and larvae produced at 1 and 3 dph were calculated. Also, for each spawn the female fecundity (egg/female), the number of eggs per spawn and the relative fecundity (eggs/female kg) were determined.

Results from the present experiment showed that feeding the experimental diets markedly affected spawning quality (**Fig. 15**). Particularly, fertilization rates were significantly higher when broodstock were fed a high histidine diet. Inclusion of histidine also produced higher percentages of viable eggs. The same trend was found in hatching and larval survival rates. The female fecundity and the average number of eggs per spawn along was highest in spawns from broodstock fed the diet rich in histidine, being over 4 and 5 times higher than in broodstock fed higher taurine or higher protein levels.

As a consequence of the higher spawning quality rates and total egg production, total number of fertilized and viable eggs, and total number of larvae produced were significantly ($P < 0.01$) higher for broodstock fed higher histidine. The amino acid composition of the fertilized eggs from broodstock fed the different experimental diets was similar. Egg diameter and total and standard length of the newly hatched larvae from broodstock fed higher histidine.

**Figure 15.** Production rates (per kg female and spawn) after feeding the diets.



The results of this study have pointed out the importance of raising histidine contents in broodstock diets from 1 to 1.5% to optimize the reproductive performance of greater amberjack, particularly to improve fecundity, fertilization rates, and egg and larval quality. Besides, the study showed that taurine levels in broodstock diets increase fecundity. Increasing protein contents over 51% lead to the lowest number of egg and larvae produced, suggesting that this dietary protein level is enough to cover greater amberjack broodstock requirements (Fig. 16).



Figure 16. Greater amberjack broodstock in the recuperation tank.

2. The optimum DHA and EPA levels as essential fatty acids for reproductive success

Hipólito Fernandez-Palacios, Marisol Izquierdo, Samira Sarih (University of Las Palmas de Gran Canaria), Ramon Fontanillas, Grethe Rosenlund (Skretting ARC)

Greater amberjack broodfish (12.19 ± 1.35 kg and 11.79 ± 2.05 kg females and males body weight, respectively) were distributed in three 40 m³ (5 m x 2.35 m) circular tanks (2♀ and 2♂ in each tank, sex ratio 1:1) in order to achieve a similar initial biomass in all tanks (1.29 kg/m³, 1.29 kg/m³ and 1.24 kg/m³) at Facilities of Parque Científico Tecnológico of University of Las Palmas de Gran Canaria (Canary Islands). Protocol followed was equal than that described previously in the protein/histidine/taurine experiment, and analyses conducted focused on egg and new hatched larvae quality as described previously. The dietary treatments used in the present study were formulated to have graded levels of DHA+EPA, ranging from 2.8 to 0.96 (% total fatty acids), and with a crude protein content of 59% and crude fat of 25 %, produced by Skretting ARC (Table 5).

Table 5. Raw material composition and proximate analysis of the diets

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

Results from the present experiment showed that the best spawn quality and production parameters were obtained from broodstock fed the diet based on 1.57% EPA+DHA, and the diet based on 0.96% EPA+DHA. The lowest fertilization and egg viability were obtained from broodstock fed diet based on



2.8% EPA+DHA). The composition of eggs were affected by broodstock diets, those reflecting the fatty acid content of the diets (**Table 6, Fig 14,**).

Table 6. Reproductive performance of broods after feeding the diets

Diet	% Fertilization	% Viable 24h	% Hatching
1	52.42±10.64 ^c	90.28±3.28 ^b	76.99±8.94 ^b
2	69.02±7.38 ^b	85.07±1.73 ^c	79.68±3.74 ^b
3	91.76±3.12 ^a	95.99±2.81 ^a	94.22±3.62 ^a
4	86.32±1.67 ^a	93.88±2.48 ^a	92.51±2.27 ^a

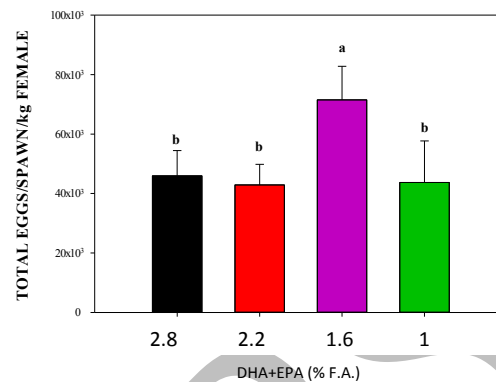


Figure 17. Total eggs produced by kg of female fed the different experimental.

3. Experimental diets with optimized Essential Fatty acids and carotenoid content

Salvador Jerez. M. Virginia Martín, Instituto Español de Oceanografía (Spain) and Covadonga Rodríguez and José Pérez, University of La Laguna (Spain). Ramon Fontanillas & Grethe Rosenlund (Skretting ARC)

The greater amberjack consisted of 50 PIT-tagged hatchery-produced F2 fish, since 2014. In February 2018, 50 broodstock reared in the facilities of Centro Oceanográfico de Canarias (IEO, P8), in Tenerife, Spain were distributed in three groups which were fed on different diets: 1) A Control Group (7 ♀ 7.6±1.2 Kg and 12 ♂ 6.0±1.1 Kg), fed a commercial pellet for turbot (52/20) (Skretting), 2) a Mackerel Group (7 ♀ 6.6±0.9 Kg and 8 ♂ 5.9±0.8 Kg), fed on frozen mackerel (*Scomber colias*) supplemented with a vitamin premix, and c) an Experimental Group (5 ♀ 6.8±1.1Kg and 10 ♂ 5.6±0.8 Kg), fed with a diet manufactured by P20 (Skretting Ltd., Norway). The latter was formulated accordingly to certain pre-requisites established by ULL(P15)/IEO(P8), to contain a lower lipid level, higher proportions of polar lipids and a different fatty acid profile, with particular emphasis to essential fatty acids (EFA; ARA, EPA, and DHA) (**Table 7**), maximizing the presence of specific marine-origin ingredients, including carotenoids.

Fish were maintained in three outdoor covered raceway tanks of 500 m³ with continuous water supply (6 renewals day⁻¹) under natural photoperiod and seawater temperature (19.8±1.1°C) and hand-feeding once a day and 3 days a week to apparent satiation. Greater amberjack breeders were sampled in June, July and August 2018 and the fish treated with an Ethylene-Vinyl acetate (EVAc) GnRH_a implant at the sampling times of July and August. In July 2 males and 2 females of each group were treated with dose of 40-50 µg GnRH_a kg⁻¹, and in August 3 males and 3 females of Mackerel and Experimental groups were implanted with a higher dose (80-100 µg GnRH_a kg⁻¹). The Control group was not treated in August, because there was a high mortality (60%) due to parasitization in the group.



Table 7. Main fatty acid composition (% of total fatty acids) of the experimental diets. +++ indicates a mayor supplementation

Groups	Mackerel	Control	Experimental
18:1n-9	5.5±1.6	26.0±0.5	11.5±0.2
18:2n-6	1.3±0.3	10.4±0.2	7.8±0.2
20:4n-6 (ARA)	2.8±0.5	0.5±0.0	1.4±0.0
20:5n-3 (EPA)	5.5±1.2	6.8±0.2	11.8±0.0
22:6n-3 (DHA)	36.0±6.3	7.1±0.1	14.6±0.3
DHA/EPA	6.5±0.6	1.0±0.0	1.2±0.0
ARA/EPA	0.5±0.1	0.1±0.0	0.1±0.0
Total Lipids	9.6±2.5	19.3±1.8	15±0.3
Polar Lipids			+++
Marine-origin ingredients			+++

The feed intake increased from April to May and declined after with mean (\pm SD) ration (Food per body weight %) of 4.6 ± 2.7 % for Mackerel Group, and 1.7 ± 1.1 and 1.8 ± 0.7 % for Control and Experimental Groups, respectively. The mean oocyte diameter decreased from June to July in all groups, but while the oocyte size of Mackerel Group continued decreasing, the size of oocyte of some females of Experimental Group was maintained or increased (**Fig 18**).

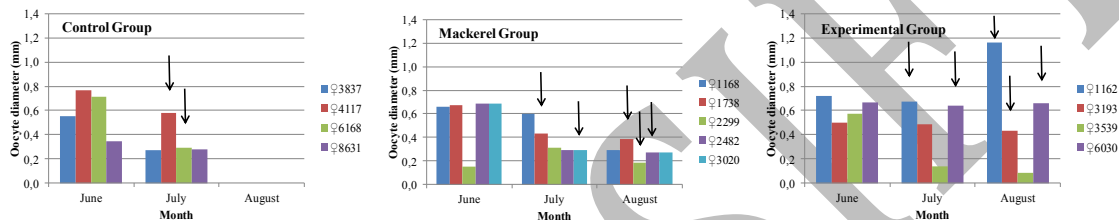


Figure 18. Oocyte diameter of individual (PIT-tag code) females biopsied in each group at different times during the reproductive season. The black arrows indicate the female implanted in each group.

Sperm quality parameters of sperm motility (%) and sperm motility duration (sec) tended to decrease at each sampling time except in the Experimental Group that maintained similar values in all sampling points (**Fig. 19**).

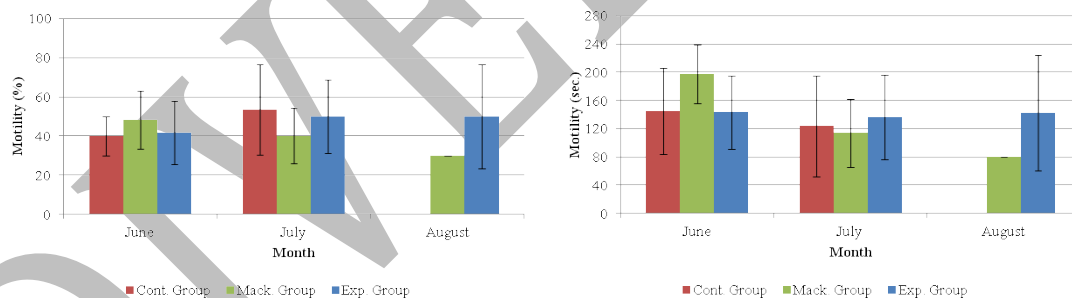


Figure 19. Sperm motility (%) and duration (sec.) of individual (PIT-tag code) males biopsied in each group at different times during the reproductive season.

So far, the three groups of bloodstock have not released eggs naturally or induced, although the biopsy of one female from the Experimental Group implanted in July showed mature eggs in August and was then implanted again with a higher dose of GnRHa. The results obtained in the recent sampling (September 4) showed a similar trend. Two females of Experimental Group implanted in August showed mature eggs ($>1100 \mu\text{m}$) while the females of the other two groups showed significantly lower oocyte diameters.

The fact that the temperature has not increased in the usual way and the fish are young (4 years old) could be related to the absence of spawns. In any case, although not conclusively, the results shown until now suggest that the Experimental diet could have positive effects on the reproduction of greater amberjack. A few samples of oocyte biopsies are being analyzed by ULL to confirm the dietary potential influence.



Larval husbandry

In order to obtain new information on the larval rearing parameters of the greater amberjack a series of studies were performed. Starting with the ontogeny of the digestion and the vision system to acquire the basic biological information, our studies focused on the prey enriching diet and feeding regime and finally to critical parameters (tank type-shape, duration of the photo phase, tank background color and light conditions, stocking density) of the rearing. The results were evaluated in terms of growth, survival, skeletal deformities, biochemical composition, stress and larval condition.

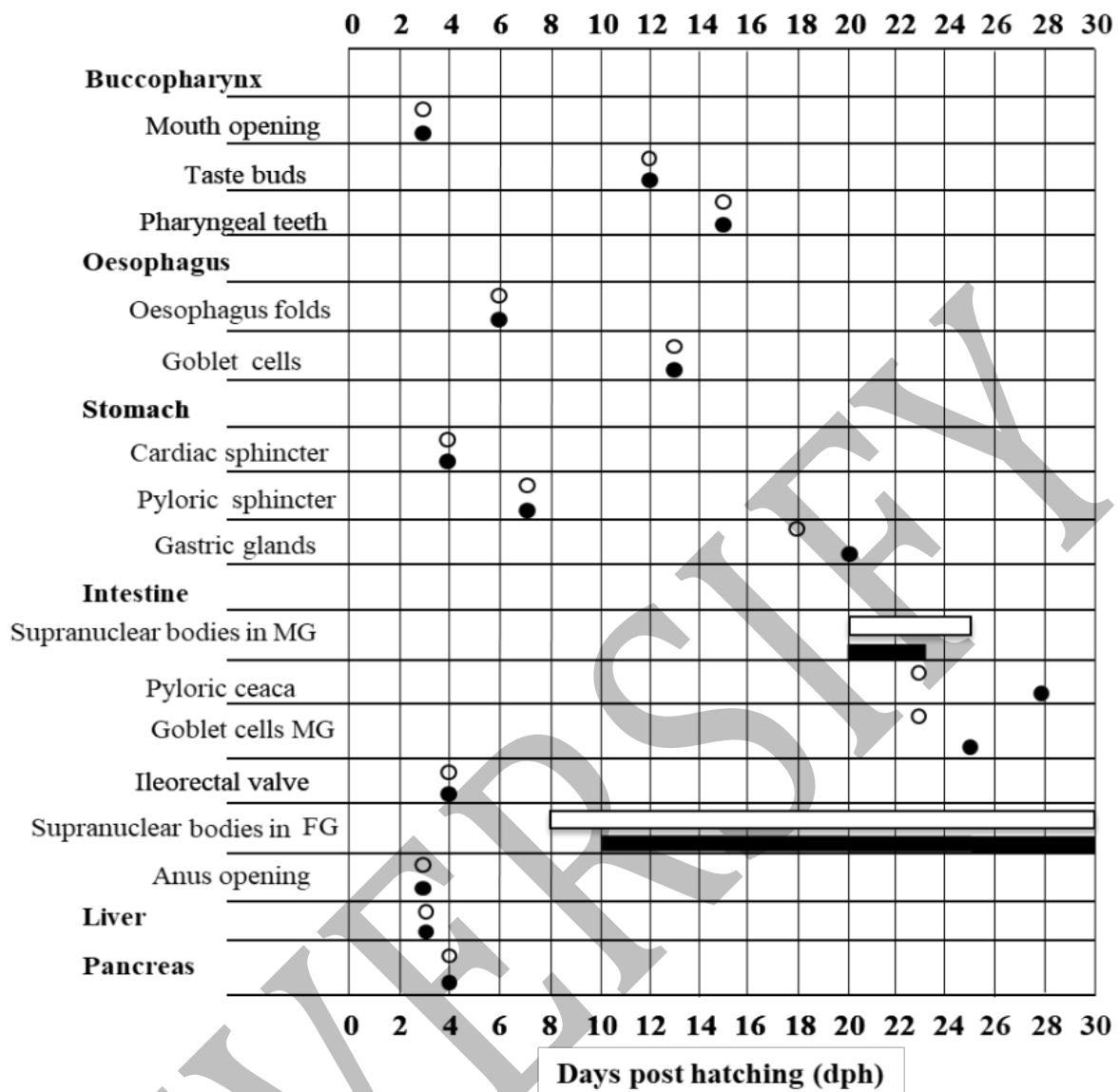
Ontogeny of digestive and vision systems

