New species for EU aquaculture

Deliverable Report

Deliverable No:	D15.5		**Delivery Month:**	59
Deliverable Title	An industrial protocol for greater amberjack larval rearing			
WP No:	15		**WP Lead beneficiary:**	P8.IEO
WP Title:	Larval husbandry - greater amberjack			
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Task Title:	Development of industrial protocol			
Other beneficiaries:	P1.HCMR	P2.FCPCT	P40.GMF	
Status:	Delivered		**Expected month:**	48
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Objective: The objective of the present study was to develop an industrial larval culture protocol incorporating the results from the performed trials.

Description:

The deliverable presents the larval rearing equipment and protocols for the larviculture of greater amberjack in order to optimize the egg-larvae stocking density, tank hydrodynamics and volume, light and photoperiod, feeding schedule combined with optimal environmental parameters and larvae management to improve the larval performance in terms of survival and growth. The information presented here takes into account the results obtained in new specific trials combined with those obtained in the studies developed in both larval husbandry and nutrition, in collaboration with other partners of the DIVERSIFY project. These include the Hellenic Center for Marine Research (HCMR), the Fundación Canaria Parque Científico Tecnológico from Universidad de Las Palmas de Gran Canaria (FCPCT), the Universidad de La Laguna (ULL), Galaxidi Marine Farms (GMF) and the external collaborator Nireus Aquaculture.

Contents

Summary

Greater amberjack (*Seriola dumerili*) is a fast growing fish and a very interesting species for the diversification of aquaculture. However, data on the rearing conditions in captivity, particularly during the early developmental stages, is scarce. For this, it is necessary to obtain new information on its rearing protocols. The main objective of this study was 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.

The effect of tank hydrodynamics showed that the higher currents occurred in the 2,000 l tanks followed by the 40,000 l tanks.

The study of the photophase (24L:00D vs 18L:6D) showed that better performance of larvae was observed with 18 hours of light resulting in higher levels of mRNA expression of IGF-I, IGF-BP1, IGF-BP3 and IGF-BP5. On the other hand, the greater amberjack larvae reared in tanks of different background color (white, green and black) exhibited the highest survival rate in those having a white background, which also showed a higher level of expression in the genes involved in the growth axis system.

The larvae showed different types of skeletal anomalies regardless of the volume of the tank (2,000 l and 40,000 l) but the histological study demonstrated a higher degree of hepatocyte vacuolization, indicative of greater stress, in 2,000 l tanks. With regard to initial stocking density (25, 50 and 75 eggs l^{-1}), the lowest density tested improved the survival, but also presented the lowest larval growth.

The aforementioned conditions (light, photophase and background color) affect the success of larvae predation i.e., food visualization. The applied feeding protocol must be based on the adequate intake of prey that provides for the development of the visual and digestive system of the larvae. In addition, the food offered has to cover the nutritional requirements in order to obtain the best growth, health and survival of the greater amberjack larvae. The frequency of prey addition (rotifers and Artemia) in the rearing tanks should ensure adequate availability of food items to the larvae and may depend also on the applied prey enrichment protocol. In this sense, there was a positive effect on the larvae performance with an experimental live prey enriching emulsion supplemented with phospholipids (PL), carotenoids, arachidonic acid (AA) and immune modulators such as *Echium* oil and black cumin oil compared to commercial enrichments. The feeding sequence (rotifers, Artemia nauplii, and artificial diet) and feeding period depended on larval development.

In general, rearing systems at higher temperature (> 23.5ºC) improved the survival and growth of the larva and the organs related to buoyancy and vision were more developed, which could influence the protocol for feeding each prey type and artificial food.

With the acquired knowledge from project DIVERSIFY, the protocols developed have been evaluated in SME hatcheries showing satisfactory and promising results.

1. Introduction

One of the major bottlenecks in the industrial production of greater amberjack is larval rearing and the availability of a sufficient number of fry. 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 protocol and the use of rearing systems to culture this species on a commercial scale.

Early trials showed that semi-intensive methods, characterized by stocking density as low as 0.25 eggs l^{-1}, seemed to be effective, but the survival rate was rather low at around 3% (Papandroulakis *et al*., 2005).

In general, low stocking densities improve growth by increasing food accessibility, water quality and providing vital space such as been cited for gilthead seabream (*Sparus aurata*) and the red porgy (*Pagrus pagrus*) (Kentouri *et al*., 1994; Hernández-Cruz *et al*., 1999; Roo *et al*., 2005a,b). In contrast, high stocking density is frequently associated with low growth due to poor water quality (Yu and Perlmutter, 1970) or increased food competition (Hagen, 1993), leading to reduced growth as was observed in other fast-growing species, such as meagre (*Argyrosomus regius*) (Roo *et al*., 2010). Moreover, the conditions of the larval rearing systems and the hatchery techniques used can be modified in order to improve the development, feeding behavior, health status (Tamazouzt *et al*., 2000; Zambonino-Infante *et al*., 2008) and survival of fish larvae (Duray *et al*., 1997; Sakakura *et al*., 2007).

Fish larvae are visual predators and the prey identification and capture dependents on the light conditions (Puvanendran and Brown, 2002) and its effect on the background colour (Duray *et al.,* 1996; Downing and Litvak, 1999; Tamazouzt *et al*., 2000; Jentoft *et al*., 2006). In addition, the duration of photo phase has a significant role in the feeding efficiency of the larvae as it prolongs the foraging period of the individuals (Papandroulakis *et al*., 2002; Papandroulakis *et al*., 2010). A prolonged photo phase has been found beneficial for the performance of some species although concerns have been expressed for the possible stress, as well as retinal damage (Migaud *et al*., 2007) that an extended photoperiod can induce (Villamizar *et al*., 2011). Lighting conditions have also been shown to affect other important developmental stages such as swim bladder inflation (Trotter *et al*., 2003).

The larval survival in marine finfish depends on timely development of vision and digestive systems and the organs required for feeding along the larval rearing (Porter and Theilacker, 1999). The stomach contents are considered as good indicators of food preference and the maximization of larval feeding should result in improved growth and survival. In addition to food intake, the larvae need to digest and assimilate it, which depends on the development and functionality of the digestive system. Moreover, the vision and digestive system development and its synchronization with larvae feeding behavior are affected by environmental factors such as temperature (Suzer *et al*., 2007; Yufera and Darias, 2007; Rønnestad *et al*., 2013).

It is important to determine the specific requirements of the greater amberjack larvae in order to optimize the rearing success. Once the basic physical conditions are determined and their effects evaluated, in combination with the feeding protocol used, the farmers can incorporate them into the design of their hatchery and adopt a feeding strategy for the larvae. Therefore, protocols for the larviculture of greater amberjack, as for any other species, include the appropriate combination of the different variables involved in the larval rearing process to increase performance during the larval stages.

In this document, we present in a coherent manner the findings from a series of trials performed during the course of the project[1] that were focusing on, (i) the tank hydrodynamics and volume, (ii) the light and photoperiod conditions, (iii) the egg-larvae stocking density (iv) the feeding schedule and (v) the nutritional supplements. In addition, the results from the industrial application in two commercial hatcheries are presented after incorporating in their everyday practice suggestions and comments resulting from the experimental trials. The above information was used to evaluate the rearing protocols and to suggest ways for their improvement.

2. Environmental conditions for larval rearing

2.1 Currents

In order to increase productivity during the larval stages, specific parameters of two different cylinder-conical tank types with volumes of 40,000 l and 2,000 l were tested in duplicates for a period of 30 days.

[1] The information used is from the following deliverables:
D15.1 Effective greater amberjack larval stocking densities.
D15.2 Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing.
D15.3 Optimum hydrodynamics and light conditions during greater amberjack larval rearing.
D15.4 Ontogeny of greater amberjack larval visual and digestive system.

These studies focused on the effect of tank hydrodynamics, as a function of volume, on larval performance in terms of growth, survival, histology, biochemical composition and skeletal deformities. The trials were performed at the facility of FCPCT.

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. The conditions regarding water exchange and air flow were similar to the ones applied during the current protocol for larval rearing. Current measurements were performed using a Vectrino velocimeter. The Vectrino is a high-resolution acoustic velocimeter used to measure 3D water velocity in a wide variety of applications from the laboratory to the ocean. The basis measurement technology is coherent Doppler processing. The water velocity measurements have a range of ± 1 cm s^{-1} with an accuracy of ±0.5% of the measured value or ±1 mm s^{-1}. The sampling volume is at a distance of 5 cm from probe with a diameter of 6 mm and a height of 7 mm (**Fig .1)**

Measurements of current field at specific depths or layers were performed. The 2,000 l tank was divided into 5 layers at 0.1, 0.65, 1.10, 1.25 and 1.5 m depth. In the first 3 layers, 17 measurements were taken, while on the fourth layer 5 measurements were taken and 1 on the last layer, at normally distributed points. For the mesocosm 40,000 l tanks, 3 layers were examined at 0.3, 0.7 and 1.5 m where 17 measurements were taken at each layer.

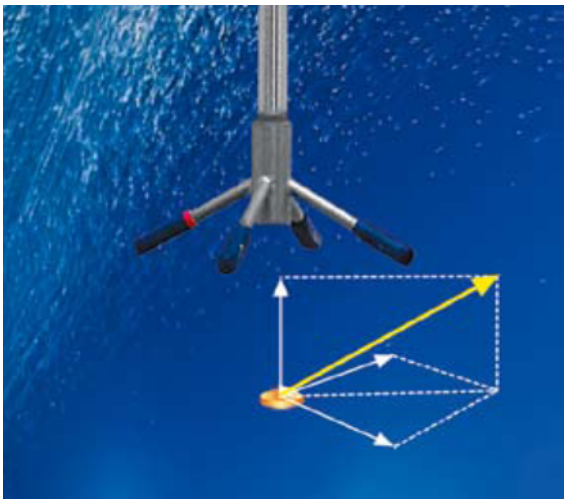


Figure 1. Representation of current sampling volume

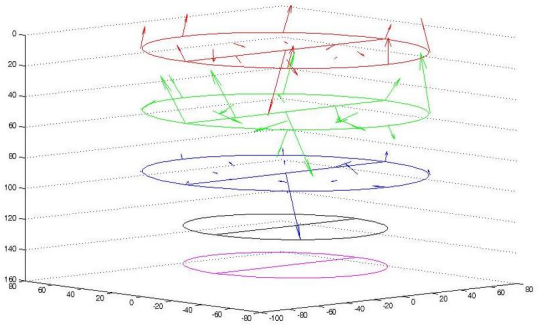
The results are shown in **Table 1**. There were differences in current profiles between the 2,000 l and 40,000 l tanks. Water currents showed that they were generally higher in the 2,000 l tanks.