I. E. Papadakis, N. Papandroulakis, HCMR, Greece and C. Rodriguez, J. Perez, ULL, Spain

The digestive system enables the fish to capture, ingest, digest and finally absorb nutrients from the food, which are then transported across the intestinal epithelium to the blood (Rønnestad *et al.*, 2013). Thus, this system and its associated organs are of special relevance to establish the feeding protocols to be used during the larval rearing, determining its qualitative and quantitative characteristics. In order to describe the ontogeny of the digestive system of greater amberjack, larvae were reared under two different rearing systems, the mesocosm (MES) and the intensive (INT) and the trials were performed in the facilities of the HCMR, Crete. Eggs from induced spawning of breeders kept in a cage farm (ARGO) were used for the rearing. For the MES rearing a 40-m³ indoor tank filled with filtered (5 µm) natural seawater (salinity 40 psu) treated with UV, which was also the water for subsequent renewal was used. Seawater temperature was maintained at 24 ± 0.7 °C and the pH fluctuated from 7.99 to 8.18 during the trial. Dissolved oxygen varied from 5.8 to 6.8 mg l⁻¹ during the larval rearing. The photoperiod was adjusted to constant light from mouth opening to 25 dph and then to 18L:06D for the remaining experimental period. Light intensity varied according to the weather conditions between 500 lux on cloudy days to 1,000 lux on sunny days, while during the night when prolonged photophase was applied, light intensity was about 250 lux. For the INT the experimental system consisted of 500-l cylindro-conical tanks, organized as closed water recirculating system coupled to a biological filter. The tanks were filled with borehole 35 psu-water. Temperature was kept at 22 ± 0.5°C during the autotrophic stage and was gradually increased to 24 ± 0.5°C after mouth opening. The pH fluctuated from 8.0 to 8.2 and the dissolved oxygen ranged from 6.8 to 7.2 mg l⁻¹. The photophase was 24L:00D from mouth opening until 25 dph and then 18L:06D for the remaining experimental period. Light intensity varied between 200 - 800 lux during the day, and was ~200 lux at night. Microalgae (*Chlorella sp*) and enriched rotifers (*Brachionus sp*) were daily added in the tanks from 3 dph to 23 dph. *Artemia* AF A₀ nauplii (12 to 14 dph) and enriched *Artemia* EG A₁ nauplii (14 to 30 dph) were offered to the larvae. In both rearing systems, artificial feeds were added progressively according to fish size (grain size 200–300 µm; and 300–500 µm) from 16 dph (MES) and 21 dph (INT). Frozen gilthead sea bream eggs in the embryo stage were also introduced in the MES rearing tanks after 20 dph. Mesocosm tanks developed also naturally zooplankton (haracticoida copepods) which potentially contributed to larval feeding.

The ontogenesis of the digestive system of greater amberjack is considered as a rapid process. The comparison of the digestive system ontogenesis between the greater amberjack larvae coming from the MES and the INT rearing systems during the trial are presented in Figure 19. The activity of digestive enzymes of greater amberjack larvae in the INT system is presented in Figure 20 while a comparison of the INT and MES systems is presented in **Fig. 20**.

In the present study, amylase, lipase and alkaline phosphatase activities were found before the onset of the exogenous feeding, suggesting the importance of egg glycogen catabolism as energy during the embryonic development. Following mouth opening (3-5 dph) the enzymatic activity seems to be more related to the degradation of substrates from the yolk sac (lecithotrophic phase) than to exogenous feeding and, the highest activity of protease with respect to that of lipase suggests that proteins are the main energy source during this stage. With increasing age, the higher lipid deposition registered in the liver of both MES and INT-larvae, together with the rise in the enzymatic activity of amylase, lipase and alkaline protease, clearly suggest the proper nutritional condition of the larvae. During this period, the feeding protocols were based mainly in rotifers, which were identified in the larval stomach content.



	0	1	2	3	4	5	6	7	8	9	12	15	17	20	23	25	28	30
INT	3.52	3.72	3.73	3.81	3.96	4.01	3.94	3.98	4.23	4.23	4.55	5.26	5.19	6.01	6.35	7.77	8.47	8.20
MES	3.52	3.67	3.75	3.83	3.99	4.05	4.03	4.02	4.05	4.09	4.53	5.00	5.69	6.49	7.2	9.23	8.54	8.74

Total length in (mm)

Figure 20. Schematic representation of the digestive system main developmental structures appearance (o MES, • INT). Horizontal bars (white MES, black INT) indicate the period of supranuclear bodies (vacuoles) presence in the mid gut and hindgut. Mean TL values are presented in the below table.

One of the most important structures of the digestive system are the gastric glands that in greater amberjack larvae appeared after 5.5 mm of TL in all the rearing systems. With the appearance of the gastric glands, from a morphological point of view, begins the development of a functional stomach marking the transition from larval to juvenile function of the digestive system. Independently of the rearing system, between 11 and 15 dph, the percentage of area covered by lipid vacuoles in the liver decreased significantly compared to the initial days of life. This lipid reduction in the liver was accompanied by a decrease in amylase, lipase and alkaline protease activities, suggesting a period of



malnutrition. During this phase, *Artemia* nauplii were offered to larvae for three days (12 to 14 dph) in both rearing systems. As in the previous period, rotifers but no *Artemia* nauplii were detected in the stomach, highlighting the reduced ability of greater amberjack larvae to assimilate this food during the transition from one to the other (Fig. 21 and 22).

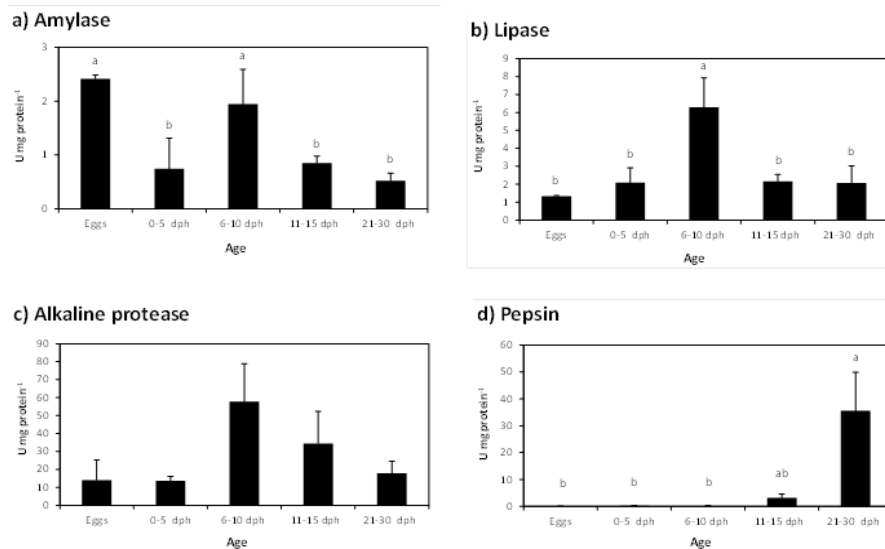


Figure 21. Activity of digestive enzymes during the ontogeny of the digestive tract of greater amberjack cultured under intensive conditions.

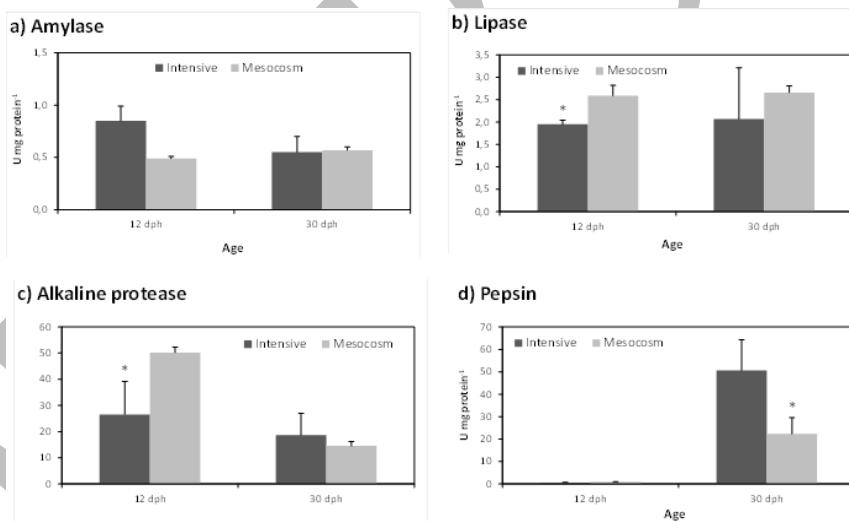


Figure 22. Comparison of digestive enzyme activities of greater amberjack larvae between intensive and mesocosm culture systems at 12 and 30 dph.

Acidic digestion rose from 20 dph, which agrees with the histologic observations where gastric glands started to differentiate at approximately 16 dph and completed its definitive histological organization at 20 dph. The increase in pepsin activity was correlated to a decrease in protease activity and related to a change in the digestive achievement of an adult-type protein digestion (Gisbert *et al.*, 2008). Our results show the relevance of protein digestion at alkaline pH, just before the onset of acidic digestion in the stomach at 15 dph where pepsin activity increased between 20 and 30 dph.

Vision system. At the day of hatching (0 dph) the retina appeared as a simple hemispherical sheet of undifferentiated neural epithelium (UNE) enclosing the lens, which comprises of a spiral of



unspecialized cells (Fig. 8a). The first differentiation was visible between 1-2 dph (Fig. 8b), when the ganglia cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL) and the photoreceptor layer (PL) appeared. From 3 dph onwards, the pigment epithelium PE appeared on the external area of the retina (Fig. 23c). The nucleus of the cone cells appeared at 3 dph in the outer nuclear layer, along with all the other neutral cells in the inner nuclear layer (amacrine, bipolar and horizontal cells), which were completely distinct.

A second population of darker skinned core cells, the nuclei of rod cells (RC), appeared in the ONL at different times for each rearing system but at the same fish total length, due to different growth rates between rearing systems. Rods first appeared at 15 dph in the INT (5.0 ± 0.2 mm) and the MES (5.3 ± 0.2 mm) rearing system, respectively. The number of rods, which were identified by their nucleus and were placed in the ONL, increased over time (Fig. 8d).

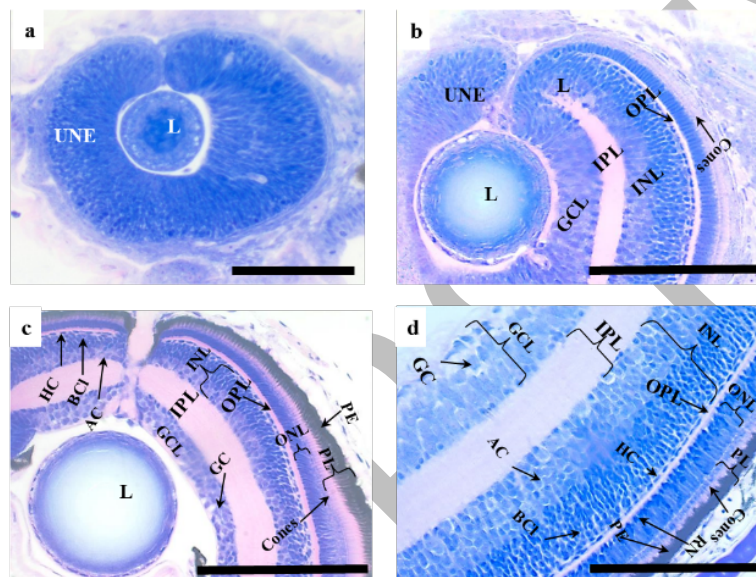


Figure 23. Histological sections (a) at hatching showing the undifferentiated neural epithelium, (b) at 2 dph showing the onset of retina differentiation, (c) at 3 dph showing the pigment epithelium of the retina, (d) at 30 dph showing the structure of the retina with the rod cells placed at the outer nuclear layer. AC= amacrine cells, BCI= bipolar cells, GCL= ganglia cell layer, GC= ganglia cells, HC= Horizontal Cells, INL= inner nuclear layer, IPL= inner plexiform layer, L= lens, OPL= outer plexiform layer, ONL=outer nuclear layer, PL= photoreceptor layer, PE= pigment epithelium, RN = Rod nuclei, UNE= undifferentiated neural epithelium. Bar represents 100 μ m.

Effect of feeding regime and probiotics

S. Jerez and M. Virginia Martín, IEO, Spain; José Pérez and Covadonga Rodríguez, ULL, Spain

Towards a feeding strategy for the larval rearing of greater amberjack a study was implemented, at IEO Tenerife Facility in collaboration with ULL, for the effect of (a) prey concentration and supply frequency and the (b) use of immune modulators substances during the rotifer administration. The results were evaluated in terms of survival, growth, physiological parameters (oxidative stress and immune system) and ontogeny of the digestive enzymes.

Following a preliminary trial for selecting products and period of enrichment, the enrichment protocols were defined. A commercial diet (T1) was compared with three experimental treatments (T2, T3 and T4) consisted of LC60/20:4n-6/10ppm carotenoids basic emulsion (T2), combined with 20% *Echium* oil (T3) and 20% black cumin oil (T4).



In a first trial two prey concentrations were tested, 5 (Low density) and 10 (High density) rotifers ml⁻¹. Fish survival was very low at the end of the feeding period independently of dietary regime and prey density treatment. There was a clear but non-significant ($P>0.05$) trend of increased survival in T3 and T4 treatments compared to T1 fish. In terms of growth, at 7 dph, larvae were significantly ($P<0.05$) smaller when fed the commercial treatment (T1). Regardless of dietary regime, the density of rotifers (5 or 10 rots ml⁻¹) did not significantly affect fish growth performance and feeding behavior.

A second experiment was carried out in order to test the combined effect of enrichment products containing immune-stimulants (T3, T4) and the feeding frequency on greater amberjack larval performance in terms of digestive enzymes activities as well as immunity and oxidative stress status.

Two different egg sources and broodstock groups were used in two trials, from IEO facilities and from FCPCT. 100 l experimental tanks were used in a flow through system. Light was continuous at 700 lux, temperature was at $22.1 \pm 0.1^\circ\text{C}$ and dissolved oxygen was $>90\%$ saturation. In both trials, from 3 dph to the end of the experimental period (12 dph), rotifers were adjusted to 5 rots ml⁻¹ in the larval culture tank. They were distributed to the larval tanks twice (10:30 h and 20:30 h) or three times (10:30 h, 15:30 h and 20:30 h) day⁻¹.

Larval growth and survival were similar in both trials. In Trial 1 no statistically significant ($P>0.05$) differences were detected in larval survival between different treatments. In Trial 2, survival was in general higher and, larvae fed 3 times a day tended to survive better than those fed 2 times for T2 and T4 treatments.

Regarding the digestive enzymes, results clearly show that alkaline proteases and lipase activities were significantly higher ($P<0.05$) in larvae receiving the T4 diet whereas no marked differences were found for amylase activity among treatments (Fig. 24).

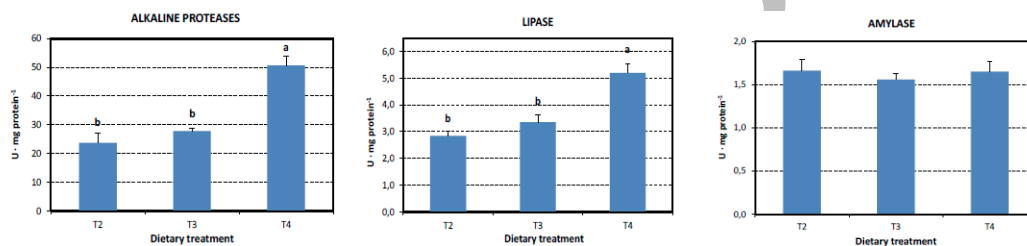


Figure 24. Alkaline proteases, lipase and amylase activities of 12 dph larvae fed rotifers from T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids+20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids +20% Black cumin oil). Values are mean \pm SD (n=3). Different letters indicate significant differences between treatments irrespective of the feeding frequency.

The levels of antioxidant enzymatic activities and lipid peroxidation measured are shown in Fig. 25. Despite a lack of differences due to the high variability of data, a general trend to increase GST activities with age is evident for all dietary treatments. A trend for a lower SOD activity is also noticeable in T3 and T4 larvae at 12 dph. Finally, lipid peroxidation levels (based on TBARS presence) also seemed to be higher at 12 dph than at 7 dph.

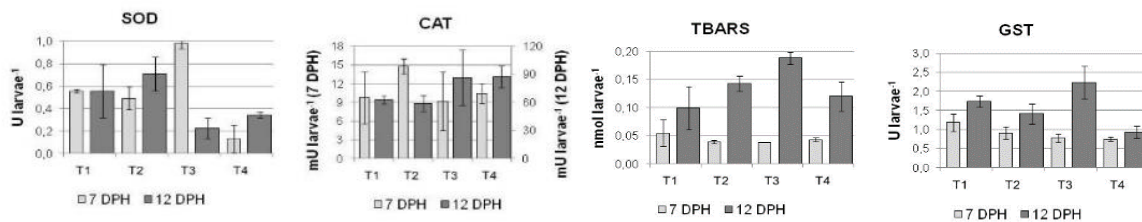


Figure 25. Antioxidant enzymes Superoxide dismutase (SOD), Catalase (CAT), lipid peroxidation (thiobarbituric acid reacting substances, TBARS) and Glutathione S-transferase (GST), of 7 and 12 dph larvae fed rotifers from different treatments (T1, T2, T3 and T4). Values are mean \pm SD (n=2).



The T4-larvae showed lower levels of several activities (Fig. 26) in the humoral innate immune results, presented significantly ($P < 0.05$) lower peroxidase activity than T1-larvae at 7 dph and the markedly lowest ($P < 0.05$) activity of all groups at 12 dph. The levels of bactericidal activity were also significantly ($P < 0.05$) lower in both 7 and 12 dph T4 larvae than in any other group. Protease activity decreased in T3 and T4 7 dph larvae compared to T1 fish while T4 larvae demonstrated a non-significant ($P > 0.05$) but clear decrease compared to the other treatments at 12 dph.

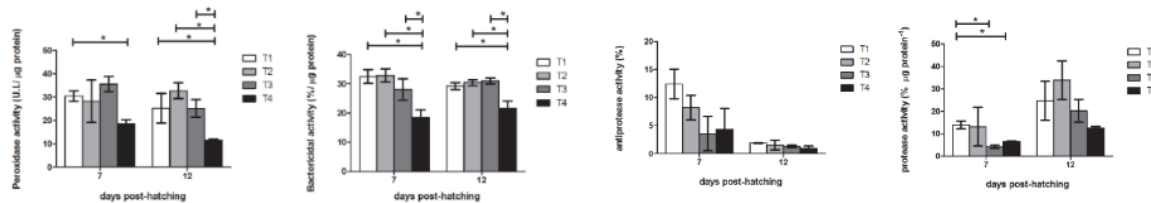


Figure 26. Humoral innate immune activities of 7 dph and 12 dph of larvae fed rotifers enriched with T1, T2, T3 and T4. Asterisks determine differences between groups.

The larvae growth was similar regarding rotifer density, showing slightly better performance at the low one (5 rot ml^{-1}) while the best growth and survival were demonstrated when the larvae were fed 3 times day^{-1} . The better survival in the T3 and T4 treatments, coinciding with the significantly higher percentages of larvae with swim bladder inflated at 12 dph that took place when the larvae were offered a lower rotifer density. Significant effect on larval length was observed with the T4 treatment. The beneficial effect of black cumin seed oil has been reported recently on growth and immune system in fish (Atwa, 1997; John *et al.*, 2007; Awad *et al.*, 2013).

For the digestive enzyme activities, they were higher in fish feeding on black cumin supplemented rotifers where higher lipase and protease alkaline activities were observed. This increased activity in better digestion and assimilation of dietary protein and lipid promoting feed efficiency. However, amylase activity, the major enzyme associated with carbohydrate digestion was not increased by the assayed immune-stimulants, which is not surprising as this fish, at all developmental stages, are strict carnivores.

The present study also investigated the antioxidant defense enzyme activities and lipid peroxidation products in response to immunostimulants. The results showed that age was an important factor to consider determining the biochemical responses to oxidative stress. An increase in some of the antioxidant activities was observed from 7 to 12 dph larvae independent of treatment. The activities of the SOD and GST enzymes were affected by immunostimulants differently depending on the age of the larvae. SOD is a primary radical scavenging enzyme while GST metabolizes lipid hydroperoxides. The activities of these two enzymes were reduced by *Echium* oil and cumin oil at 12 dph but only SOD was reduced by cumin oil at 7 dph.

Effect of environmental parameters during rearing

C. M^a. Hernández Cruz and A. La Barbera, FCPCT, Spain; N. Papandroulakis, A. Tsalafouta and P. Anastasiadis, HCMR, Greece

The main objective of this study was to define some environmental and husbandry parameters towards an optimum larval rearing methodology. The tank hydrodynamics in two different cylindro-conical tank types were tested, the effect of three different background colors as well as two photophase regimes on larval performance was studied and also the effect of initial stocking density was studied.

The effect of tanks hydrodynamics and stocking density on larval performance.

Two different tanks types: 40,000 l and 2,000 l were tested in duplicates for a period of 30 days. In the 2,000 l tanks two initial stocking densities (10 and 20 larvae l^{-1}) were also tried. The effects on larval performance in terms of growth, survival, histology, biochemical composition and skeletal deformities



was determined.

The applied water exchange rates (as % of total water volume) in 2,000 and 40,000 l tanks were 10% and 4% per hour, respectively while air flow was 350 and 1,400 ml min⁻¹, respectively, similar to the ones applied during the current protocol for larval rearing. Current measurements were performed using a Vectrino, a high-resolution acoustic velocimeter used to measure 3D water velocity. Measurements of current field at specific depths and layers were performed.

Larval rearing was conducted with eggs from a natural spawning from a broodstock maintained in the facilities of FCPCT de la ULPGC, Spain. The eggs were stocked at two different densities in four 2,000 l tanks (10 and 20 eggs l⁻¹) while two 40,000 l tanks were stocked with 10 eggs l⁻¹ each. Photoperiod was natural (14:10 h, L:D), the salinity 37 psu, and temperature 25-27°C. Water DO was at 5–8 g l⁻¹. The green water technique was used, adding live phytoplankton (*Nannochloropsis sp.*) at 250,000 cells ml⁻¹ in the rearing tanks during feeding with enriched rotifers (1-30 dph), while enriched *Artemia* was delivered from 12-30 dph. From 13-30 dph, the fish were fed progressively larger microdiets of 75, 150 and 300 µm.

Results showed differences in current profiles between the 2,000 l (2.0x10⁻² to 5.0x10⁻² cm s⁻¹) and 40,000 l (2.0x10⁻² to 3.0x10⁻² cm s⁻¹) tanks, being generally higher in the 2,000 l tanks.

A graphical representation of the current profile is presented in **Fig. 27**.

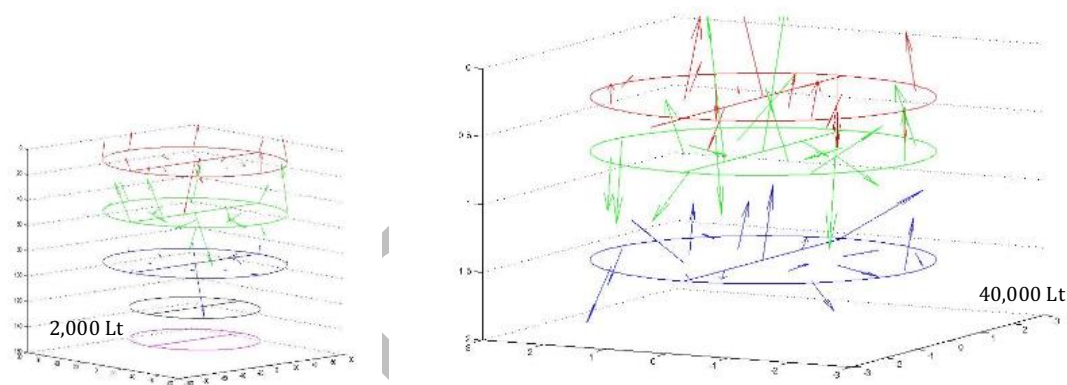


Figure 27. Current profiles in the different layers of the various tanks. Arrows are in 3-d representation and the observed size does not represent the actual velocity value.

Larval performance

Significant differences, in total length and body weight, were observed (**Figs. 28**) between treatments.

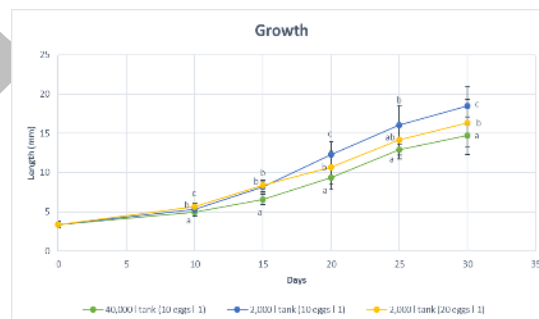


Figure 28. Larvae total length at different tanks and densities. Different letters indicate significant ($P < 0.05$) difference.

The larvae of 2,000 l tanks stocked at 10 eggs l⁻¹ grew better than the ones stocked at 20 eggs l⁻¹ as well as larvae from the 40,000 l tanks stocked with 10 eggs l⁻¹. Severe cannibalism and size dispersion were observed. Survival was higher in 2,000 l tanks compared to 4,000 l tanks but there was no difference



between the 10 and 20 eggs l⁻¹ treatments in the 2,000 l tanks. The proximate analysis of the samples showed no significant difference in lipid, protein and ash contents between the larvae of all treatments.

The fatty acids contents of the rotifers and *Artemia* presented no significant difference. The fatty acids analysis of the larvae showed that larvae of 2,000 l tanks stocked with 20 eggs l⁻¹ had the highest DHA (22:6n-3) content, while the larvae of 2,000 l tank 10 eggs l⁻¹ showed higher ARA (20:4n-6). In addition, the larvae of mesocosm 10 eggs l⁻¹ showed higher 18:3n-3. The liver of larvae cultured in 40,000 l tanks and 2,000 l tank (10 eggs l⁻¹) showed regular hepatocyte morphology with few cytoplasmic lipid vacuoles that did not alter hepatocyte size or shape.

The anomalies evaluation showed different types in all treatments as shown in **Fig. 29** but were independent of their effect as no significant difference was found ($P < 0.05$).

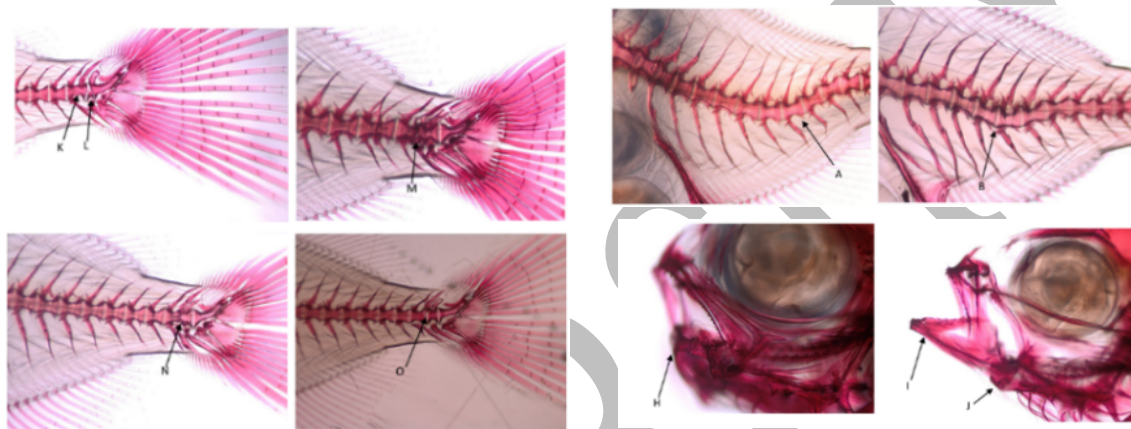


Figure 29. Different types of anomalies observed in greater amberjack larvae at different tanks and densities.

This study showed higher survival in the 2,000 l tanks, independent of egg stocking density, compared to the 40,000 l tanks, which was also true for growth, measured in total length and body weight. This was particularly apparent in 2,000 l tanks stocked with 10 eggs/l⁻¹. The histological study showed a regular hepatocyte morphology with few cytoplasmic lipid vacuoles in the mesocosm denoting better digestion and absorption of dietary lipids but larvae in 2,000 tanks with density 20 eggs l⁻¹ had a higher degree of vacuolization denoting poor digestion and absorption of dietary lipids that could be due to the stress of the high density, as observed in other species (Salhi *et al.*, 1999; Ramírez-Bolaños, 2016).

The results of anomalies evaluation showed a markedly appearance of different types in all treatments through the larval stage as lordosis, vertebral body fusion, and anomalous dentary, that could lead to a lower survival. In conclusion, according to the previous results, the adequate rearing density of greater amberjack is 10 eggs/l⁻¹ in 2,000 l tanks.