Table 1 Mean velocity at the different layers of the experimental tanks (mean and standard deviation, n=1,500)

Tank volume (l)	Layer	Mean velocity value (cm s^{-1})
2,000	surface	$1.51x10^{-2}$ ±$1.55x10^{-2}$
	medium1	$0.92x10^{-2}$ ±$1.38x10^{-2}$
	medium2	$0.84x10^{-2}$ ±$3.32x10^{-3}$
	medium3	$0.23x10^{-2}$ ±$2.07x10^{-2}$
	bottom	$1.30x10^{-2}$ ±$3.65x10^{-2}$
40,000	surface	$9.10x10^{-3}$ ±$3.22x10^{-2}$
	medium	$8.70x10^{-3}$ ±$3.05x10^{-2}$
	bottom	$7.50x10^{-3}$ ±$8.69x10^{-2}$

A graphical representation of the current profile is presented in (**Fig. 2).** Arrows are in 3-d representation and the observed size does not represent the actual velocity value.

(a) 2,000 l



(b) 40,000 l

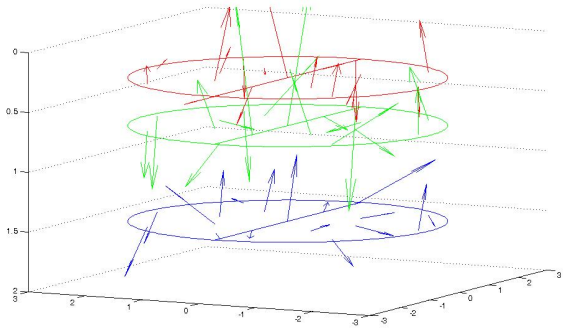


Figure 2. Current profiles in the different layers of the 2,000 l (a) and 40,000 l (b) tanks. Arrows are in 3-d representation and the observed size does not represent the actual velocity value.

2.2. Light

Fish larvae are visual predators and their capacity to identify prey is dependent on the light conditions in their environment (Puvanendran and Brown, 2002). The effect of light has an important role in the growth and survival of fish larvae (Duray *et al.,* 1996; Downing and Litvak, 1999; Tamazouzt *et al*., 2000; Jentoft *et al*., 2006). In addition, the duration of photo phase has a significant role in the feeding efficiency of the larvae as it prolongs the foraging period of the individuals especially at the early stages (Papandroulakis *et al*., 2002; 2010).

During the course of the project, the effect of three different background colors as well as two photophase regimes on amberjack larval performance and on the expression of genes implicated in the growth axis was characterized for the first time during early ontogeny in amberjack.

The trials were implemented during two years as results obtained during the first year were poor and did not allow deriving any conclusions. The experimental trials were performed at the hatchery of IMBBC, HCMR with eggs from the Argosaronikos SA cage farm. The methodology applied was the intensive rearing method, characterized by controlled conditions of water quality, light intensity, photophase and feeding. A variation of this methodology is the so-called "pseudo-green" water approach that is based on the frequent addition of phytoplankton and zooplankton in the larval rearing tanks. The tanks used were 500 l, organized in couples in a closed water system with a biological filter. The temperature was kept at 24±0.8°C, the pH fluctuated from 7.81 to 8.18 and the dissolved oxygen from 4.92 to 7.42 mg l^{-1}. A skimmer was installed

during the appropriate period (5 to 15 dph) to keep the surface free from lipids. Light intensity varied between 200 - 800 lux during the day, and was about 200 lux during the night.

Feeding was based on daily administration of enriched rotifers (from 3-21 dph), Instar II Artemia nauplii (from 12 dph onwards), and artificial diet (from 21 dph). Phytoplankton was added daily from 3 to 22 dph at 300 ± 100 x 10^3 cells ml^{-1}. The administration of the zooplankton was implemented with the use of an automated feeding system allowing continues administration of food.

The performance of the larvae was compared by measuring the growth and the survival while the expression of growth hormone/insulin-like growth factor axis related genes was also used.

Regarding the **photophase**, two light:dark conditions were tested; 24:00 and 18:06 hours d^{-1}. 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 (P>0.05) 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 photoperiod (24L:00D vs 18L:6D), 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. 3).**

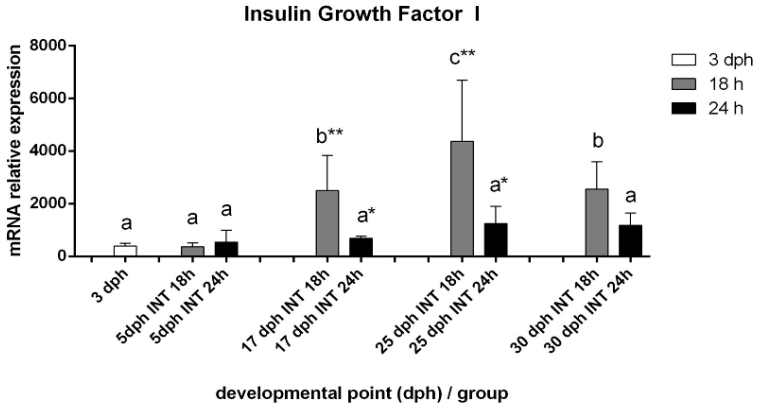


Figure 3. mRNA relative expression levels of IGF-I between the different photoperiod regimes during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 4). Means with different letters indicate differences between the different developmental points whereas asterisks indicate differences between the photoperiod schemes (P<0.05).

For the **light intensity,** tanks with three different colors (black, green and white) were used for larval rearing, in duplicates. For this, white or green fabric that completely covered the inner walls was used, while black tanks served as controls. Furthermore, underwater lights were used to improve light intensity in the water column without significantly changing the intensity on tank's surface. Underwater lighting was applied from 8:00 to 20:00 imitating the increased brightness during summer months. In **Fig. 4** the tanks with different backgrounds are shown.

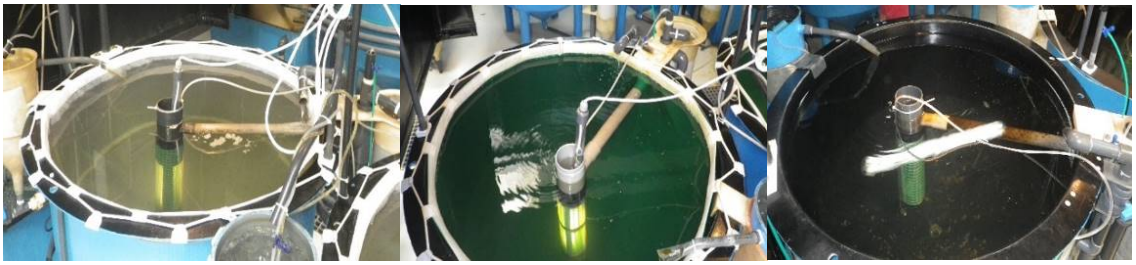


Figure 4. Tanks with different color backgrounds.

No statistically significant (P>0.05) differences were observed in the growth of the larvae in terms of total length and body weight 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 rates among the different groups with the larvae in the white tanks exhibiting the highest rate (22.2±0.7%) compared to green (16.5±0.9%) and to black (8.2±3.1%).

Furthermore, the fish reared in the white background showed increased levels of the genes implicated in the growth axis system compared to the fish reared in the black and green backgrounds. In particular, IGF-I showed generally higher levels of expression as development proceeds and also it appeared affected by the background color as higher levels were observed in fish reared in the white background at 17 dph and 30 dph compared to fish reared in the black and green backgrounds (*P*<0.05 ; **Fig. 5**).

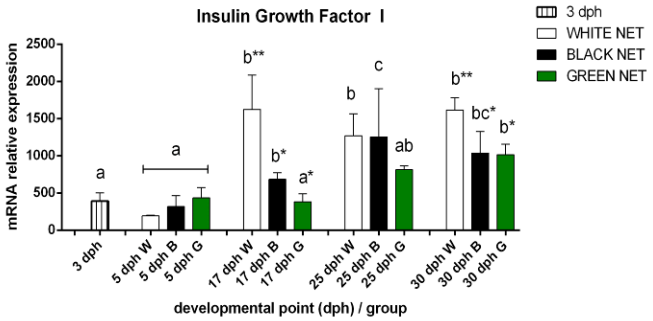


Figure 5. mRNA relative expression levels of IGF-I between the different background colours during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 4). Means with different letters indicate differences between the different developmental points whereas asterisks indicate differences between the background colours (P<0.05).

GH expression levels showed no differences throughout development but they were affected by the color of the tank as at 17 dph fish in the white background showed higher levels compared to the fish reared in the green background (**Fig. 6**).

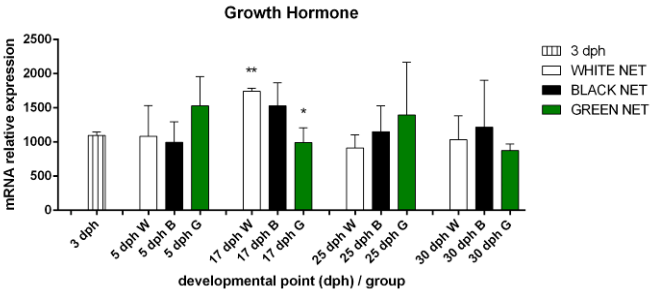


Figure 6 mRNA relative expression levels of Growth Hormone between the different background colours during early ontogeny of *Seriola dumerili*. Values are means ± standard deviation (n = 4). Asterisks indicate differences between the background colours (*P*<0.05).

Although the studies did not reveal any significant effect of background color on larval growth (total length, body weight) there was a catalytic effect on their survival. The difference between the white background and all the other conditions was apparent. 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 with 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.

Additionally, the analysis carried out on the expression of growth hormone/insulin-like growth factor axis related genes during the early ontogeny of *Seriola dumerili,* showed that IGF-BP1 and GH expression are affected by the background color of the tank. There were lower expression levels of IGF-BP1 at 5dph and higher levels of GH at 17dph (flexion) in the white group compared to the other two groups (data not shown). Additionally, 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 the white background compared to the other two backgrounds, particularly the green color, which coincides with the differences observed in the survival rates.

The presented results from the trials of tanks with the modified "light environment" demonstrated an improvement by an order of magnitude than previously reported confirming the validity of the tested hypothesis and indicate a significant technological step forward in the larval rearing of the greater amberjack.

2.3 Effect of volume and light intensity on the performance of the larvae including immune response and oxidative stress

In general, larger tanks and the use of a semi-intensive approach have been linked to successful results of marine fish larval rearing (Duray *et al*., 1997; Sakakura *et al*., 2007; Russo *et al*., 2009), especially when knowledge of the specific biological requirements of a species is scarce or not available (Divanach and Kentouri, 2000; Papandroulakis *et al*., 2004). This approach however is difficult to support an industrial activity and proper feeding protocols should be developed.

The ability of the larva of greater amberjack to see the prey, like many teleost, depends on the vision system (i.e., the eye) development and light conditions. After yolk sac exhaustion, there is a positive allometric increase of organs related to prey detection (i.e eye) and capture in the first days of feeding (Osse *et al*., 1997; Rodríguez and Gisbert, 2002; Yúfera and Darias, 2007) as well as increased visual sensitivity (Olla *et al*., 1995; Privileggi *et al*., 1997).

The growth, development and survival of the larvae are influenced, directly or indirectly, by a wide range of environmental variables such as temperature, light and oxygen. The inadequate inflation of swim bladder can affect the feeding behavior due to the alterations of buoyancy, equilibrium and position of fish in the water (Hadley *et al*., 1987; Battaglene and Talbot, 1990; Itazawa, 1991; Trotter *et al*., 2003) as well as severe anomalies in larval development, which can impact on feeding and growth (Hashimoto *et al*., 2013).