The effect of stocking density and tanks type on larval rearing

A specific study was performed for the effect of egg stocking density and the type of tank, on larval performance in terms of growth and survival. The trials were performed at the facility of FCPCT.

Greater amberjack eggs were stocked at different densities: 25, 50 and 75 eggs l⁻¹ in two different cylinder-conical tank types with volumes of 40,000 l and 2,000 l tanks and they were tested in duplicates, for a period of 30 days. Larval rearing was performed according to the protocol of the GIA, which requires a natural photoperiod (14:10 h, light:dark), as well as recommended salinity (37 psu) and temperature conditions ($26 \pm 1^\circ\text{C}$). Water renewal flow was increased progressively from 25% d⁻¹ to 200% h⁻¹. Water entered the tank from the bottom and was let out from the top; its quality was tested daily. Water was continuously aerated (125 ml min⁻¹), attaining 6.78 ± 0.5 ppm of dissolved O₂, saturation ranging between 60% and 80%. All tanks were equipped with a surface skimmer for



removing buoyant organic material. The green water technique was used adding live phytoplankton (*Nannochloropsis* sp.) to maintain a concentration of 250,000 cells ml⁻¹ in the rearing tanks during feeding with enriched rotifers and *Artemia*). Subsequently they were progressively fed microdiets of 75, 150 and 300 µm. Rearing and feeding conditions followed the protocol of the institute. Severe cannibalism and size dispersion were observed from 10-15 days post hatching (dph).

The treatment with 75 eggs l⁻¹ showed significantly increased TL (17.43 ± 4.19 mm) (**Fig. 30A**), whereas the treatment with 25 eggs l⁻¹ showed significantly increased survival (11.25%± 4.92) compared to the other treatments (**Fig. 30B**).

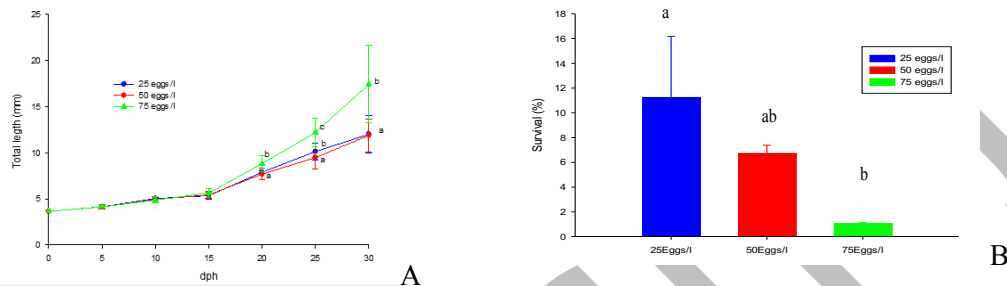


Figure 30. Total length (A) and Survival (B) of greater amberjack *Seriola dumerili* larvae (30 dph) at different densities; 25, 50 and 75 eggs l⁻¹. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The effect of light (intensity and duration) on larval rearing.

The studies were implemented at HCMR with eggs from induced spawns from broodstock kept in the Argosaronikos SA cage farm. The methodology applied for larval rearing was the intensive method, characterized by controlled conditions of water quality, light intensity, photophase and feeding. A variation, the so-called “pseudo-green” water, is based on the frequent addition of phytoplankton and zooplankton in the larval rearing tanks. Cylindro-conical 500 l tanks were organized in duplicates as closed water system with biological filter. Mechanically filtered borehole water of 35 psu was used to supply the tanks. Temperature was kept at 22±0.5 °C during the autotrophic stage and was gradually increased to 24±0.8 °C after mouth opening. The pH fluctuated from 7.8 to 8.2 and the dissolved oxygen from 5.0 to 7.4 mg l⁻¹. Light intensity varied between 200 - 800 lux during the day, and was about 200 lux during the night.

Feeding was based on enriched rotifers (from 3-21 dph), Instar II *Artemia* nauplii (from 12 dph onwards), and artificial diet (from 21 dph). The concentration of rotifers in the tank was maintained at 3.0 individuals ml⁻¹, while *Artemia* was added at 0.1 individuals ml⁻¹. Phytoplankton was added daily from 3 to 21 dph at 300 ± 100 x 10³ cells ml⁻¹. The plankton delivery was implemented with the use of an automated feeding system allowing continues administration of food.

The growth was estimated with regular measurements of total length and wet weight and at the end of rearing period (~25dph) populations were counted. In addition, we investigated the *somatotropic axis* that represent the endocrine and autocrine regulators for skeletal muscle growth and are known to play key roles in the regulation of metabolism and physiological processes. Samples taken at 3, 5, 17, 25 and 30 dph were used for the gene expression analysis.

Photoperiod trial. Two Light:Dark conditions (24:00 and 18:06 hours d⁻¹) were tested. The survival of the larvae varied between 6% and 13.6%. The mean survival for the 18L:06D photo phase was higher (10.6±4.2%), although not significantly than the 24L:00D one (8.2±3.1%). In terms of total length, larvae grew with an exponential rate of 0.310 d⁻¹ independent of photophase.



The trials revealed that the photoperiod (24L:00D vs 18L:06D) affected the mRNA expression levels of IGF-I with higher levels for the 18L:06D group at 17 dph and 25 dph compared to the 24L:00D group. Additionally, there was a statistically significant gradual increase ($P < 0.05$) in mRNA levels as development proceeded, which was observed only in the 18L:06D group with peak values at 25 dph (Fig. 31).

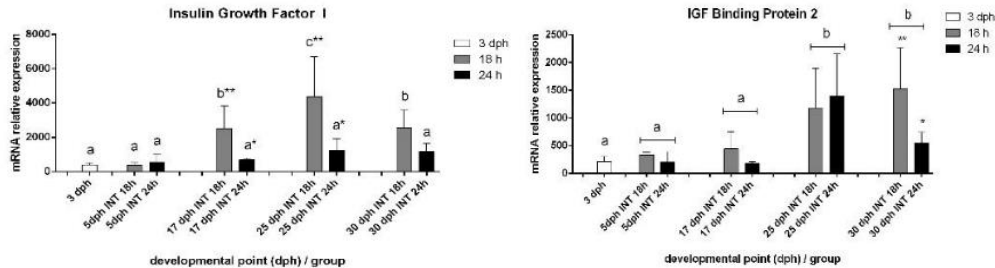


Figure 31. mRNA relative expression levels (means±SD; n= 4) of IGF-I and IGF Binding Protein 2. Different letters indicate differences between developmental points, asterisks between photoperiod schemes ($P < 0.05$).

The IGF-BP2 expression showed a gradual increase throughout development with statistically higher levels at 25 dph and 30 dph ($P < 0.05$). Additionally at 30 dph an effect of the photoperiod regime was observed with higher expression levels in the 18L:06D group compared to the 24L:00D group ($P < 0.05$; Fig. 31).

Light intensity trial

Tanks with three different colors (black, green and white; Fig. 32) were used in duplicates, while submerged lights (from 8:00 to 20:00 imitating the brighter period of the day) increased light intensity in the water column without significantly changing the intensity on tank's surface.



Figure 32. Tanks with different color backgrounds.

No statistically significant differences were observed in the growth of the larvae between the different tank colors. Fish growth was exponential in terms of TL (Black: 0.0481 d^{-1} , White: 0.0393 d^{-1} , Green: 0.0355 d^{-1}) and wet weight (Black: 0.1260 d^{-1} , White: 0.1970 d^{-1} , Green: 0.171 d^{-1}). However, significant differences were observed in the survival during the trial. White background resulted in significant higher survival rate ($22.2 \pm 0.7\%$) compared to green ($16.5 \pm 0.9\%$) and to black ($8.2 \pm 3.1\%$).

Furthermore, results showed that fish reared in the white background had increased levels of the genes implicated in the growth axis system compared to the fish reared in the black and green backgrounds. In particular, IGF-I showed generally higher levels of expression as development proceeds and also it appeared affected by the background color as higher levels were observed in fish from the white tank at 17 dph and 30 dph compared to fish reared in the black and green tanks (Fig. 33). GHRH expression levels remained low and stable throughout development in fish reared in the green background whereas in fish reared in the white one there was a statistically significant upregulation at 30 dph and for fish reared in the black background at 25 dph (Fig. 33). Additionally, background color affected the mRNA levels of GHRH with higher levels for the white background at 17dph, 25 dph and 30 dph (Fig. 35).

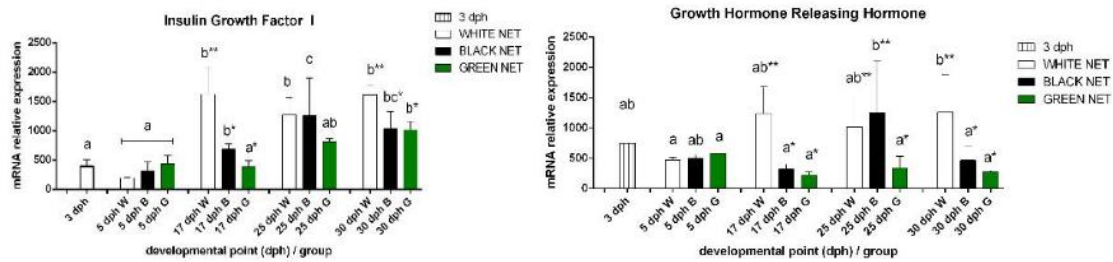


Figure 33. mRNA relative expression levels (means \pm SD; $n = 4$) of IGF-I and GHRH between the different background colors. Different letters indicate differences between the developmental points whereas asterisks between background colors ($P < 0.05$).

This study showed a catalytic effect of the tank background color on larval survival. The beneficial effect of the brighter environment on the performance of the greater amberjack may reflect requirements related to the pelagic nature of the species. Larvae of greater amberjack are found in nature in open seas sometimes associated to floating objects such as medusas etc. (personal observations of N. Papandroulakis and GMFMC 2004). The light conditions there are characterized by the high intensities and the associated transparency of the water, which is hardly imitated in rearing conditions. The use of white background and the addition of a submerged light changed the rearing environment allowing the better adaptation of the larvae to the rearing conditions.

At 30dph higher mRNA expression levels of several genes (IGF-I, IGF-II, IGF-BP2, IGF-BP3 and IGF-BP5) were observed in the larvae reared in tanks with white background compared to the other two backgrounds and especially the green color, which coincides with the differences observed in the survival rates. This preliminary study provides for the first-time information on the regulation of the various components of the IGF signaling pathway in greater amberjack and serve for better understanding the complex relationship between background color and fish performance at early ontogeny.

The presented results from the trials with the modified “light environment” of tanks are better at one order of magnitude of any previous reported showing the validity of the tested hypothesis. They are significant because the achieved survival rates are reported for first time, indicating a substantial technological step in the larval rearing of the greater amberjack.

Towards an industrial protocol

S. Jerez, C. Rodríguez (ULL), C.M. Hernández Cruz (FCPCT) and N. Papandroulakis (HCMR)

The results developed obtained were validated and tested in two commercial hatcheries in Greece during two successive years. The hatcheries had a small experience with the species. Both hatcheries used eggs from breeders kept in GMF and Argosaronikos SA farm.

In the first hatchery, incubation was directly in the larval tanks at a density of around 120 eggs l^{-1} . Following hatching the density of the larvae was about 75 ind l^{-1} , indicating a survival rate of 62%. Phytoplankton was added in the larvae tanks since day 2 and until day 15 post hatching. Light intensity was 800 lux on 3 dph, increased to 1200 lux on 6 dph until 12 dph when it was decreased to 1000 lux and gradually to 500 lux until 20 dph. The photophase was continue (24L:00D) from mouth opening to 20 dph when it was decreased to 18L:06D until 30 dph when it was set to natural. Feeding was based on enriched rotifers and subsequently with Artemia and dry feeds. Frozen eggs were also added in the tanks after 20dph. Following the hatchery phase, individuals were transferred for weaning and selected in size. The final number of juveniles transferred for pre-growing was around 15.000 that were classified in 4 size-classes between 0.3 and 2.5 g.



The trials in the second hatchery were implemented again with direct incubation of eggs in the larval rearing tanks. According to the standard protocol of the hatchery eggs after transport are incubated and transferred only after mouth opening to larval rearing tanks, a procedure proved to be lethal for the larvae. During the 2017 period, the hatchery received 4 batches of eggs of 1.0, 1.2, 0.65 and 0.5Ms respectively. The rearing temperature was set at 24.5 to 25.0 °C. Larval rearing was performed following the standard protocol and the feeding was based on enriched rotifers, instar I and enriched instar II *Artemia* nauplii followed by artificial diets. The light conditions in some of the tanks were modified in order to increase the light intensity on the surface of the tanks at >1000 lux, resulting in significant higher survival. Following 20 dph, fish were selected in size and grouped accordingly, improving thus significantly the performance of the larvae and in particular their survival rate. The hatchery finally transferred in cages 48.300 juveniles of 25-50 g.

General recommendations

Rearing system

The larvae rearing system based on large tank and low initial stocking of eggs-larvae improves the growth performance and survival of greater amberjack.

Stocking density

Egg stocking densities upper than 25 eggs l⁻¹ affects negatively the results during the larva rearing of greater amberjack.

Light conditions

Photo phase of 24 L:00 D from 1 to 20 dph and 18 L: 06 D between 21 and 30 dph, light intensities of 800, 1200, 1000 and 500 lux at 3, 6, 12, and 20 dph, respectively.

Water quality

A renewal of filtered seawater (5µm) at an increasing rate ranging from 15-40% day⁻¹ at 1 dph, 30-40% at 10 dph, 100-120% at 20 dph, and 200-240% at 30 dph ensures a good quality of the rearing environment, maintained the basic physical conditions during the larvae rearing:

Dissolved oxygen ranged between 4.9 and 8.2 mg l⁻¹, preferably upper than 6.0 mg l⁻¹, salinity between 35 and 40 psu, pH between 7.8 and 8.5, and temperature ranged from 22 to 27°C, preferably between 23.5 and 25.0°C.

Feeding

The feeding protocols used have to be coordinated with the rearing conditions and the larval development. The larva has to be able to see, ingest and digest the food, and therefore needs the coordinated development of vision and digestive system. Larva rearing developed under conditions that allow faster growth have to consider the time of beginning and duration of the feeding periods with the different items. In general, the addition of live microalgae at 150-300 x 10³ cell ml⁻¹ from 1 dph, enriched rotifers (*Brachionus plicatilis*) two times a day, from 3 to 25 dph, at densities between 3 and 10 rot ml⁻¹, *Artemia* AF nauplii at 12 dph, during 5-7 days and enriched *Artemia* EG 1-day at 14-18 dph, and weaning diet (200-800 µm) from 18 dph look like a good sequence.

Moreover, the enriched emulsions of prey supplemented with phospholipids (PL), carotenoids, araquidonic acid (AA) and immune modulators such as *Echium* oil and black cumin oil improve the



larval rearing of greater amberjack, so enriching that tend to have these characteristics would give better results in the larval performance of greater amberjack.