Access to information available on the morphological and functional changes is essential for the implementation of the successful larval rearing protocol of greater amberjack (Zambonino-Infante *et al*., 2008), in terms of improved survival and growth.

A trial to evaluate the effects of a feeding protocol on growth, survival and development of larvae of greater amberjack reared in two different tank volumes and light intensities was carried out and assessed in terms of the development of organs involved in swimming, food detection, prey intake and health status.

Spawned eggs obtained from F1 *Seriola dumerili* broodstock at the IEO-COC facilities were collected with a passive egg collector placed in the outflow of the spawning tank, rinsed and placed in 90 l cylinder-conical tanks. The fertilized eggs were incubated with constant exchange of filtered seawater and slightly aeration.

The newly hatched larvae were stocked (3 larvae l^{-1}) in circular rearing tanks of 32 and 40 m^3 with different surface-volume ratios (0.5 and 0.7 m^{-1}, respectively) and light conditions (2500 and 1000 lux, respectively). The tanks, with a black background, were previously filled with filtered (5µm) seawater at ambient temperature (23-25ºC) and the rate of renewal was increased progressively during the larva rearing as follows: 15-40% day^{-1} at 1 dph, 30-40% at 10 dph, 100-120% at 20 dph, and 200-240% at 30 dph.

Aeration was also provided in the tanks by means of pipes distributed in the perimeter and the center of the tank in order to maintain the larvae in gentle rolling suspension. The oxygen saturation ranged between 89 and 95% (6.3 to 6.6 mg l^{-1}).

The photoperiod, light intensity and temperature used in the larval rearing trials in IEO facilities were natural. The photoperiod at our latitude was 14L:10D, while the light intensity depended on the tank location in our facilities and its dimensions (depth). In 32 and 40 m^3 tanks the surface light intensity was about 2,500 and 1,000 lux, respectively. The light intensity decreased with depth in both tanks by 50% at 20-40 cm and about 80% at 100 cm.

During the larval rearing, live microalgae (*Chlorella sp*.) cultured at the IEO facilities, was added daily from 1-4 dph to 15-25 dph at 150-300 x 10^3 cells ml^{-1}. Feeding was based on daily administration of rotifers (*Brachionus plicatilis*) harvested from the stock culture and enriched with DHA Protein Selco (INVE S.A., Belgium) distributed two times a day (8.00 and 16.00 h) from 3 to 25 dph. The initial density of rotifers in the rearing tank was 3 rotifers ml^{-1}, increased progressively to 5-10 rotifers ml^{-1} at 8-10 dph. The remaining rotifers in the larval rearing tank was estimated daily before adding the newly enriched rotifers. During the rotifer feeding, copepods were introduced to the rearing tanks due to the natural productivity in the rotifer culture, which potentially contributed to larvae feeding.

At 12 dph, and during 5-7 days, Artemia AF nauplii were added to rearing tanks and Artemia EG 1-day enriched with A1 DHA Selco (INVE S.A., Belgium) were offered between 14 and 18 dph. The Artemia was supplied two times daily, and density in the rearing tanks ranged between 0.05 and 0.5 Artemia ml^{-1}.

The time to start of feeding with artificial feeds depends on the larva size and development. Nevertheless, in all trials it began at 18-20 dph. Size, frequency and quantity of artificial feeds increased progressively according to fish size (NRD 2/4 size of 200–300 µm, and NRD 3/5 size of 300–500 µm, INVE S.A., Belgium).

During the trials, larvae were sampled periodically to evaluate growth parameters. Total length and eye diameter were measured with a Nikon Digital Sight DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) and the percentage of larvae with inflated swim bladder as well as swim bladder large and height was also determined. Prey intake was determined by analysis of stomach contents. The presence of ingested rotifers can be easily recognized look for whole rotifers or for their components such as mastax, lorica, or eggs. At the same time, the ingested copepods and copepodits, can also be visible in the stomach contents. To confirm the ingestion rate of Artemia nauplii in older larvae, it is sufficient to visually identify the deep orange nauplii by checking digestive tracts. The survival of larvae was calculated based on the number of surviving fish that were individually counted at 30 dph.

Larvae samples at 12 and 25 dph were collected from 32 and 40 m^3 tanks, flushed with N_2 and kept frozen at -80 °C until analyzed for the nutritional condition, oxidative status and immune system parameters.

Antimicrobial activities were determined in larvae homogenates from samples collected from each rearing tank at 12 and 25 dph. Pools of larvae were weighed and mechanically homogenized in 1 ml of 0.01 M PBS (9 mM sodium phosphate dibasic, 2 mM, sodium phosphate monobasic and 0.15M NaCl), and centrifuged at 10,000 g during 10 min at 4ºC to avoid cell debris. The supernatants of larvae homogenates were used for lysozyme, peroxidase, protease, anti-protease and bactericidal activity assays. Protein concentration of larvae homogenates were estimated by the Pierce BCA protein assay reagent using BSA as a standard. Lysozyme activity was measured according to a turbidimetric method that uses the lysis of *Micrococcus lysodeikicus* for determination of the lysozyme activity using hen egg-white lysozyme as the standard (Parry *et al*., 1965). The peroxidase activity was measured with a method previously described (Quade and Roth, 1997). Protease activity was determined as the percentage of hydrolysis of azocasein by 2 mg ml^{-1} of proteinase K (Charney and Tomarelli, 1947). Total anti-protease activity was determined as the percentage of inhibition of the hydrolysis of azocasein by 2 mg ml^{-1} of proteinase K (Ellis, 1990). Serum antibacterial activity was determined by evaluating the inhibition on the bacterial growth of *Vibrio harveyi* curves with a method modified from (Sunyer and Tort, 1995).

Pooled samples of whole larvae were homogenized in (1:5, w:v) ice-cold 20 mM Tris-Cl containing 1X cOmplete™ protease inhibitors cocktail (Roche Diagnostics GmhbH, Mannheim, Germany) at a pH 7.4.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the method of Marklund and Marklund (1974) based on the inhibition of the autoxidation of pyrogallol (1,2,3-trihydroxybenzene) to purpurogallin. Briefly, 100 µl of homogenate was mixed with tris-cacodylic buffer (50 mM Tris-HCl, 50 mM cacodylic acid, 1mM diethylenetriamine pentaacetic acid, pH 8.2), and 2 mM pyrogallol. The autoxidation of pyrogallol and the inhibition of this reaction was monitored spectrophotometrically at 420 nm. Superoxide dismutase activity was calculated using the molar extinction coefficient of purpurogallin (2640 M^{-1} cm^{-1}). One unit of SOD activity is equivalent to the amount of enzyme that produces a 50% inhibition of the auto-oxidation of pyrogallol. The activity of SOD is expressed as units mg $protein^{-1}$.

Catalase (CAT, E.C. 1.11.1.6) activity was quantified by monitoring the rate of enzyme-catalysed decomposition of 5 mM H_2O_2 in 10 mM potassium phosphate buffer, pH 7.0, and measuring the absorbance at 240 nm. Catalase activity was calculated using the molar extinction coefficient of hydrogen peroxide (ε = 42.6 M^{-1} cm^{-1}). One CAT unit was defined as the decomposition of 1 mmol H_2O_2 per minute, and was expressed as units mg $protein^{-1}$.

Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined following the conjugation of 5mM GSH with 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and the absorbance of the Mesenheimer complex

produced measured at 340 nm. Glutathione S-transferase activity was determined using the molar extinction coefficient of Mesenheimer complex (Ɛ = 9.6 mM¬cm¬) (Habig *et al*., 1974). One unit of GST activity was defined as nmol GS-DNB originated per minute and was expressed as units mg protein^{-1}.

Lipid peroxidation was determined by the thiobarbituric acid reacting substances (TBARs) method (Ohkawa *et al*., 1979), using TMP (1,1,3,3-tetramethoxypropane) as standard for calibration curves. Briefly, larval homogenates was mixed vigorously with sodium dodecyl sulfate 8.1%, 20% acetic acid (w/v) containing 0.05% BHT in methanol and freshly prepared 50 mM thiobarbituric acid solution before heating for 60 min at 95 °C. After ice-cooling, the mixture was centrifuged at 10000 g for 3 min, at 4 °C, and supernatant read fluorimetrically (Applyskan, Thermo Scientific, Milan, Italy) with 485 nm (excitation)/535 nm (emission) wavelengths. TBARS content was expressed as nmol MDA mg protein^{-1}.

All the data were statistically treated using a SPSS Statistical Software System 19.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA followed by Tukey *post-hoc* test unless otherwise stated. When variances were not homogeneous, a non-parametric Kruskal–Wallis test was accomplished.

The newly hatched greater amberjack larvae (3.50±0.16 mm TL) started to feed at 4 dph (3.63±0.32 mm TL). The TL increased exponentially during the larval rearing (**Fig. 7**). The growth rate and the percentage of larvae with inflated swim bladder (**Fig. 8**) were higher in tanks of 32 m^3 than in tanks of 40 m^3. In 32 m^3 tanks, all larvae showed the swim bladder inflation at 7 dph (3.84±0.30 mm TL), and in 40 m^3 tanks, at 14 dph (4.59±0.29 mm TL).

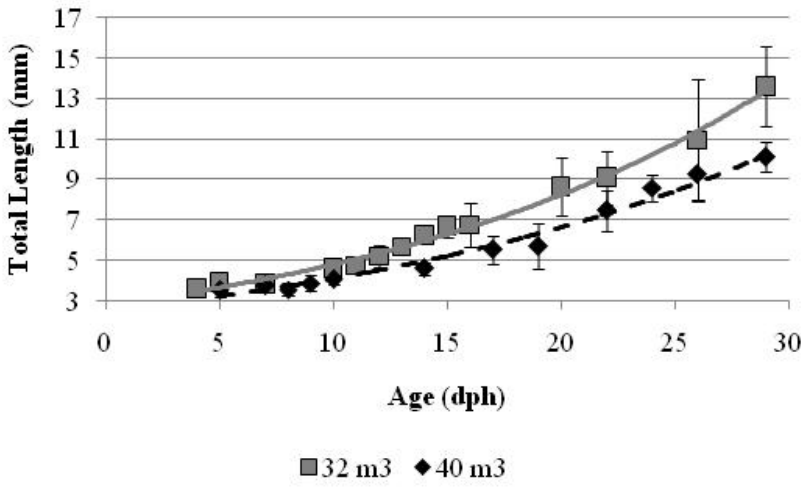


Figure 7. Total length (mm) of greater amberjack larvae reared in 32 and 40 m^3 tanks. Values are mean ± SD (n=10).