Husbandry

During larval rearing and especially following 20dph high size variability occur in all rearing systems tested until today. The reasons for this variability are not known but a study is already in progress (HCMR, National project) that may identify the causative biological mechanisms. This high variability is confronted until now with early sorting of the reared groups in to appropriate size classes. Applying standard methods and equipment available in all hatcheries the sorting procedure although with losses results in significant higher survival compared to unsorted groups. Unsorted groups between 20 and 30 dph present mortality of more than 90% while for the sorted groups is limited to apx 10%.

During sorting, transport of the individuals is also a requisite. Individuals of less than 15 mm do not tolerate netting and transfer should performed with care. Avoiding air exposure. After reaching 20 mm in TL individuals can be netted normally.

Husbandry practice with larger individuals (>0.5-1 gr) are easier although in some cases light anesthesia may help.



Grow out husbandry

Development of rearing method in cages

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Cage rearing is important for the industrial application, but appears to be challenging. Several trials have been performed in industrial scale to define the rearing conditions and develop appropriate methodologies and practices.

A group of 12.000 individuals was transferred to cages at a mean weight of 10g in September 2016. Two groups were transferred in two circular cages with net of different depth (2,800 and 1600 m³) in order to study the effect of stocking density during rearing. Feeding was performed with automatic feeders and or manually during the personnel's presence. During the first month in the cages the groups exhibited a high growth rate reaching a mean weight of 218 ± 56 g and 205 ± 65g for the deep and shallow cage respectively. However, significant mortality accounting of 25% and 34% of the groups respectively was observed. Also, the presented high variability within groups, did not permit the application of appropriate husbandry practices, especially to ones related to feed size. Following sorting, a group of 5,000 individuals with a mean weight of 460±20g and a second group of 3,500 individuals of mean weight 263±19g were created. The performance of the "big" and the "small" group was monitored in terms of growth, feeding efficiency and survival as well as for pathologies and especially for parasitism outbreaks. Methodologies for treatment were developed.

During the course of the 2016 trial several incidence occurred causing significant mortalities. Mortality, associated with the presence of gill parasites (*Z. seriolae*), resulted in losses for the group with the larger individuals apx 25% while it was significant lower (apx 6.5%) for the group with the smaller fish. The incident was confronted with repeated baths with hydrogen peroxide that efficiently eliminated the parasites. Efficient methodology was developed for the application of the peroxide bath that was repeated when required i.e. in case warms were observed during sampling.

The rearing continued until June 2017 when 4,900 individuals with a mean weight of 914±150g and 3,090 with mean weight of 631±120g were remaining in the cages. In **Figure 34** the growth performance of the two groups is shown.

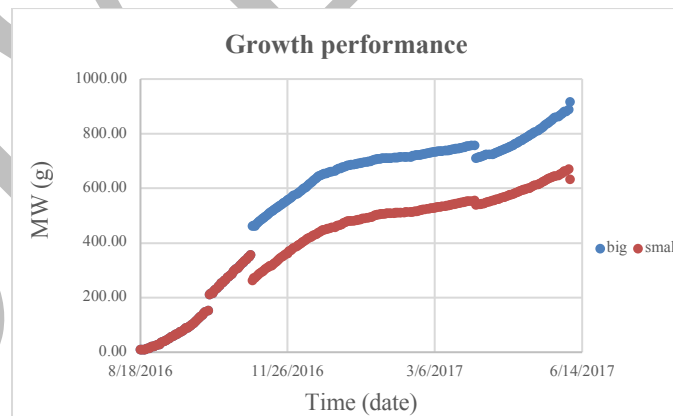


Figure 34. Growth performance observed in the experimental period.

During the trial the growth rate was 2.07 g d⁻¹ for the small group and 2.86 g d⁻¹ for the big one. Before sorting the groups have a similar growth of 5g d⁻¹ and the estimated FCR was 1.2. Following sorting and until the end of the trial, the two groups presented a similar growth performance with a linear rate of 1.42 g d⁻¹ and the estimated FCR was 2.03 and 1.86 for the small and big individuals respectively.



The trial was repeated in 2017 with 26.500 individuals transferred in two rectangular cages of 10x10x8 m in groups of 12.000 individuals of mean weight 23g and 14,500 of 15.5g respectively. Groups were fed manually, 3 times per day, with commercial diets.

A significant incidence of parasitism occurred with *Z. seriolae* during November resulting in the loss of more than 50% of one group. The second group was successfully treated with hydrogen peroxide following the procedures gained during the first trial. Since, both groups have developed high heterogeneity in size sorting was required. In the following **Fig.35** the size distribution before the sorting is presented.

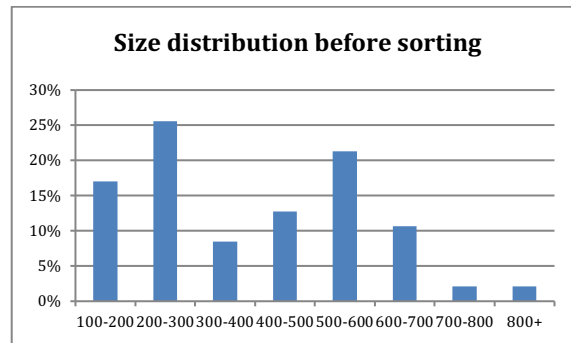


Figure 35. Size distribution

In January 2018, two groups were formed of 4,700 each with a mean weight of 406±40g and 607±23g. They were transferred in two cages with similar density (2.2 Kg m⁻³) and their performance was monitored until July 2018. During the first course of the trial (until the sorting) individuals presented a linear growth of 3.45g d⁻¹ and the mean FCR was 1.47. At the end of the trial 4,870 individuals with mean weight of 597±191g and 4,500 with mean weight of 955±189g were remaining in the cages. In **Fig. 36** the growth performance of the two groups is shown.

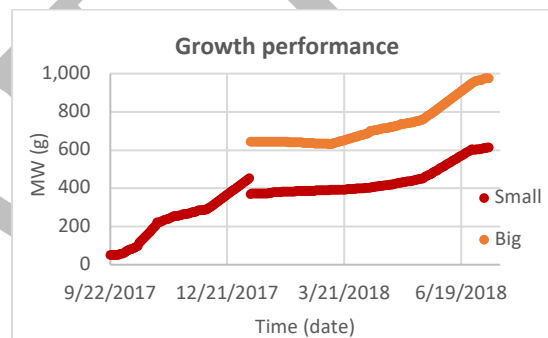


Figure 36. Growth performance observed in the experimental period.

During the second part of the trial the growth rate was 1.25 g d⁻¹ for the small group and 1.83 g d⁻¹ for the big one and the estimated FCR was 2.46 and 2.35 respectively. During both trials, fish accept without problem commercial feeding of appropriate composition i.e. high protein (of fish origin) prepared at commercial scale. There was no problem during the standard husbandry practices of net cleaning/changing and although the stocking density was not high, a value of apx 5 kg m⁻³ is considered acceptable for a pelagic fish.

The main difficulty stems from the species specific parasites that significantly affect the rearing. Even though the treatment of the parasite with peroxide is well established and confirmed, still the application is not easy and appropriate methodologies especially for big cages should be developed. The species is also facing bacterial infections and in the case of Greece incidences with *V. harvey* were reported causing significant mortalities (HCMR unpublished data).



This study provides a first estimation of the innate immune status of amberjack, greater amberjack kept in commercial settings, i.e. with the stressful conditions and pathogenic infections which often accompany them. A parasitological infection occurred at the beginning of the trial before the first sampling, and fish were sorted by size. The lasting increase in lysozyme and myeloperoxidase activities (**Fig. 37**) in small fish compared to large fish could be either due to the size of the fish or also due to a reaction to this infection.

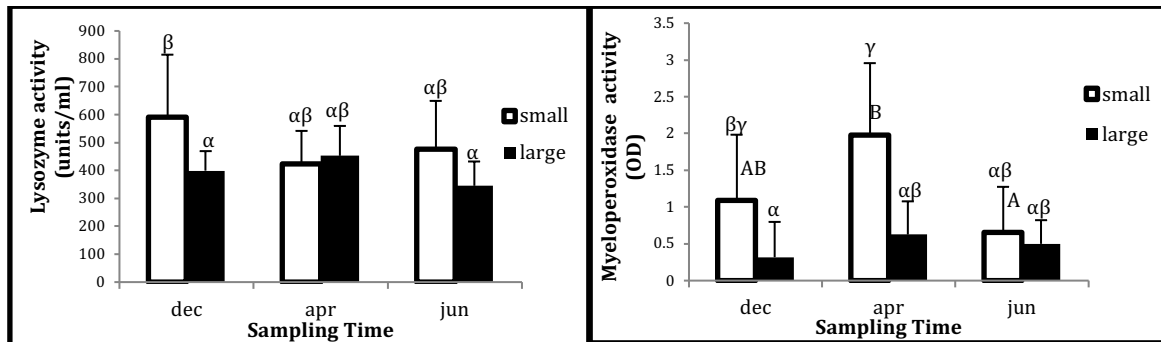


Figure 37. Serum lysozyme antibacterial activity and myeloperoxidase activity of small (250-500g) and large (600g-1kg) greater amberjack kept in sea cages for 0, 3 and 6 months. Different capital letters shows significant differences between different sampling times (General Linear Method, $P < 0.05$). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, $P = 0.00005$, Tamhane's t-test), $n = 10$.

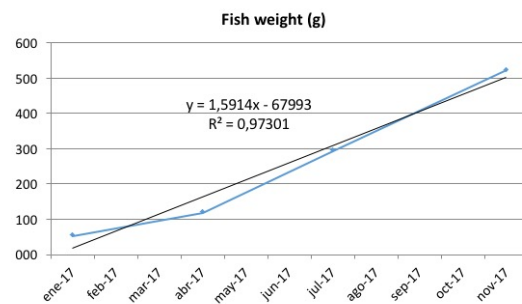
The present study suggested that the parasitological infection of amberjack could have long-term effect on the health of the fish and care should be taken to treat quickly the fish in such event to minimize the future adverse effects in either short-term such as mortalities, or long-term such as secondary infections.

Another cage trial for about 1 year was also done under the Canarias weather and sea current conditions. After amberjack spawning and the subsequent grown up from larvae to juveniles at the FCPCT laboratories, fish from 52.92 ± 23.86 g were transported to Taliarte harbour for their transference to CANEXMAR company and stocking in their experimental cages, according to a previous agreed protocol. Fish transport scheme from lab to sea was developed according to next protocol: A track was prepared with 500L fish transport boxes; Fish density during transport was around 20-22 kg/m³ and oxygen level maintained close to 6.5 using an oxygen bottle; Timing: initial to final fish picking from the tanks at the FPCT (11:00-13:00) and arriving to the port (13:30), total 2:30 horas fish management.

During the growth in the cages, a sampling schedule for proximately every 90 days was agreed with the company, although it was determined by sea overview and water current: 1) Wt 3 batches of fish at the cages and determine medium weight & size to determine/adjust fish feeding; 2) Take 15-20 fish to the FCPCT laboratories- for the individual fish sampling; 3) Weight; length; observations & photos; 4) Parasites observations; 5) Eviscerate & wt again the fish; 6) Dissect the 2 fillets and weight (1 by 1) and stored 1 whole fillet for biochemical analysis; 7) Remain fillet and the rest of the whole fish for health analysis (**Fig. 38**)

**Figure 38.** Intermediate samplings at CANEXMAR cages.

Along the trial fish were fed a daily evening meal during 30 min proximately with a commercial high protein diet. Fish responses during meals were normal and no important mortalities observed along the assay. The sea overview and the current water conditions were defined as those for the medium levels according to company daily recorded scales. Fish growth along the trial showed a slow increase during the first period with a higher slope response after April (**Fig 39**), which means that fish need an acclimation period after stocking in the sea, while moreover cage was better covered to avoid too much light incidence and daily feeding properly adjusted and managed. Recorded water temperature did also start increasing after April. Table 8 show the results summary of the individual fish sampled at the FPCT at the different samplings along the trial.

**Figure 39.** Fish weight (g) along the whole cage trial at CANEXMAR.**Table 8.** Different measure parameters along the trial at the CANEXMAR experimental cages.

Sampling	Temperature (°C)	weight (g)	Growth (g from the initial)	Growth (% initial)
Jan-17	18	52.91±23.86	-	-
April-17	19	119.00±25.44	66.09±25.44	124.91±48.08
July-17	21	293.70±57.35	240.79±57.35	455.10±108.40
Nov-17	23	521.82±103.73	468.91±103.73	886.24±196.05
Sampling	Total length (cm)	K	Evisc weight (g)	VSI
April-17	21.01±1.46	1.27±0.07	105.83±15.04	11.33±0.94
July-17	29.66±1.72	1.11±0.07	275.70±55.72	9.19±2.47
Nov-17	35.31±2.68	1.16±0.08	546.75±91.22	7.14±1.65
Sampling	Right fillet (g)	Left fillet (g)	Fillet (%)	HSI
April-17	26.33±6.09	25.94±4.74	55.77±3.75	1.82±0.41
July-17	48.92±11.01	56.20±14.73	65.80±3.00	1.11±0.42
Nov-17				1.55±0.35



Development of feeding methods

Salvador Jerez, Daniel Montero, M. Virginia Martín and Alvaro Fernández Montero, IEO, Spain

An appropriate feeding strategy is of paramount importance to produce quality fish size in grow-out operations. Feed management strategies govern ration size, meal frequency, and temporal and spatial distribution of the feed, among other topics. Among the different feed management practices proven to maximize the benefit of feeding, feeding frequency and ration size play an important role in regulating the feed intake, growth and waste outputs of fish (Silva *et al.*, 2007).

A few previous studies on greater amberjack grow out have been implemented and results of growth performance, fish condition and feed efficiency have been obtained. However, most of these studies have focused on the effects of feed with different diets on grow out, the impact of sampling frequency and only a few included feeding strategy, frequency and ration, on growth performance of greater amberjack juveniles (Jerez, 2013).

Our study aimed at optimization of feeding strategy according to the appetite variability for 5 g and 200 g greater amberjack juveniles by the alteration of feed delivery rate, fixed versus continues, by allowing fish to dictate the timing and size of their daily ration for growth performance, feed conversion, survival, welfare, condition and juvenile quality of greater amberjack.

Definition of feeding pattern for 10 g individuals. An experiment was conducted to define the best dietary regime for greater amberjack juveniles (early juveniles during 120 days), combining two parameters dietary regime: a) feeding rate (% of body weight per day) and feeding frequency (n° of meals per day in which the defined feeding rate is distributed).