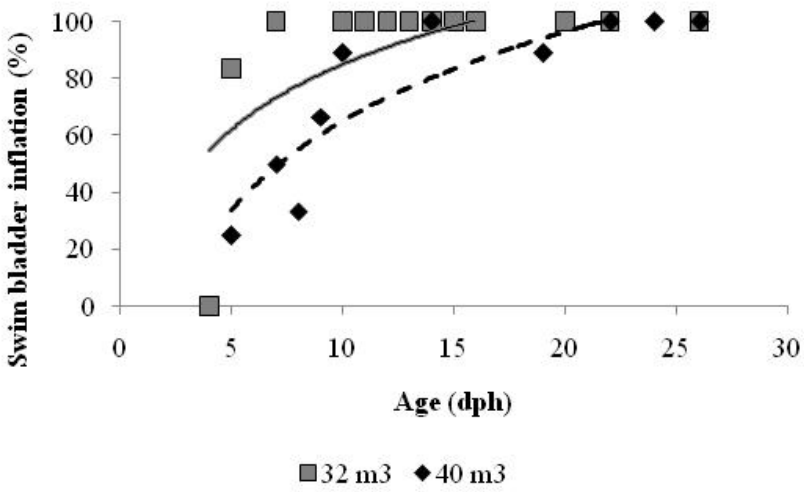


Figure 8. Swim bladder inflation (%) of greater amberjack larvae reared in 32 and 40 m^3 tanks. Values are mean ± SD (n=10).

Similar to the percentage of inflation, the swim bladder volume of larvae reared in 32 m^3 tank was greater longer than cohorts reared in 40 m^3 tank at the same age, although TL was similar (**Fig. 9 a, b**).

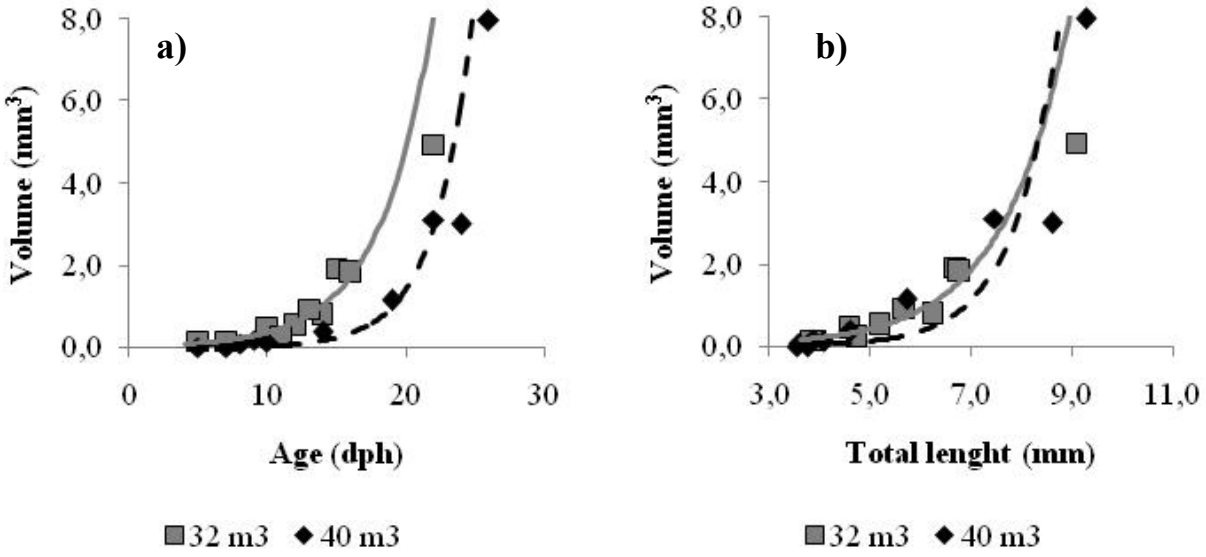


Figure 9 a, b. Swim bladder volume of greater amberjack larvae reared in 32 and 40 m^3 tanks with respect to Age (a) and Total length (b).

In general, the eye diameter was higher in larvae reared in 32 m^3 than 40 m^3 tanks at the same age (**Fig. 10a**). However, the ratio eye diameter-TL was similar in both rearing tanks, increasing linearly from about 7% to 10%, until the larva was about 6.0 mm TL (**Fig. 10b**) at age 13 and 17 dph for 32 and 40 m^3 tanks, respectively (**Fig. 10c**). No changes were observed after this point.

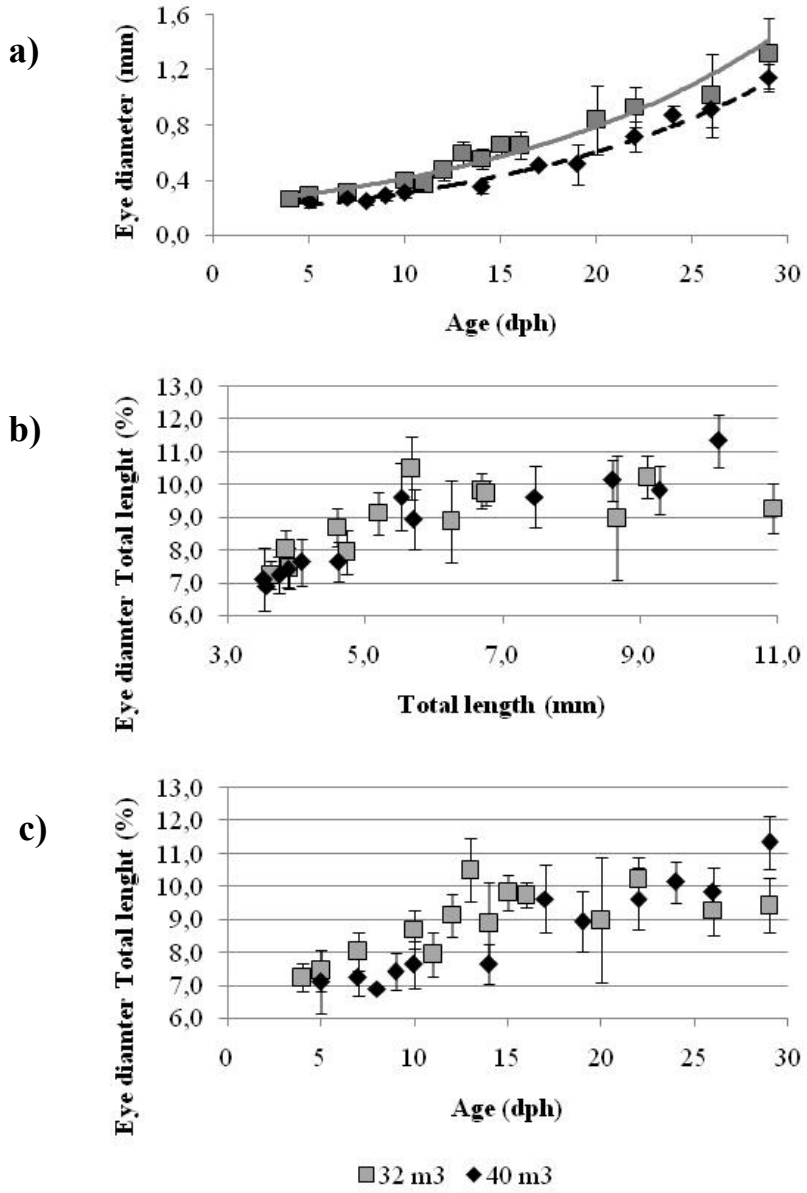


Figure 10. Eye diameter (mm) (a) and ratio of Eye diameter to Total length, expressed as percentage (%), respect to TL (mm) (b) and Age (dph) (c) of greater amberjack larvae reared in 32 and 40 m^3 tanks.

In this study, the prey items in the stomach were related to age (dph) and TL of larvae. The rotifers intake showed a decrease coinciding with an increase of Artemia intake, irrespective of the rearing larvae tank volume. Copepods at different developmental stages were ingested together rotifers and Artemia from initial feeding (4 dph) to more than 20 dph.

Larvae ingested a higher number of rotifers at 7-10 dph (between 3.7 and 4.2 mm TL) and they did not show rotifers in their stomach after 16-19 dph (about 6.4 and 4.6 mm TL in larvae reared in 32 and 40 m^3 tank volume, respectively) (**Fig. 11 a, b**).

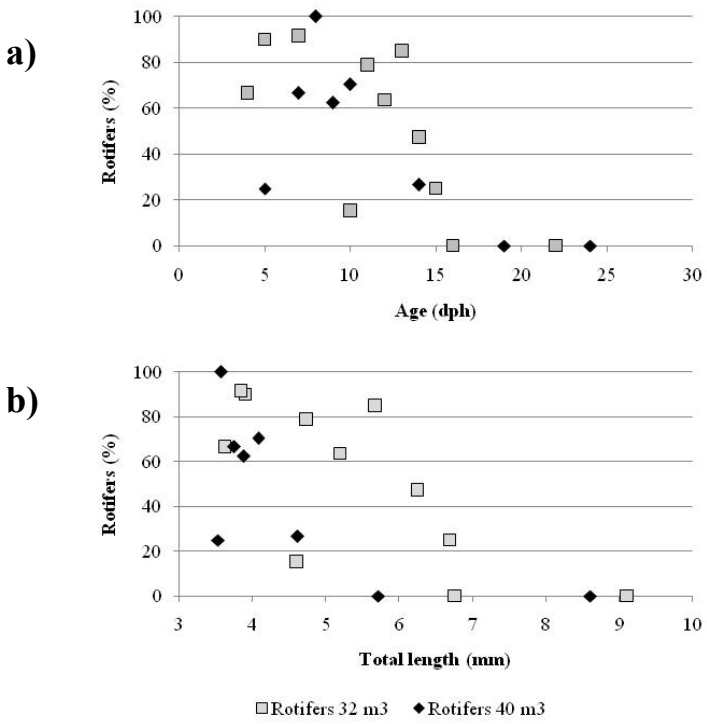


Figure 11a, b. Relationship between rotifers intake and Age (dph) (a) and Total length (mm) (b) in greater amberjack larvae reared in 32 and 40 m^3 tanks.

Copepods in different developmental stages were present in the stomach of larva from first feeding (> 3.5 mm TL) to more than 20 dph. This prey item supposed from 10% to more than 50% of total prey intake in 32 and 40 m^3 tanks (**Fig. 12 a, b**).

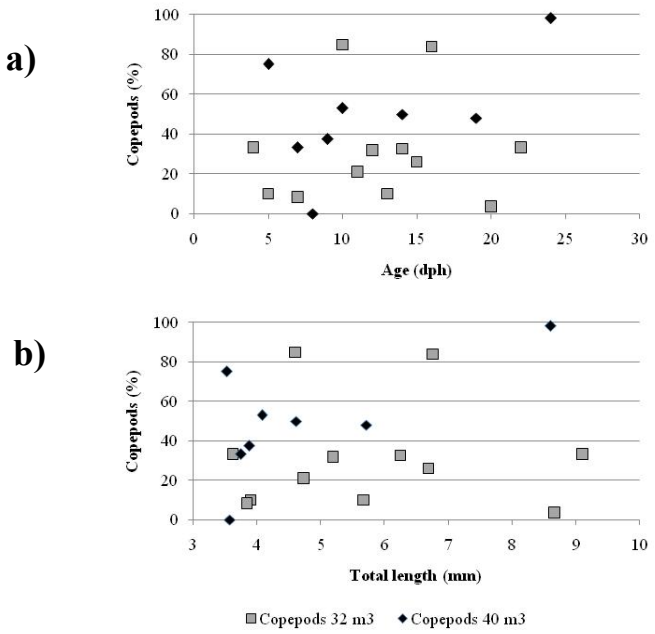


Figure 12 a, b. Relationship between copepods intake and Age (dph) (a) and Total length (mm) (b) in greater amberjack larvae reared in 32 and 40 m^3 tanks.

Moreover, Artemia nauplii were added to rearing tanks from 12 dph to 26 dph and found in the larvae stomach immediately after being offered. Artemia nauplii were found in larvae stomach from 5.2 mm TL in 32 m^3 tank and from 4.3 mm in 40 m^3 tanks, respectively (**Fig. 13 a, b**).

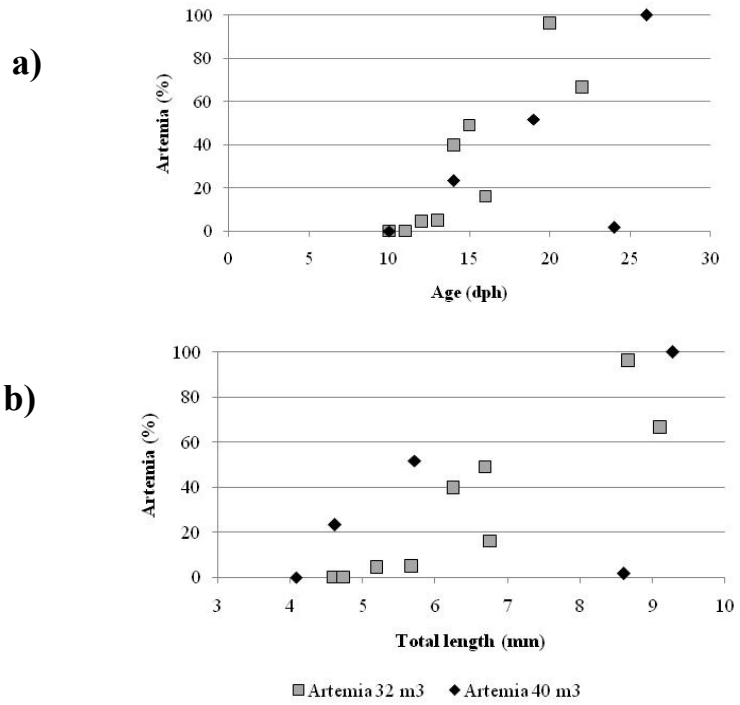


Figure 13 a, b. Relationship between Artemia intake and Age (dph) (a) and Total length (mm) (b) in greater amberjack larvae reared in 32 and 40 m^3 tanks.

The offering of artificial feeds started at 20 and 22 dph in 32 and 40 m^3 tanks, respectively, and was found in larvae stomach at 22 and 24 dph (9.1±1.1 and 8.6±0.7 mm of TL), respectively.

The humoral immune activities studied showed similar levels in 32 and 40 m^3 tanks at 12 and 25 dph (**Fig. 14**). Overall, activities were similar or decreased with age for larva reared in the 32 m^3 tank, and maintained or increased with age for larvae in the 40 m^3 tank, except bactericidal activity that increased with age in both tanks but was lower in larvae reared in the 32 m^3 tank than in the 40 m^3 tank.

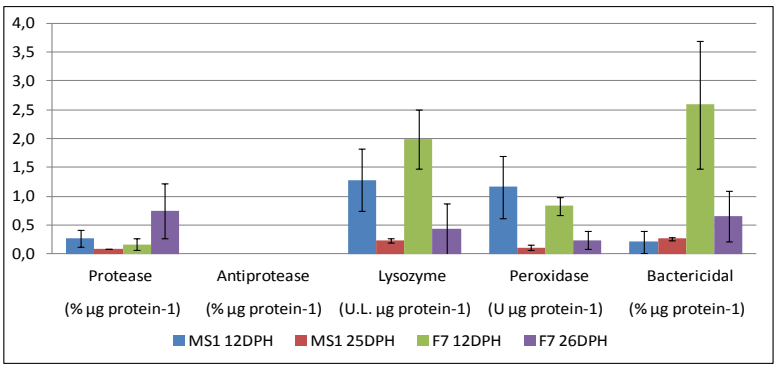


Figure 14. Humoral innate immune activities of 12 dph and 25 dph greater amberjack larvae from 40 m^3 and 32 m^3 tanks. Values are mean ± SD (n=2).

The levels of antioxidant enzymatic activities and lipid peroxidation measured in larvae at 12 and 25 dph are shown in **Fig. 15**. A general trend to decrease antioxidant activities with age is evident for both tanks. In addition, larvae from 40 m^3 tank seemed to show lower Catalase and SOD activities than 32 m^3 tank larvae. Finally, lipid peroxidation levels (based on TBARS presence) also seemed to be higher in larvae from tank 32 m^3 compared to 40 m^3 at 12 dph.

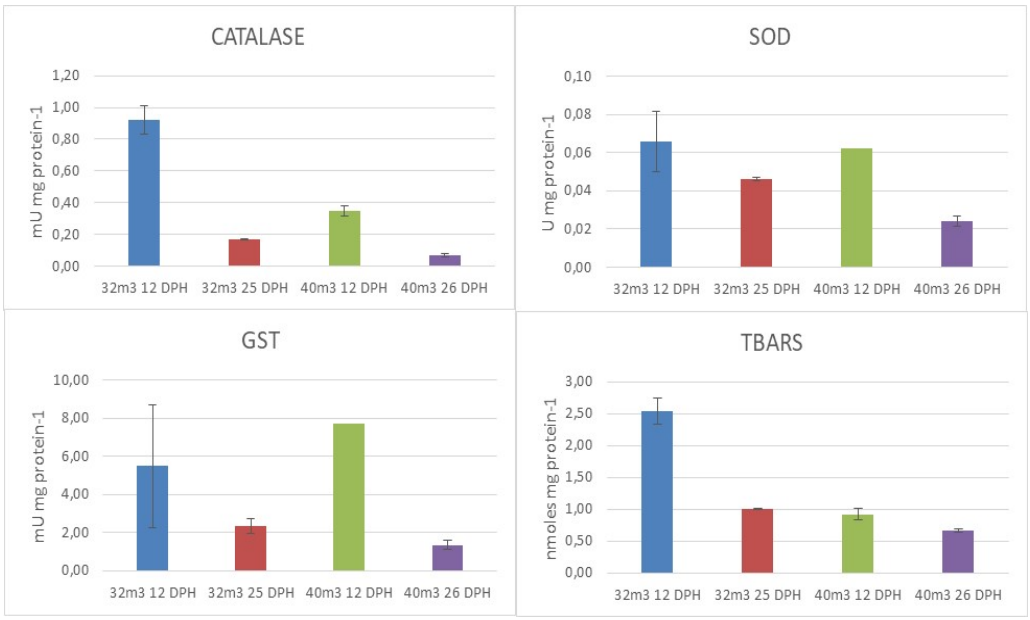


Figure 15. Antioxidant enzymes Catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) of 12 and 25 dph greater amberjack larvae from 32 and 40 m^3 tanks. Values are mean ± SD (n=2).

The larvae rearing trials in the facilities of IEO have been developed in large tanks (32 and 40 m^3) combined with low initial stocking density of newly hatched larvae.

Larval growth, development and survival are influenced by a wide range of environmental factors of the rearing system, such as volume, temperature, and light conditions, among others. Hatchery techniques using large tanks at low larval rearing density are an approach for juvenile production, especially when the biological requirements of the species are scarce or unknown (Divanach and Kentouri, 2000; Papandroulakis *et al*., 2004; Russo *et al*., 2007; Shields, 2001). In fact, the first success in greater amberjack larva rearing was obtained using an initial stocking density as low as 0.25 eggs l^{-1}, in semi-intensive rearing larvae system getting a 3.4% of survival rate at 40 dph (Papandroulakis *et al.*, 2005).

The growth of larva reared in 32 m^3 tanks was faster and the survival higher than those reared in 40 m^3, reaching 13.6±1.9 and 10.1±0.7 mm TL, and 2.5 and 1.8 %, respectively, at 30 dph. The size of larva obtained in both tanks were intermediate than reported for greater amberjack reared in mesocosm and extensive mesocosm rearing systems, but also coincided with an intermediate temperature (Papandroulakis *et al*., 2005).

The capacity of larva to detect and identify food items is dependent on the light conditions in the rearing environment (Puvanendran and Brown, 2002). It appears that during very young stages, light direction and intensity play a major role in most fish species (Boeuf & Le Bail, 1999) and feeding response of fish marine larvae could be triggered by the presence of visual stimuli (Kolkovski *et al*., 1997). In Carton's (2005) study, the feeding intensity of yellowtail kingfish (*Seriola lalandi*) larvae increased with both larval age and light intensity and feeding incidence was severely depressed in total darkness, indicating that yellowtail kingfish are primarily dependent on vision for feeding.

The lower light intensity registered in 40 m^3 tank compared with the light recorded at the same time in 32 m^3 tank might have been an advantage in growth and development for the larvae reared in the 32 m^3 tank.

The inflation of swim bladder may depend on environmental factors aforementioned (Battaglene and Talbot, 1990; Hadley *et al*., 1987; Trotter *et al*., 2003). Therefore, abiotic factors, such as access to the air–water surface, photoperiod, and light are particularly important factors in the success of swim bladder inflation. The light cues appear to be essential for triggering a larva's response in rising to the water surface to gulp air (Woolley and Qin, 2010). However, the threshold of light intensity and sensitivity to light clearly vary between species and probably stage of development. In addition, for the same species, the light sources (natural vs artificial with different spectral composition), the range of rearing systems (tank dimensions) and the husbandry protocols (green vs clear water) used influence the success of larval culture (Villamizar *et al*., 2011). Incomplete inflation or non-inflated as well as overinflated swim bladder affects the survival because it can cause different anomalies distributed throughout the body (Chatain and Ounais-Guschemann, 1990; Kitajima *et al*., 1994) and limited or negative buoyancy (Kurata *et al*., 2012; Papandroulakis *et al*., 2005; Takashi *et al*., 2006) can affect the equilibrium and position of fish in the water (Itazawa, 1991). The successful inflation as well as its volume contributes to improved feeding and growth of greater amberjack larvae (Hashimoto *et al*., 2013).

All larvae of greater amberjack reared in 32 m^3 tank inflated their swim bladder at 7 dph (3.8 mm TL) while the larvae reared in 40 m^3 inflated at 14 dph (4.6 mm TL). The inflation of swim bladder of greater amberjack has been cited earlier during 3 to 6 dph (Imai *et al*., 2011; Teruya *et al*., 2009), and smaller size (Papandroulakis *et al*., 2005). Therefore, the highest light intensity and its vertical distribution in the water column in the tank of 32 m^3 could encourage the swim bladder inflation at a younger age.