Six hundred fish of 12.01 ± 1.5 g were distributed in twenty-four tanks (25 fish/tank) (flow-through system) and fed on the eight dietary strategies (in triplicate) during 120 days: Apparent satiety 3 intakes d^{-1} (S3) and apparent satiety once a day (S1). 3.5% of the biomass divided in 3 intakes d^{-1} (3.5/3), 3.5% of the biomass divided in 4 intakes d^{-1} (3.5/4) and 3.5% of the biomass in a unique intake d^{-1} (1 intakes/day). 2.5% of the biomass divided in 3 intakes d^{-1} (2.5/3), 2.5% of the biomass divided in 4 intakes d^{-1} (2.5/4), and 2.5% of the biomass once a day (2.5/1).

Animals were fed on a commercial diet with high protein content (52% crude protein, 20% crude lipids). Dissolved oxygen was 7.5 ± 0.6 and temperature was 2.1 ± 1.4 for the whole period. Specific Growth Rate and Feed Conversion Ratio (FCR), Protein efficiency ratio (PER) and condition factor (k factor) were calculated.

Fish fed on apparent satiety 3 intakes d^{-1} presented significantly higher growth compared with fish fed on apparent satiety 1 intake. Animals fed on 2.5% biomass were significantly smaller than those fed on 3.5% treatment at any of the daily regime. Besides, animals fed on 3.5/3 and 3.5/4 feeding regime showed similar growth than apparent satiety group (Fig.40).

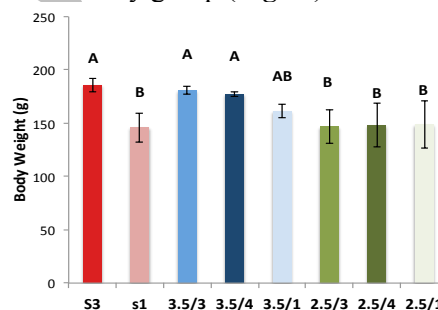


Figure 40. Final weight (g) for each treatment at the end of the trial. Different letters indicate significant differences among dietary treatments ($P < 0.05$).

K factor was not affected by dietary treatment, which denotes no deformities and no fasted animals due to the dietary regime assayed, and just only less weight gain due to the dietary regime applied.



Figure 41. Juvenile of greater amberjack after 120 under the different dietary regimes.

No fin erosion or other external sign of illness or low welfare were observed in the experimental fish, showing all of them the typical shape and morphology of healthy greater amberjack juveniles (**Fig. 41**). Feed conversion ratio (FCR), for fish fed on apparent satiety 3 intakes per day was significantly lower when compared with fish fed on apparent satiety 1 intake. For those animals fed on 2.5% regime it was significantly higher than those fed on 3.5% treatment at any of the dietary regime used. Besides, animals fed on 3.5/3 and 3.5/4 feeding regime showed similar FCR than apparent satiety group. The protein efficient ratio (PER) measured was affected by the dietary regime. Fish fed on apparent satiety 3 intakes per day presented significantly higher ($P < 0.05$) PER when compared with fish fed on apparent satiety 1 intake. Those fish fed on 2.5% biomass feeding regime were significantly ($P < 0.05$) lower PER than those fed on 3.5% treatment at any of the dietary regime used. Besides, those animals fed on 3.5/3 and 3.5/4 feeding regime showed similar PER than apparent satiety group.

Definition of feeding pattern for 200 g individuals. 1

80 juveniles (262.1 ± 55.5 g and size 23.0 ± 1.7 cm) were randomly divided into 12 homogeneous groups of 15 fish each. The groups were maintained in tanks (1m^3 cylindrical and 4m^3 square) during the first and last two month, respectively, with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu), temperature ($18.8 \pm 0.4^\circ\text{C}$; decreasing from 19.4°C to 18.1°C throughout the experiment) and oxygen saturation ($92.4 \pm 4.8\%$). Fish were fed a commercial pellet (52% crude protein, 20% crude fat). The fish were fed daily *ad libitum*. Triplicated fish groups were fed at a feeding frequency of 1 (at 08:00 h), 2 (08:00 and 18:30 h), 3 (08:00, 13:30 and 18:30 h) and 7 (08:00, 10:00, 12:00, 13:30, 15:00, 17:00 and 18:30 h) meals d^{-1} . Feed left uneaten was recovered from the bottom of the tank 30 minutes after its administration to quantify the daily feed intake (FI).

The Specific Growth Rate (SGR) tended to increase with the increasing of the feeding frequency at day 60, showing a higher SGR fish groups fed 7 and 3 meals per day compared to fish groups fed 1 meal per day (**Fig. 42**). No differences were observed in the period 60-90 while lower SGR was obtained in fish fed 1 meal per day, between 60 and 90 days. In the overall period (0-120 days), the fish fed 1 meal per day showed a significantly lower SGR. The other three feeding strategies (2, 3 and 7 meals per day) presented a similar SGR. Results indicated significant differences between fish fed at different feeding strategies at 120 days, mainly on cranial and several body regions.

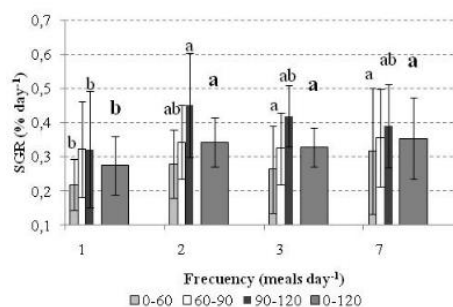


Figure 42. Specific growth rate (SGR, $\% \text{ day}^{-1}$) at different periods and overall duration of fish fed at 1, 2, 3 and 7 meals day^{-1} . Different letter indicates significant differences ($P < 0.05$).



More than 53% of variation was correlated with longitudinal and transversal body measurements while a smaller percentage of the variability was related to peduncle and eye measurements. Analysis demonstrated a significant separation of fish fed at 1 meal per day from other groups ($P < 0.05$).

Antioxidant enzymes were determined at the beginning (0 days) and at the end (120 days) of the trial in liver, muscle, gill and brain from fish fed with different feeding frequencies. The results showed several differences in antioxidant defenses comparing among feeding frequencies groups for all tissues analyzed. Thus, catalase activity was lower in 1 meal per day group in both liver and gills. Several differences among feeding groups were also observed at GPx and GST for all tissues analyzed. Immunological parameters from serum of fish fed with different feeding frequencies at 60, 90 and 120 days are shown in **Fig.43**. Bactericidal and peroxidase activities were significantly lower in 1 meal per day fish after 90 days as well as the protease activity at the end of the assay (120 days).

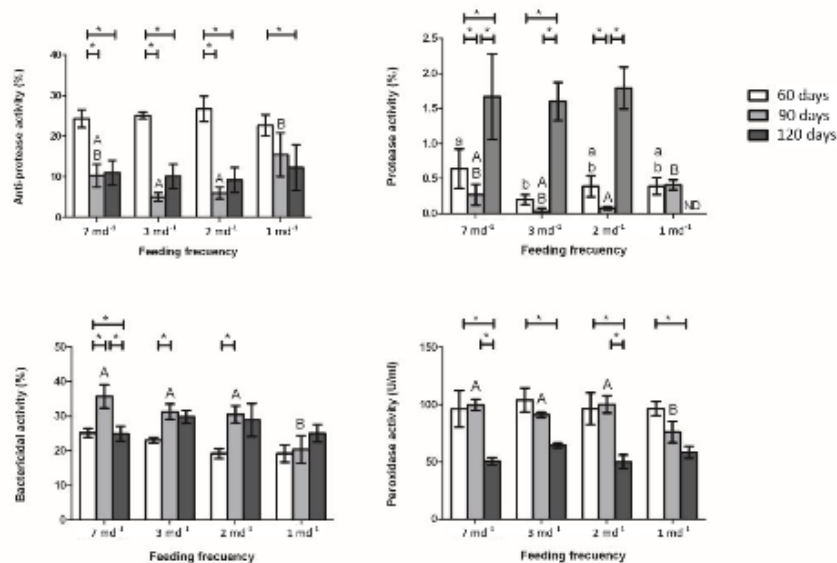


Figure 43. Effect of feeding frequencies on antiprotease (%), protease (%), bactericidal (%) and peroxidase. Different letters denote statistically significant differences between feeding frequencies at the same time point and asterisk denote statistically significant differences between different time points at the same feeding frequency (ANOVA, Fisher's LSD post-hoc test $P < 0.05$).

For 200 g greater amberjack juveniles, the better results in growth and feed conversion rates have been obtained when they are fed from 2 to 7 meals per day. The absence of changes among the hematological and biochemical parameters suggests that greater amberjack juveniles were able to adapt to the different feeding frequencies under the particular culture conditions. However, results from immunological parameters reveal differences in the immune status among fish subjected to different feeding frequencies that could influence the health status of fish. The findings of the current study have practical significance for establishing greater amberjack rearing practice.

Development of appropriate husbandry

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The definition of the appropriate temperature ranges for the rearing of the greater amberjack is of great importance in order to properly select the geographical areas for industrial production. The study included different size classes of individuals. A second parameter studied was the stocking density again in two different size classes



Temperature tolerance

Juveniles

Two hundred and twenty-five greater amberjack juveniles of 19.5 ± 4.1 g body weight and 9.8 ± 0.7 cm total body length were distributed in 9 cylindrical 500 l tanks (25 individuals per tank). The three temperature treatments, 17, 22 and 26 °C, were assayed by triplicate. Systems with three tanks of a given temperature were controlled by one RAS. Oxygen levels were similar among the different tanks around 7.8 mg l^{-1} . Fish were fed to apparent satiety three times per day during 120 days with a commercial diet with 52% of crude protein and 20 % crude lipids. Fish held at 26° C showed significantly higher body weight compared with fish held at 22°C. The differences between these two groups were significant already after 60 days of trial. Fish held at 17°C showed the lowest final body weight, showing differences with both groups after 30 days of trial (**Fig. 44**).

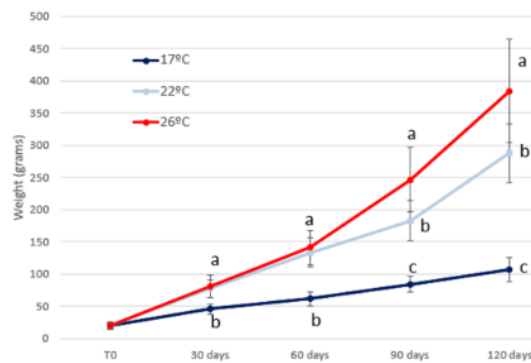


Figure 44. Growth rate of greater amberjack fingerlings during 120 days of trial at the three different temperatures

Significant differences among groups were found in the centroid size and shape. The centroid size was correlated to fish length indicating a change shape linked to fish growth. The PCA analysis, showed that the increase of temperature led to elongated shape of fish body, especially of the head, differentiating clearly specimens reared at 17°C and 26°C. The mean values of caudal propulsion efficiency (CPE) differed among groups, noting higher propulsion of fishes with a temperature increase (**Fig.45**).

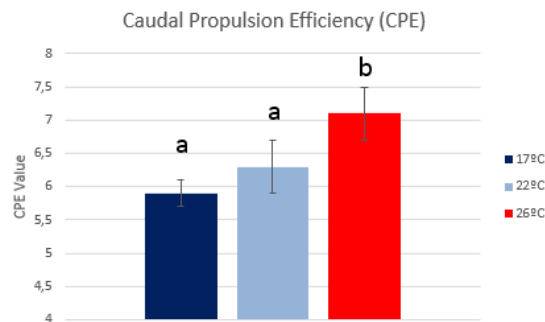


Figure 45. Caudal propulsion efficiency (CPE) value of the three treatments; relation between caudal fin depth (CFd) and caudal peduncle minimal depth (CPd)

The specimens reared at 26°C showed significant swimming differences with the individuals reared at 17°C and 22°C; whilst, it was similar between the individuals reared to 17°C and 22°C. After one month of growth, there were significant differences on FCR, being this parameter higher for fish held at 17°C when compared with fish held at 26°C. For the next sampling point, the differences among fish held at 17°, 22 and 26°C increased, being significantly higher for fish held at 17°C, when compared to fish held at 22°C and fish held at 26°C respectively. Regarding total FCR for the whole on-growing period, fish held at 26°C showed the highest FCR, being this value below one (**Fig. 46**).

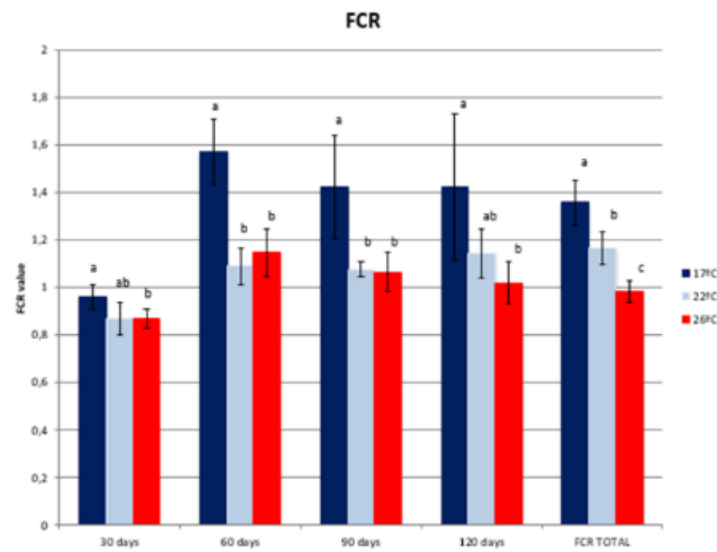


Figure 46. FCR values at each sampling point for the three temperatures and the total FCR.

The amount of feed intake was significantly higher in fish held at 26°C when compared to fish held at 17°C and similar to those held at 22°C. There were no significant differences in the protein or lipid retention among groups held at different temperatures. However, the protein gain was significantly higher in fish held at 26°C when compared to those held at 22°C, being the protein and lipid gain significantly lower for those fish held at 17°C.

Individuals of 350g

Three water temperatures were tested (16°C, 21°C and 26°C) in juveniles of greater amberjack during 98 days and individuals were sampled for blood and growth parameters three times throughout the experimental period. A total of 108 juveniles of greater amberjack (325.6 ± 24.2 g) were randomly distributed among 9 circular 500L indoor rearing tanks ($n = 12$ per tank) at an initial temperature of 15 °C (ambient). Over the following week, seawater temperature was gradually adjusted from ambient to temperature regimes: 16°C (Group A), 21°C (Group B) and 26°C (Group C). The experiment was performed in triplicates, where each temperature regime composed a semi closed recirculating water system with mechanical and biological filtration. All groups were fed standard commercial diet manually *ad libitum* twice a day (09.30 h and 12.30 h) and, in addition, a simple automatic belt-feeder was used to distribute the food between approximately 14.00 h and 20.00 h, when the lights switched off. Starting from 325.6 ± 24.2 g body weight in all groups, individuals reached 395.1 ± 67.7 g, 483.7 ± 64.3 g, and 441.7 ± 95.6 g for groups A, B and C respectively.

Best growth was observed in fish reared at 21°C, where individuals gained an average of 161.5 g during the 98 days of experiment. Although some low intra-group variability was observed, there was no significant differences between replicates. For larger juveniles as the ones used in this study, 26°C was a condition that exhibited high instability, with the lowest survival rate observed ($75 \pm 14.4\%$) and an apparent longer acclimation period. However, individuals in this treatment seemed to display compensatory growth throughout the last month of experiments. Moreover, the greater coefficient of variation registered for 26°C body weight values ($21.7 \pm 0.7\%$) indicated a higher size heterogeneity in this group.

Significant changes were observed on the measured parameters. Cortisol levels, presented in **Figure 3a**, showed a high inter-individual variability throughout the experimental period, supporting the existence of individuals with low (LR) and high (HR) cortisol responsiveness (Temperature did not have an effect on lactate, while plasma glucose was affected and concentrations increased with temperature and sampling time (**Fig. 47F**).

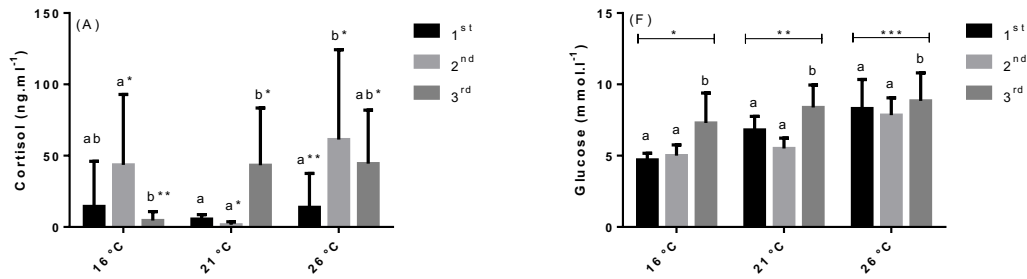


Figure 47. Changes in plasma cortisol (A), and glucose (F) concentrations between and within treatment groups and sampling points. Different letters indicate statistically significant differences between sampling points within the same temperature treatment, while asterisks indicate significant differences between treatment groups within the same sampling point ($p < 0.05$).

The increase observed in glucose values with increasing temperatures, especially at the last sampling point, could be explained due to a higher feed intake in all groups during the last month of experiments. In order to better clarify the observed temperature differences fish responses between small and high size amberjack, two other consecutive trials were developed with fish from 200g to proximate 1000g. Some other specific parameters like the effect of the temperature and digestion time on protein digestion during the on-growing were also studied.

Semi-closed recirculated system tanks (6 tanks, 10m³ each) were used. In a first assay, triplicate groups of 160 fish from 203.18±20.70g were submitted to 23°C and 26°C during 105 days, while in the 2nd trial fish from 450-550g were acclimated and feeding at 20°C and 23°C (FCPCT facilities). In both cases fish were manually feeding to apparent satiation 2 times per day (08:30 and 14:30), from Monday to Saturday, with a commercial high protein diet (51% protein, 20% lipid). The effect of the diet quality on fish growth response at higher temperatures was moreover monitored during last part of the first trial, being fish changed to fed a commercial lower protein diet (40% protein, 20% lipid). A summary of the growth results from both trials is shown in **Fig. 48**.

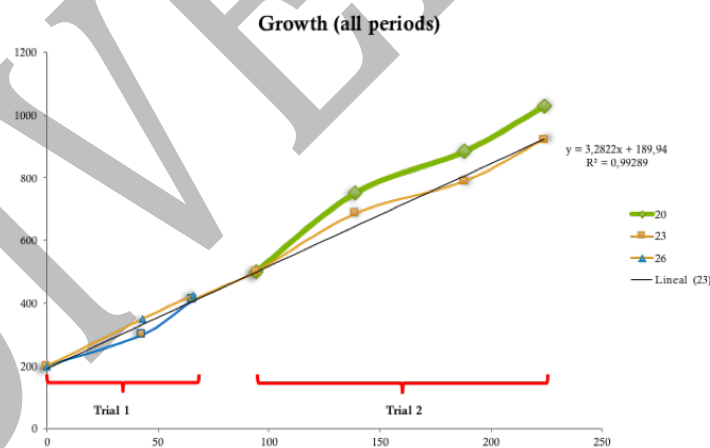


Figure 48. Growth curve for the amberjack growth at 20°C, 23°C & 26°C from around 200g to 1000g in the 2 consecutive trials (X/Y axis =days of feeding/fish weight in g)

Survival rates were high, around 95%, being not significantly influenced by temperature over the duration of the trials. For similar feed intake (1.43 % day⁻¹), significant higher growth in weight and length ($P < 0.05$) were observed after 43 days at the temperature of 23°C (346.48 ± 44.99g) respect to 26°C (308.53 ± 39.82g). Better SGR and significantly lower FCR were obtained for 23°C (SGR=1.24±0.15 % day⁻¹; FCR=1.30±0.21), respect to 26°C (SGR=0.97± 0.11 % day⁻¹; FCR=1.94 ±0.25). Similar statistic for the growth results were observed when lowering protein level from 50% to



40% with higher feed intake (1.60) for both temperatures; some better FCR response in this case for 26°C (1.39) respect to 23°C (1.73) together with significantly lower PER in the latter (1.23 while 1.47 for 26°C). Parasites appearance at the end of the first trial were mostly observed under 26°C. Regarding fish composition along the first trial, lipid content was observed to increase about 100% respect to initial fish; significantly higher lipid content was observed for 26°C fish, in the whole fish nor in the fillet which agreed with the significantly higher VSI showed for the 26°C fish. Temperature was shown also to affect whole fish fatty acid composition promoting lower ω -3 y ω -3 HUFA in 26°C fish, and n3/n6 ratio in favor for the lowest temperature (23°C). Lower unsaturated fatty acids at 26°C could reflect an increase in the lipogenesis, while higher values in 23°C fish may respond to a higher phospholipids demand for cells construction at higher growth rates. On the other hand, color measurement in 3 different parts on fish skin and fish fillet showed higher values at 26°C respect to 23°C for the Chrome values in both tissues at any sampled part, which could mean that higher temperature promotes fish colorless.

For the second trial, no significant differences were observed for both temperatures although 20°C fish showed some better growth responses with some lower feed intake. For fish over 500g the feed intake lowered down below 1, to about 0.84 and 0.69 for 23°C and 20°C, respectively; the observed FCR values were 1.36 (23°C) and 0.95 (20°C). As a conclusion, and with no significant effects observed in the 2 trials for the temperature on the fish feed intake, lower temperatures seem to promote in general better results in higher size amberjack, being the poorer results in general for 26°C (may be sub-optimal temperature with an increase of the protein turn-over), and similar and better in the range from 20°C to 23°C.

The effect of temperature and total reaction time on protein digestion

For the digestion analysis, and to test the effect of the temperature and total reaction time in the hydrolysis of protein in the intestine and stomach of greater amberjack during the on growing culture phase, the routine digestive enzymatic techniques were adapted to specific fish conditions to latter evaluate the effects in the experimental fish under test. After 105 days feeding, stomach and liver from final fish from the different temperature tested in a previous trial (17, 22, 26°C) were taken at 0-4-8-12-18 and 24 hours after a morning feeding, and subsequently in vitro analysed.

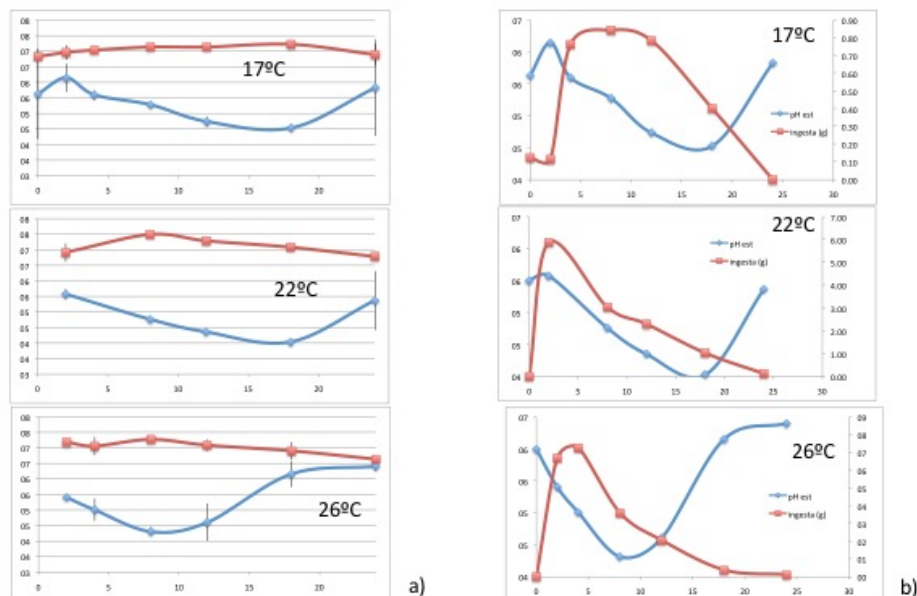


Figure 49. a) Intestine (red) and stomach (blue) pH values at the 3 temperatures; b) Stomach pH (blue) versus gastric evacuation.



Results showed no significant variation pattern post intake in the intestine, not also been affected by temperatures (Fig. 49). Stomach pH was reduced at a maximum of 4.0. Slow acidification was observed in all cases, being about 18h in those fish maintained at 17 & 22°C and much quicker (8h) in those maintained at 26°C, which would correspond with a higher velocity for the gastric evacuation in the latest. In any case, stomach pH did not reach below 4, does not down enough to transform significant quantities of pepsinogen into pepsin which denote a limited gastric protein hydrolysis in this specie. pH values and stomach gastric evacuation appears in parallel for fish maintained at 22 & 26°C. This observation was not shown for 17°C group. As a conclusion from these preliminary results, the optimal range for the digestion of this specie is between 22°C and 26°C and the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the enzymatic activity in that range is maximum, while in the intestine the maximum activity range between 12 and 18h. All these results related to protein utilization are of high interest and need to be better studied in order to promote an efficient culture of the greater amberjack, meanly in this grow out phase where high volume of feed is needed.

Stocking density

To achieve this objective, rearing trials at 3 different stocking densities (9 groups) were performed with individual size of 5 g in 500 l tanks during 2015, and at 4 different stocking densities (12 groups) with fish initial size of 150 g in 4000 l tanks during 2017, for a period of 4 months.

The results, for the 5 g groups, showed that at day 30, the Specific Growth Rate (SGR) decreased significantly with the increasing of the density, but no significant differences were found between fish groups at different density assayed in the following periods. However, the SGR in overall period (0-120 days) decreased as stocking density increased. In the overall period, the fish maintained at higher density (HD) showed the lower SGR and dispersion (Fig. 50).

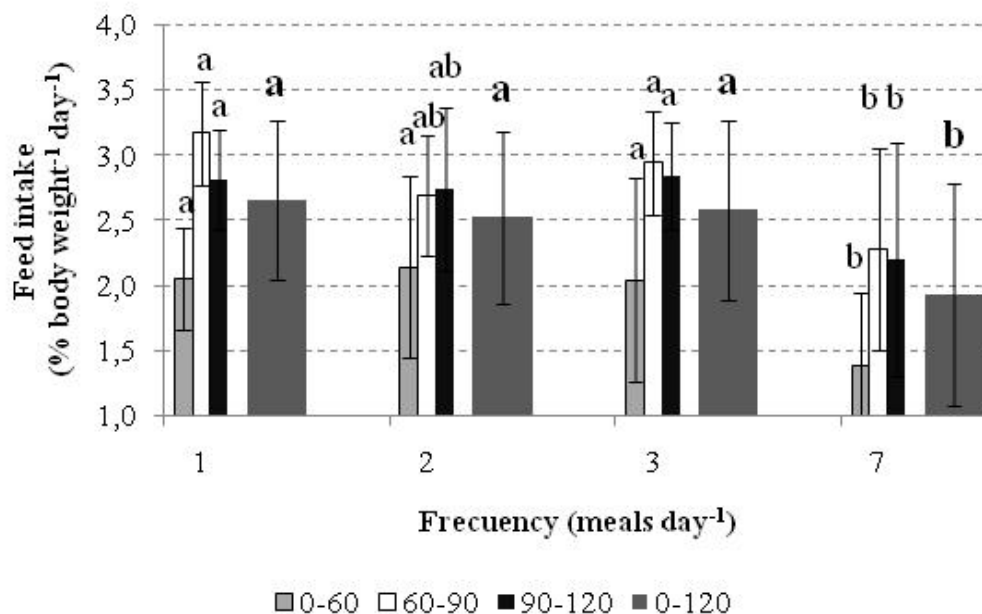


Figure 50. Specific growth rate SGR (% d⁻¹) at the different periods and overall duration (120 days) at Low (LD), Medium (MD) and High (HD) density. Different letter indicates significant differences among different stocking densities (P<0.05).

Feed intake (% body weight day⁻¹) decreased significantly during experimental period in all stocking density assayed (Fig. 51).

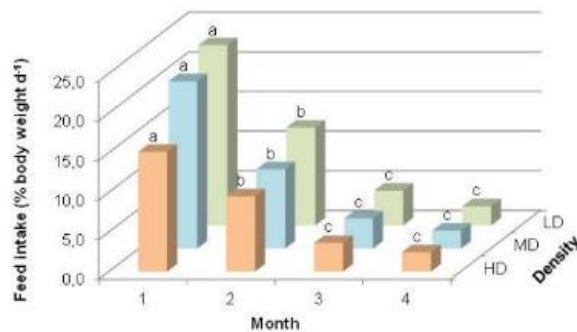


Figure 51. Feed intake (% body weight d^{-1}) at High (HD), Medium (MD) and Low density (LD) during the trial. Different letter indicates significant differences among different periods ($P < 0.05$).

Results of two-way ANOVA showed that both factors time (month) and stocking density influenced the Feed intake being significantly lower at High density than at Low density. This influence was greater during the first two months. The trial with the 150 g individuals showed that the Specific Growth Rate (SGR) of the fish stocked at HD was significantly higher in the periods 30-60 and 60-90 days ($P < 0.05$). In the period 90-120 the tendency changed and the SGR decreased with increasing of the fish density. Thus, although the SGR tended to rise with the increasing of the fish density, not significant differences were observed in the overall period (0-120 days) (Fig. 52). The results showed that stocking density can affect growth rate and feed intake in greater amberjack juveniles in an apparent fish initial size-dependent way.

For instance, and for 5 g fish, initially stocked at 0.5 kg m^{-3} , which was translated in a final stocking density of 7.4 kg m^{-3} after 120 days, the fish showed lower specific growth rate and condition index than the other groups. Some differences found in certain immunological parameters may also reflect a negative influence of high stocking densities in the health status of fish.