The success of the capture of the prey by the larvae is related to the contrast of the prey to its background and the visual ability of the larva. Larval growth and survival are affected by inappropriate conditions or poor development of the organs (eyes) involved (Duray *et al.,* 1996; Downing and Litvak, 1999; Tamazouzt *et al*., 2000; Papandroulakis *et al*., 2002; 2010). After exhaustion of the yolk-sac, larvae shift from endogenous to exogenous feeding, and the ability to detect the prey in the first days of feeding is linked to the positive allometric increase of organs (Osse *et al*., 1997; Rodríguez and Gisbert, 2002; Yúfera and Darias, 2007). The greater eye size also provides for better visual sensitivity to the fish and assists in prey capture (Lim *et al*., 2014; Olla *et al*., 1995; Privileggi *et al*., 1997). In many fish larvae, the appearance of the photoreceptor cells type depending on the age and light conditions (Britt *et al*., 2001; Kusmic and Gualtieri, 2000; Lenkowski and Raymond, 2014; Pankhurst and Hilder, 1998; Shand *et al*., 1999), and there is larger lens size with increased eye diameter (Fernald, 1985; Higgs and Fuiman, 1998; Miyazaki *et al*., 2000; Margulies, 1997; Poling and Fuiman, 1998). In this study, allometric growth pattern of the ratio of eye diameter to TL was positive and higher in 32 m^3 than 40 m^3 tanks up larvae of 6.0 mm TL, and it became isometric and similar after this larva size. In our facilities, the different location of the larva rearing tanks (indoor or outdoor) as well as the different tank depth (volume) leads to a higher light intensity (surface and depth) in the 32 m^3 than 40 m^3 tank. This could explain the earlier inflexion point in eye diameter-TL ratio at younger age and higher growth in larvae reared in 32 m^3 compared to 40 m^3 tank. The optimal and faster development of eye could improve the initial consumption of zooplankton.

The stomach contents can be considered a good tool to understand the larva feeding preferences, although it should be considered in conjunction with digestibility and assimilation. In this study, the stomach content of greater amberjack larvae showed prey items at 4 dph, once the yolk-sac has been absorbed, eyes pigmented, mouth opened and jaw development completed. The intake remained low over the first days, and increased considerably with age in both tanks. The larvae reared in 32 m^3 tank showed the maximum number of rotifers ingested at a younger age (3 days before) and smaller size than larvae reared in 40 m^3 tank. Also, the larvae reared in 32 m^3 tank did not show rotifers in their stomach contents 3 days before than the larvae reared in 40 m^3 tank, but they show a larger size (6.4 mm TL and 5.7 mm TL for larvae reared in 32 and 40 m^3, respectively). The higher growth rate observed in the tank of 32 m^3 with respect to the 40 m^3 tank could also facilitate a greater dispersion of the size of the larva, an aspect to be taken into account in the larval culture.

In this study the onset of cannibalism was detected (i.e. dead larvae at the bottom) later than previously reported, at 17 and 21 dph in 32 and 40 m^3 tanks, respectively, with larval TL around 7.0 mm in both tanks. These differences could be due to unequal environmental conditions between the tanks. The aggressive behavior of greater amberjack larvae is affected by light intensity, stocking density, feeding level, starvation, and the size heterogeneity (Miki *et al*., 2011). The grading of larvae could be necessary in order to improve the survival.

Lysozyme, peroxidase, antiprotease and proteases enzymes play an important role in the fish immune functions. Moreover, these enzymatic activities have been related to pathogens, stress or several environmental factors. Fish lysozymes possess a high potential for bactericidal or bacteriolytic activity against Gram-positive and Gram-negative bacteria (Saurabh and Sahoo, 2008). The role of proteases and antiproteases has been related with the defense against bacterial or parasite infections, while the peroxidase acts as an important microbicidal agent (Guardiola *et al*., 2014). In this study, the levels of humoral immune activity were quite similar between tanks and age of the larva, and the few variations found in bactericidal activity and lysozyme level do not respond to a clear pattern.

The results of present study showed that age was a crucial factor to consider determining the biochemical responses to oxidative stress. A decrease in some of the antioxidant activities was observed from 12 to 25 dph larvae independent of tank volume. The catalase activity was affected by rearing conditions differently depending on the age of the larvae. The activity of this enzyme seemed reduced in 40 m^3 tank compared to 32 m^3 at 12 dph. The effects of tank volume on the peroxidation status of the larvae were evident at 12 dph. Higher values of TBARS were observed in larvae from 32 m^3 tank. In general, an amelioration of enzymes' antioxidant activities was observed in 25 dph larvae compared to 12 dph and in 40 m^3 tank compared to 32 m^3. The results are according to literature for Atlantic cod (Hamre *et al*., 2014) observing a general decrease of the enzymes antioxidant activities after flexion stage and in general less marked in 40 m^3 tanks compared to 32 m^3 tanks.

3. Husbandry practices

3.1 Density

In general, low stocking densities such as in the gilthead seabream (*Sparus aurata*) and the red porgy (*Pagrus pagrus*), improves growth by increasing food accessibility and providing vital space. On the other hand, protocols for the larviculture of amberjack have to be developed to maximize the egg stocking density in order to achieve suitable production levels for a successful industry. These studies included the effect of egg stocking density and the type of tank on larval performance in terms of growth and, survival. These trials were performed at the facility of FCPCT.

Greater amberjack eggs were stocked at different densities of 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. They were tested in duplicate tank types 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), a salinity of 37 psu and a temperature of 26 ± 1°C. Water renewal flow was increased progressively from 25% d^{-1} to 200% min^{-1}. Aerated water entered the tank from the bottom and exited from the top at a flow rate of 125 ml min^{-1}), attaining 6.78 ± 0.5 ppm of dissolved O_2, saturation ranging between 60% to 80%. All tanks were equipped with a surface skimmer for removing buoyant organic material. The green water technique was used by adding live phytoplankton (*Nannochloropsis* sp.) to maintain a concentration of 250,000 cells ml^{-1} in the rearing tanks during feeding with rotifers and Artemia enriched with Ori-Green (Skretting ^{TM}, France). Subsequently they were progressively fed microdiets of 75, 150 and 300 µm Gemma (Skretting, France). Rearing and feeding conditions followed the protocol of GIA**.**

Severe cannibalism and size dispersion were observed from 10-15 days post hatching (dph).

The treatment with 50 eggs l^{-1}, during the first trial, showed significantly increased growth in terms of total length (TL, 5.471 ± 0.64 mm) at 15 dph. However, during the second trial, the treatment with 75 eggs l^{-1} showed significantly increased TL (17.43 ± 4.19 mm), whereas the treatment with 25 eggs l^{-1} showed significantly increased survival (11.25%± 4.92) compared to the other treatments (**Fig. 16**).

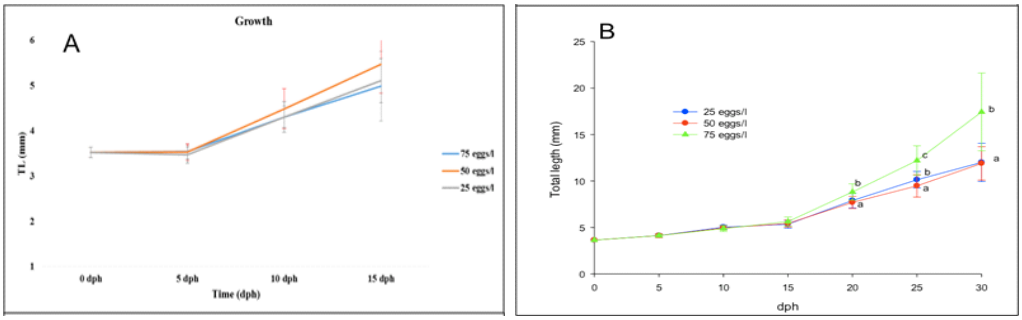


Figure 16: Total length of greater amberjack *Seriola dumerili* larvae of the first trial **A** (15 dph) and the second trial **B** (30 dph) at different densities; 25, 50 and 75 eggs l^{-1}. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

When analyzing the type of tank or volume we observed that, the larvae of 2,000 l tanks had the highest total length compared to the other treatments (**Fig. 17**).

The results of survival showed that the treatment with 25 eggs l^{-1} showed significantly increased survival (11.25 %) compared to 75 eggs l^{-1} treatment, while there was no significant difference between the treatments 50 and 75 eggs/l and between the treatments of 50 and 25 eggs l^{-1} (**Fig. 18**). However, if we analyze the results according to the type of tank or volume we observed that, survival was higher in 2,000 l tanks, independent of density.

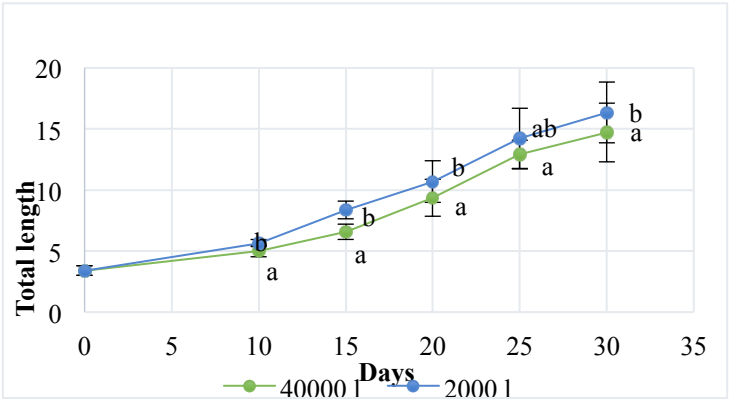


Figure 17. Total length of greater amberjack *Seriola dumerili* larvae at different tanks. Values of same age larvae having different letters were significantly (P<0.05) different

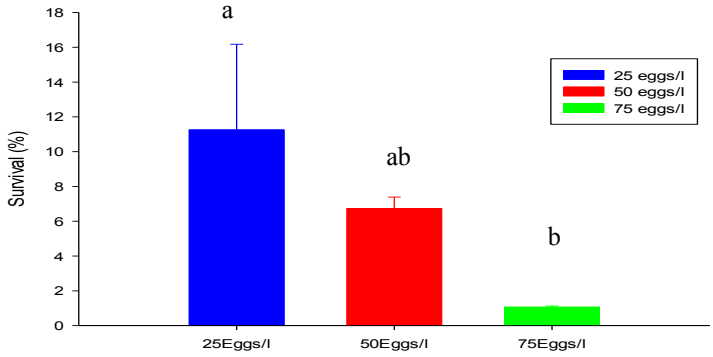


Figure 18: Survival of greater amberjack *Seriola dumerili* (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1}. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

In other species, the influence of larval density on growth is not so clear because other factors such as the availability of food and the deterioration of water quality are involved (Baskerville-Bridges and Kling, 2000). The first trial (30 dph) showed that the treatment with 50 eggs l^{-1} increased growth significantly ($p < 0.05$) in terms of total length and dry weight at 15 dph compared to 25 eggs l^{-1} and 75 eggs l^{-1} treatments. In contrast, the second trial demonstrated that the treatment with 75 eggs l^{-1} showed significantly increased total length and dry weight at 30 dph compared to 25 eggs l^{-1} and 50 eggs l^{-1} treatments. This higher growth could be attributed to the low larval survival rate in these treatments, which means more food availability as has been indicated in red porgy (*Pagrus pagrus*) (Roo *et al*., 2010).