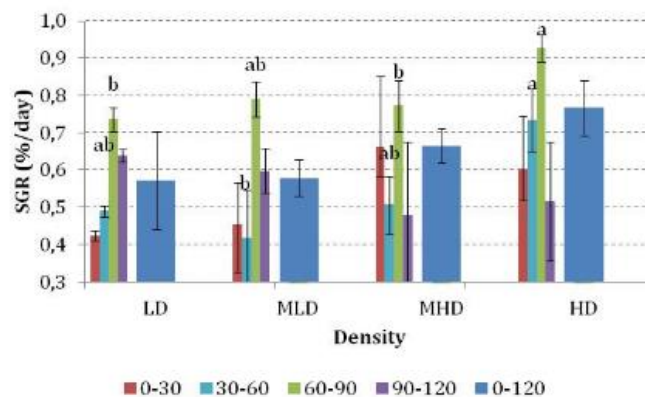


Figure 52. Specific growth rate (SGR, $\% d^{-1}$) at the different periods and overall trial (120 days) of fish stocked at different densities (kg m^{-3}). Different letter indicates significant differences among treatments ($P < 0.05$).

However, for 150 g fish (initial density of 3.2 kg m^{-3} and final stocking density of 6.8 kg m^{-3}), these negative effects on growth as well as on hematological, biochemical and immunological parameters were not observed in fish stocked at high density with respect to other stocking densities, and the overall period (0-120 days).



Independently of the fish initial size, oxidative stress, specifically, CAT, SOD, GST, and TBARS measured in muscle and liver, did not experience any significant change. Since availability of oxygen and fish movement capacity is affected by the increasing stocking density, our findings support the hypothesis that under the assayed conditions, greater amberjack juveniles have mechanisms to cope with the ROS production induced by metabolic changes associated to high density conditions, and that they are able to adapt to the increasing stocking densities without showing an immunosuppressive state typically observed in chronically stressed fish. The findings of the current study having practical significance for establishing greater amberjack rearing practice.

DIVERSIFY



Fish health

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Insights into the immune system

As the greater amberjack culture is foreseen to grow dramatically in the very near future, disease management will be based on the modulation of the immune system through the development and use of vaccines but also through the use of immunostimulants. Therefore, knowledge of the immune system function has been recognized as an extremely important gap to be filled by the DIVERSIFY project.

Through a series of studies, a panel of relevant greater amberjack immune genes have been sequenced, to allow future study of mucosal immune responses in this species. qPCR assays have been optimized for each gene. In vivo and in vitro studies using PAMP stimulation have demonstrated that the expression of these genes can be modulated by such stimulants, and so these molecules are good markers for the effects of treatments (eg dietary or other) to increase disease resistance. The list of the respective genes include IL-1 β , IL-8, IL10, IL-17A/F, IL-17D, IL-22, TNF α , Mx, IFN1, IFN γ , iNOS, IgM, IgT, RAG2. Moreover, the list contains the housekeeping genes EF-1a, β -Actin and the antimicrobial peptides Piscidin, Defencin and Hepsidin.

Rearing effect on mucosal defense of greater amberjack

Fish, being in constant contact with the water, are continuously exposed to various pathogens including parasites. It is known that fish mucosal surfaces in gills, skin and intestine is the first line of defense against pathogenic microorganisms (**Fig.53**). In DIVERSIFY project, the effect of various stressors which are common in intensive fish farming such as high stocking density and intense manipulations was studied with regard to the innate immune response of the fish



Figure 53. Histological section of greater amberjack gills showing the distribution of the mucous-secreting cells.

It was shown that high stocking density and handling stress, adversely affect the innate immune response of greater amberjack as expressed in fish mucus. This was clearly shown with respect of lysozyme activity, which is a principal antimicrobial enzyme of fish mucosa. Bactericidal activity of lysozyme was also found to be higher when fish were reared at 26°C compared to 22 and 16°C. Through this work in the DIVERSIFY project, the basal transcriptional values of the main genes involved in the mucosal defense of greater amberjack were evaluated and the physiological distribution of mucus cells were mapped on the fish skin through histology. This work demonstrated the immune potential of skin mucus of amberjack, and showed that relative to other species the mucosal surfaces include a full repertoire of antimicrobial defenses. Furthermore it was shown that these defenses can vary with certain



environmental conditions, and that they were especially sensitive to aquaculture-associated stressful conditions.

Epitheliocystis disease

This is an infectious disease, characterized by multiple cysts in the gills that has been shown to cause significant problems and mortality if it occurs at the early life stages of the fish, or during the transition of the fish from the hatchery to the on-growing cages. Despite the fact that the disease is one of the first described in fish generally, little is known about the causative agents and the route of infection. Until recently, the disease was thought to be caused exclusively by chlamydia, however recent studies have expanded the range of the types of bacteria that can cause the disease including representatives from the β - and γ -proteobacteria. In the DIVERSIFY project we have developed and assessed molecular tools for the early diagnosis of the disease. The tools include molecular PCR probes for all major epitheliocystis-causing agents including *Chlamydia*, *Endozoicomonas* spp. and *Ichthyocystis* spp. A nation-wide survey was conducted in Greece in order to collect data and samples regarding the epitheliocystis outbreaks in the major farmed fish species that include, European seabass, gilthead seabream and of course greater amberjack. It was shown that at least in Greece, the major pathogen causing epitheliocystis belongs to the newly described genus *Ca. Ichthyocystis*. In bass and bream aquaculture, the pathogenic species are either *Ca. Ichthyocystis sparus* or *Ca. Ichthyocystis hellenicum*, while in greater amberjack the infectious agent is a related but different and possibly a novel species of the same genus. The disease has been observed at the first months of the fish in the cages, following the same pattern as in other reared species. It can cause mortality that may reach 4-5%, however it can contribute to significantly higher mortality if it co-exists with other pathogens such as *Vibrios* and monogenean parasites. The disease causes massive granulomatous inflammatory response in the gill tissues which is unique in this species that results in significant impairment of breathing capacity (**Fig. 54**). Although, there are anecdotal reports that antibiotics can be used as a treatment, epitheliocystis lesions usually resolve without intervention if the host is not immunocompromised within a couple of weeks. Therefore, it is highly recommended that fish are monitored throughout rearing and especially during their first three to four months in the sea-cages. If epitheliocystis is observed, care should be taken to reduce stress and prevent in other disease that may co-infect the host.

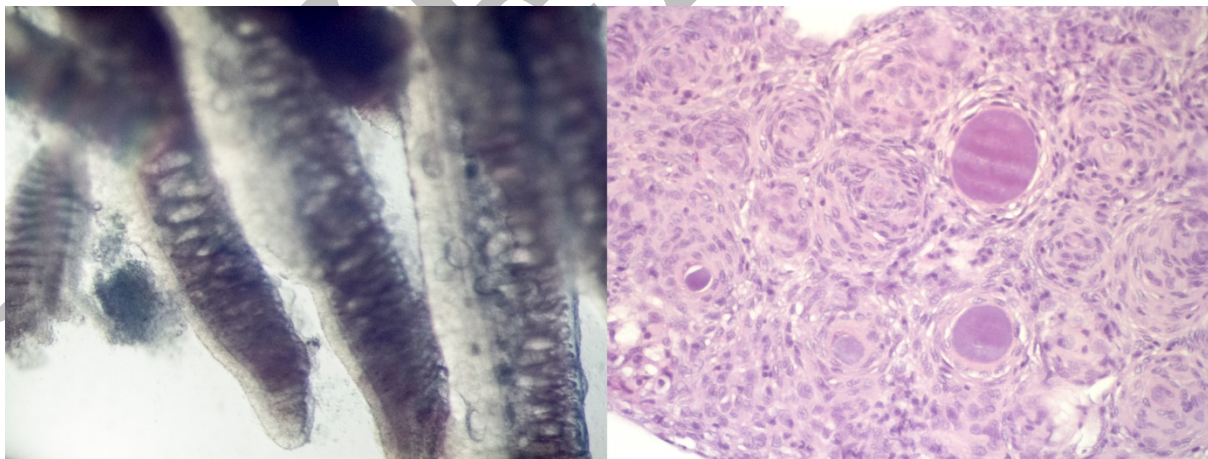


Figure 54. Left: fresh squash preparation of epitheliocystis-affected greater amberjack showing multiple cysts in the gills. Right: histological section of the same gills showing massive hyperplastic response, bacterial inclusions and granulomatous inflammation (Histological picture by Dr. Maja Rueten, Pathovet AG)

Vibriosis

Greater amberjack is susceptible to vibriosis with the principal species being *Vibrio harveyi*. The disease presents the typical signs of a bacterial septicemia with haemorrhages in the skin mainly in the tail, anus and behind the opercula (**Fig.55**). Skin ulcers are often seen at the progressed stages of the disease. The



onset of the infection coincides with changes in the water temperature, mostly when temperature is above 23°C. Losses may reach 40% if the disease is not treated early. Since this is a bacterial infection, antibiotics can be of value, however *Vibrio harveyi* may develop antibiotic resistance rapidly, therefore it is extremely important to select the appropriate antibiotic based on antibiogram.



Figure 55. Juvenile greater amberjack infected by *V. harveyi*.

Parasitic infections

The most significant parasitic diseases in cultured greater amberjack are caused by the monogeneans *Zeuxapta seriolae* and *Neobenedenia girallae*, the first infecting the gills causing sever anemia and the second the skin causing ulceration and promoting secondary bacterial infections (**Fig.56**). Both parasites may result in huge losses if they remain untreated.



Figure 56. Left: Anemic gills of greater amberjack due to massive infection by the gill fluke, *Zeuxapta seriolae* (arrow). Right: *Neobenedenia girallae* on the skin of greater amberjack.

Zeuxapta seriolae can be found throughout the year however it becomes extremely problematic in the summer months. This is because the propagation of the parasite is temperature-dependent and in temperature above 22°C it can complete its life-cycle within 20-25 days. Bath treatments with hydrogen peroxide have shown great potential to reduce the impact of infestation and control the parasite. Dose as low as 75 ppm of hydrogen peroxide for a 30-min bath can be effective. However, hydrogen peroxide can be highly toxic to the fish and its toxicity increases with the water temperature. Therefore, this intervention should be applied with great cautious and under veterinary supervision. The parasite can be controlled only with repeated treatments with a two-week interval in order to break the life cycle. However, if wild greater amberjack are near the culture area, it is very possible to have a re-infection of the cultured fish very fast. Therefore, it is highly recommended to monitor gill health at a monthly



basis. The most important factor for the appropriate management of the disease is to avoid year-class overlap, and if this is not possible, to treat all stocks simultaneously in order to eliminate all possible reservoirs of reinfection. Monogenean eggs form entangled masses that are easily attached in fouled nets, therefore cleaning the nets will significantly help the control of the disease. Moreover, various natural ingredients are being tested as possible antiparasitic treatments.

Regarding *Neobenedeniagirellae*, promising results have been produced by the administration of mannan oligosaccharides (MOS and cMOS) as immunostimulants in the fish diet. These ingredients have shown to enhance mucus production which is a first line of defense against external parasites and to promote innate immune response of the fish. Administration of cMOS in healthy fish resulted in significant lower parasite load compared to control fish following challenge with *Neobenedeniagirellae*. cMOS not only prevented parasite attachment, but also reduced the growth and development of the parasites concomitant with increased immune responses. A mobilization of fish defences to the skin mucus has been described as an effect of prebiotics, and could affect the correct development of parasites as they attempt to overcome the first physical and chemical barriers of the host.

Other parasites that have been observed include the blood fluke, *Paradeontacylix* sp. and the copepod *Pennella* sp., however their significance as pathogens is probably lower to that of *Zeuxapta seriolae* and *Neobenedenia girralae*.



Market, Consumer perception, new products and business model

Gemma Tacke, 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. 57**), value adding and market development. The studies have been performed across five largest 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.* greater amberjack) in relation to the current species in the market. Species such as greater amberjack are 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. Although different, tuna related species might easily be seen as the closest and, in some cases, as a cheaper direct competitor.

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) and
- 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 and 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 greater amberjack (**Fig. 17**): ready-made fish tartar with additional soy sauce; fresh fish fillet for grilling in the pan and fresh fillet with different healthy seasoning and marinades. Fresh greater amberjack fillet grilled was the product with the highest score (mean acceptability value) in the overall liking test in blind and fully informed conditions.



Figure 57. Greater amberjack new products developed in Diversify: ready-made fish tartar with additional soy sauce (top left); fresh fish fillet for grilling in the pan (top right) and fresh fillet with different healthy seasoning and marinades (bottom).



Sensory characterization of new fish species and consumer acceptance of new product development

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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 having the product information in advance on the consumer acceptance degree (**Fig. 58**). This test learns that greater amberjack needs some extra clarification to consumers before introducing the products in the market.

In the case of greater amberjack, it is presented as a natural fillet, a seasoned fillet and a ready-made fish tartar with additional soy sauce. The amberjack products were perceived as having equally high intensities with other processed products in several attributes. Specifically, the steak was perceived as having amongst the highest green aroma, potato and sardine flavor, teeth adherence and secondary fibrous and chewy texture. This implicates that the products are rated positively and interesting.

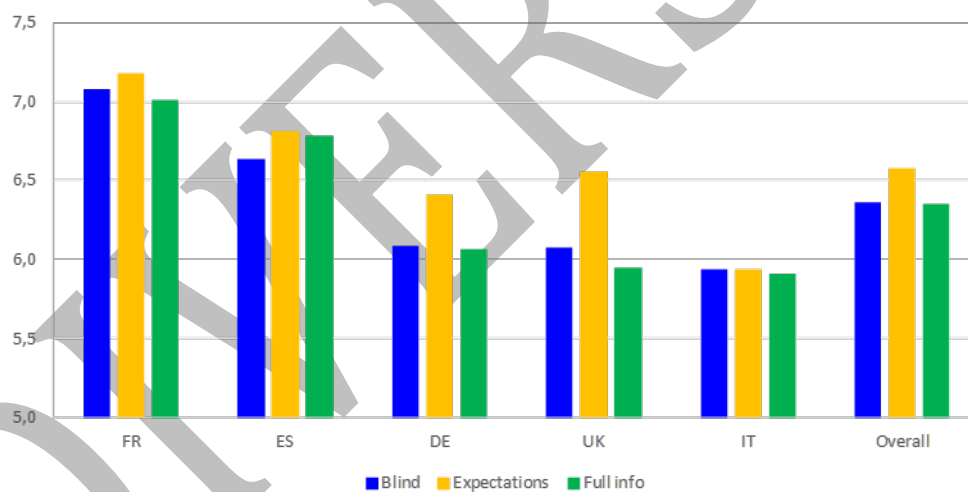


Figure 58. Results of the consumers' acceptance tests for new developed products performed in 5 European countries. Consumers were not informed about the product (blue bar), then knowing the product to be tested, they were asked about their expectation (orange bar) and finally they had the full information before tasting the product (green bar).



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