3.1.1. Skeletal Ontogeny

Similarly to other species, in *Seriola dumerili*, skeletal anomalies constitute one of the most important bottlenecks during the hatchery phase (Cobcroft and Battaglene, 2013). The above trials (3.1 Density) were also used to study bone ossification, and evaluate skeletal anomalies. During and at the end of each experiment one hundred larvae were sampled

To study the bone ossification, all specimens were fixed in 10% buffered formalin. These analyses were carried out using the method of Mesa-Rodríguez *et al.* (2014). Single staining was performed to dye cartilage. Whole-mount staining of bone was performed using a modification of the method of alizarin red (Park and Kim, 1984). Different regions of the axial column were identified and examined according to Boglione *et al.* (2001). Observations were performed on the right side of the stained samples under a stereomicroscope. The data obtained was processed to calculate incidences for each descriptor (anomaly typology) and treatment.

The bone mineralization of the vertebral column was completed at 30 dph as illustrated in **Fig. 19** denoting the early bone mineralization of greater amberjack.

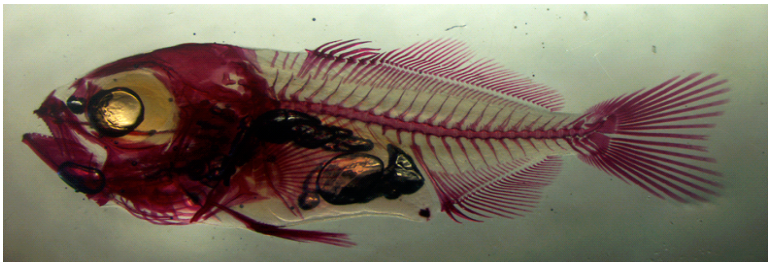


Figure 19. Image of alizarin red staining of greater amberjack *Seriola dumerili* (30 dph).

The cranial structures began to ossify by 3.38 ± 0.15 mm (**Fig. 20)**, with the calcification of upper jaw (premaxilla) and cleithrum. The jaw structures differentiated in two regions, the upper maxilla and premaxilla and the lower jaw.

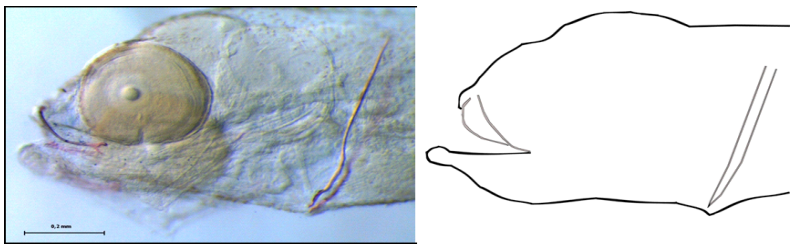


Figure 20. Cranial development to a size of TL (mm): 3.38 ± 0.15

The first visible structure in greater amberjack larvae was the maxilla, followed by the dentary and the premaxilla, and the last structures were the Angulo-articular and the retro-articular by 4.49 ± 0.14 mm TL, (**Fig. 21)**. At this time, small premaxillary teeth were first seen, whereas the dentary teeth developed.

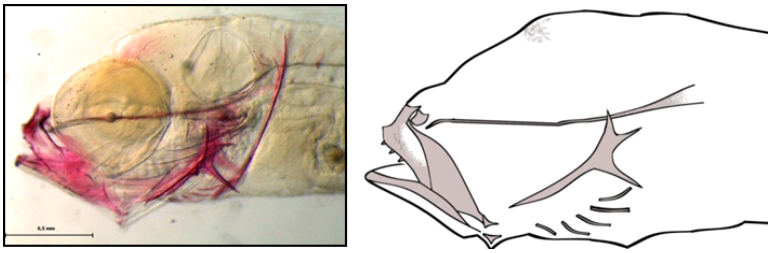


Figure 21 Cranial development to a size of TL (mm): 4.49 ± 0.14

The last bones to calcify were registered in the otic region at 10.15 ± 1.86 mm (**Fig. 22).**

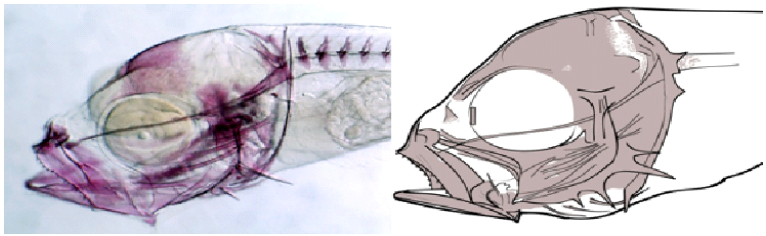


Figure 22. Cranial development to a size of TL (mm): 10.15 ± 1.86

In the vertebral column and fin ontogeny of greater amberjack, the first structures that began to ossify were the neural arches (4.49 mm) (**Fig. 23)** and continued with the haemal arches and the centrum.

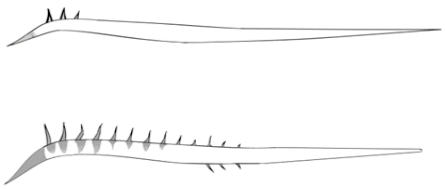


Figure 23. The vertebral column ontogeny of greater amberjack to a size of TL (mm): 4.49 mm± 0.14

The caudal fin began to ossify with the calcification of the caudal rays (Lepidotrichia) by 5.29 mm, (**Fig. 24)** and continued with flexion of the notochord by 5.36 mm.

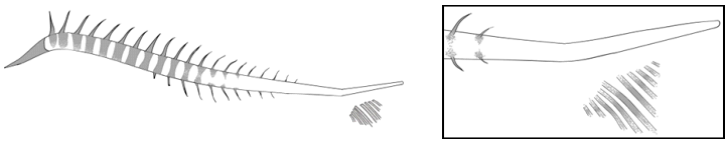


Figure 24. The caudal fin and the flexion of the notochord of greater amberjack to a size of TL (mm): 5.36 mm± 0.2

The last bones to calcify were the haemal arches (up to 12.87 mm) and the caudal fin was totally ossified by 13.03 mm, **(Fig. 25** and, **Fig. 26)**.

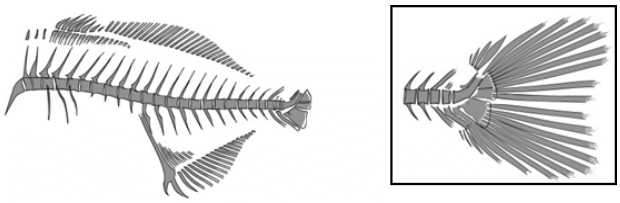


Figure 25. The caudal fin ontogeny of greater amberjack to a size of TL (mm): 12.87 mm± 0.12

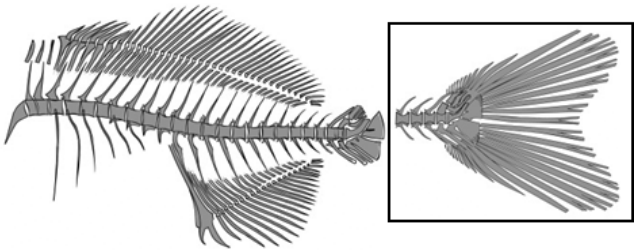


Figure 26. The caudal fin ontogeny of greater amberjack to a size of TL (mm): 13.03 mm± 0.09

There was a high percentage of severe anomalies found in all larvae of greater amberjack *Seriola dumerili* (30 dph) cultures regardless of the type of treatment (**Fig. 27),** where the highest percentages corresponded to the cranium and the haemal area (**Fig. 28)**.

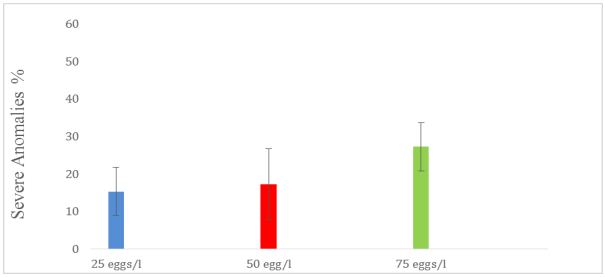


Figure 27. Severe anomalies percentage of greater amberjack *Seriola dumerili* (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1} (P>0.05).

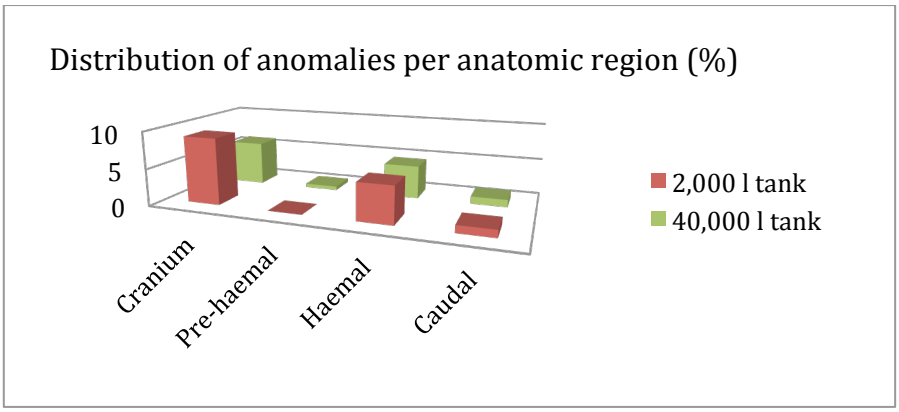


Figure 28. Distribution of anomalies (%) per anatomic region of greater amberjack *Seriola dumerili* larvae in different tanks and densities. Values among treatments having different letters were significantly (P<0.05) different

The results showed a marked appearance of different types of severe skeletal anomalies in all treatments throughout the larval stages such as lordosis, vertebral body fusion, and anomalous dentary, that could lead to a lower survival (**Fig. 29).**

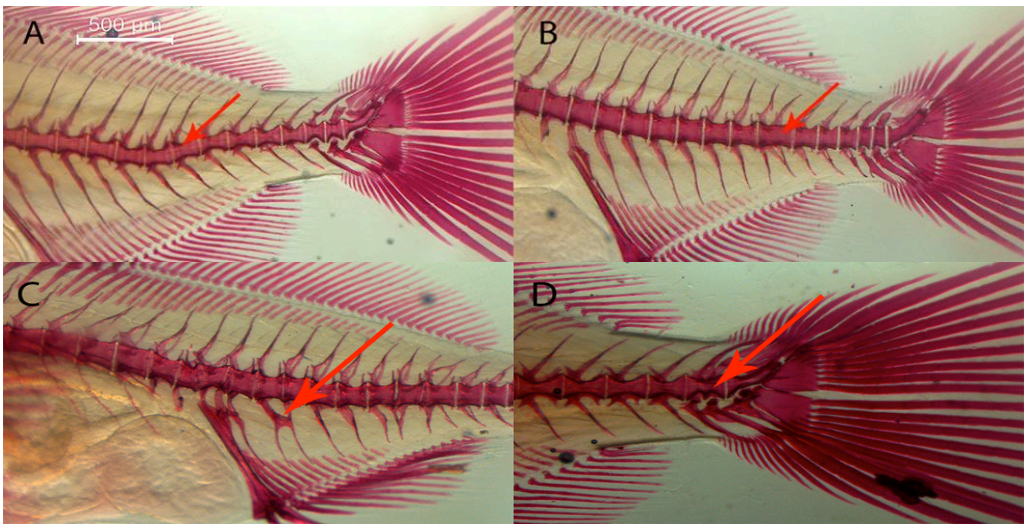


Figure 29. Different anomalies in greater amberjack *Seriola dumerili* (30 dph) **A**: Lordosis, **B**: Vertebral fusion, **C**: Fusion of neural arch and spines, **D**: Fusion of caudal vertebrae.

4. Nutritional requirements and Feeding

4.1. Nutritional requirements

The scarce knowledge on greater amberjack larval nutritional requirements, metabolic capacities and immune response to handling, results in inadequate larval feeding protocols that lead to very poor overall larval survivals. The importance of essential fatty acids (EFA) for marine fish larval performance has been widely discussed (Izquierdo and Koven, 2011). Specifically, long chain highly unsaturated fatty acids (HUFA; 22:6n-3, DHA; 20:5n-3, EPA and 20:4n-6, AA) are essential components of cellular membranes that modulate many physiological processes. DHA plays an important role in maintaining normal larval neural development and visual function, which is directly involved in predation efficiency. Several findings have shown that dietary phospholipids (PL), particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are a more efficient source of HUFA for some fish larvae at the rotifer and *Artemia* feeding stages. However DHA-rich marine PL sources are scarce and have only occasionally been used to transfer this EFA into young larval tissues (Li *et al*., 2015; Olsen *et al*., 2014). EPA, AA and other 20 carbon fatty acids derived from direct elongation of stearidonic acid (SDA; 18:4n-3) and gamma linolenic acid (GLA; 18:3n-6) are precursors and modulators of eicosanoid production, including prostaglandins and leukotrienes, involved in osmoregulation and immune modulation and stress resistance. Although certain levels of AA are present in wild fish tissues, including greater amberjack eggs, larvae, and gonads (Rodríguez-Barreto *et al*., 2012, 2014; Zupa *et al*., 2017), commercial enrichment preparations and fish diets tend to be deficient in this EFA, whereas much higher proportions of 18:2n-6 and EPA than those present in wild fish tissues are used for aquafeed formulations.

Viable greater amberjack wild eggs have around 17% of total lipid (TL) in dry matter (DM). Of this 30% is triacylglycerol (TAG) and 20% is phospholipids (PL), with around 25% of DHA, 5% of EPA and 3-4% of AA being present in both TL and PL (Rodríguez-Barreto *et al*., 2014). Carotenoids, mainly astaxanthin, are also present in the viable eggs. Therefore, a similar combination of all these essential nutrients should be considered as components of an enrichment protocol of rotifers at first feeding.

Although lipid emulsions are arguably the most extended enrichment products (Sorgeloos *et al*., 2001), their use has been related with detrimental autoxidation of HUFA and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched live preys have been described (Monroig, 2006). Among antioxidants, carotenoids including astaxanthin, with high oxygen quenching abilities, can inhibit HUFA peroxidation (Guerin *et al*., 2003; Atalah *et al*., 2011; Betancor *et al*., 2012; Hamre *et al*., 2013). Carotenoids are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Guerin *et al*., 2003). There is evidence that carotenoids mitigate deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. Specifically, carotenoids are found to be a determining factor for good egg and larval quality in greater amberjack (Watanabe *et al*., 2003).

From this perspective, the combined effect of novel HUFA-rich polar lipids and carotenoids included in enrichment products for live prey (rotifers), was studied by IEO and ULL in several trials performed under WP9 frame, in order to determine the optimum formula to improve greater amberjack larval performance and to be used in further rearing protocols.

In a **first enrichment trial** the rotifers (at an initial density of 300 rotifers ml^{-1} in 10 l triplicate tanks) were enriched with three experimental emulsions added at 8g 100 l^{-1} concentration to the enrichment tanks. The emulsions were designed with different proportions of lipid sources varying in their chemical structure; phospholipids (PL) or triacylglycerol (TAG), and combined to supply high LC-PUFA levels and DHA/EPA/ARA ratios resembling those present in total and polar lipids of wild amberjack eggs.

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 low supplementation of soybean lecithin was included to help emulsification and absorption of lipids. Finally, the E2 emulsion was formulated as 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). The commercial protocol consisted of a rotifer conditioning with the Spresso product, followed by DHA-PROTEIN SELCO enrichment (INVE-AQUACULTURE, Belgium). Triplicate samples of rotifers were taken at 3, 6, 10 and 24 h after enrichment for evaluation of lipid and fatty acids and compared to greater amberjack eggs composition. The best enrichment combination in terms of rotifer PL absolute content and proportions of DHA, EPA and ARA was achieved with the marine lecithin used in treatment E1 for 3h. This treatment better resembling the targeted composition of wild viable eggs.

In a **second preliminary experiment**, the selected emulsion (E1) was mixed with three different proportions of carotenoids (50, 100 or 150 ppm Naturose ~2% astaxanthin), and added to the enrichment tanks at 6g 100 l^{-1} concentration, under the same rearing conditions and sampling times, for evaluation of rotifer survival and total lipid and carotenoid content assessment. Regardless of the treatment, maximum absorption of carotenoids was also reached after 3h**.**

From these two preliminary enrichment experiments and according to the carotenoid and lipid and fatty acid composition of wild greater amberjack female gonads and eggs, it was concluded that rotifers enriched for short periods (3-6h) with 6% of the marine lecithin with a slight supplementation of AA (E1) in combination with a range of carotenoids (< 50 ppm), might improve the species larval performance at early life stages.

Then, the effect of new combinations of PUFA-rich lipids and carotenoids formulated based on these preliminary enrichment assays, were assessed on greater amberjack larval performance, welfare and body composition. To this purpose **a third experiment** was carried out which a rotifer enrichment commercial protocol (C) was compared with three experimental emulsions (E1; E1,10 and E3,10) added at a 6% concentration for 3h to the rotifer enrichment tanks, under the same rearing conditions. E1,10 and E3,10 consisted of previous emulsions combined with 10 ppm (mg l^{-1}) of Naturose astaxanthin.

Newly hatched larvae of greater amberjack were randomly distributed in 12 experimental tanks of 100 l (5000 larvae per tank). Water exchange and continuous light conditions were 0.30 l min^{-1} and 700 lux (surface of the water) during the feeding trial. Average seawater temperature and dissolved oxygen during this period were 22.4 ± 0.4°C and 7.56 ± 0.12 mg l^{-1}. Enriched rotifers were added twice a day and adjusted to 5 rot ml^{-1} from 3 to 11 dph and increased to 10 rot ml^{-1} until the end of the trial.

Total length and eye diameter were measured (at 6, 10 and 14 dph) with a digital system (Nikon Digital Sight) and the percentage of larvae with swim bladder inflated was determined. At the end of the trial (14 dph) larvae of each tank were counted and the percentage of survival calculated. At 14 dph samples of larvae were collected in triplicate from each treatment and protein, cortisol and body levels of glucose, lactate, sodium and potassium as well as the larvae lipid profiles were determined.

Levels of essential n-3 fatty acids and phospholipids are often lower in rotifers than in a natural prey such as copepods (van der Meeren *et al*., 2008). This can be improved by the use of high levels of n-3 fatty acids in the form of phospholipids such as the marine lecithin LC60 used in the present study or together with other phospholipid sources such as soybean lecithin for live prey enrichment (Coutteau *et al*., 1997; Cahu *et al*., 2009; Li *et al*., 2014; Olsen *et al*., 2014). Rotifers enriched with polar rich emulsions containing a marine natural lecithin LC60 combined with 10 ppm of Naturose also resulted in a significant advantage in larval growth, survival and welfare compared to rotifers enriched with other emulsions (**Fig. 30**).

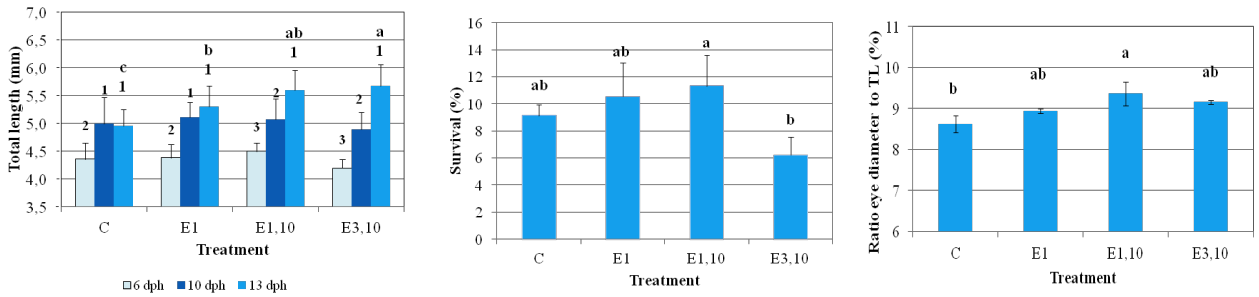
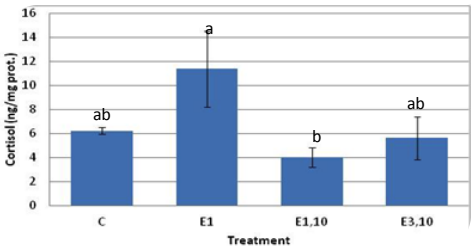


Figure 30. Total length (mm) at 6, 10 and 13 dph (a), final survival percentage at 13 dph (b) and eye diameter to total length ratio (%) (c) of greater amberjack larvae, fed with rotifers enriched with commercial and experimental (E1, E1,10 and E3,10) emulsions.

Carotenoids represent a group of micronutrients that may be deficient in rotifers for adequate larval nutrition (Hamre et al., 2008). Carotenoids perform a biological role similar to that of α-tocopherol (as a naturally occurring antioxidant), protecting tissues and reactive compounds from oxidative damage hence its importance in preventing LC-HUFA peroxidation and general larvae condition (Guerin *et al*., 2003).

In the present study, lower levels of cortisol were observed in the larvae fed with treatment E1, 10 with respect to the other treatments (**Fig. 31**). The lower cortisol response coincides with the lower mortality of E1,10 larvae and with a comparable good growth. Stress in teleosts is characterized by the immediate release of catecholamines and cortisol; both hormones are concerned with energy reallocation from anabolic activities such as growth toward activities to restore homeostasis. The primary stress response in fish is known to further trigger and leads to sequential secondary responses (e.g., increases in glucose, lactate, and changes in plasma chloride, sodium, potassium) (Barton *et al*., 2002). Whole body levels of glucose have been observed to increase at post stress. In the present study, no differences were found in whole body glucose levels but the slight increased values in the larvae from Control and E3,10 treatments could be an indication of the fact that these fish might also be under a certain degree of stress. Tissue lactate levels in fish are known also to increase at post stress (Barton *et al*., 2002). The elevation of lactate concentrations immediately after stress is likely due to muscle glycolysis and lactate may be used for gluconeogenesis in the liver. In the present study, whole body lactate levels were seen to increase in the larvae from control group with respect to the other treatments (**Fig. 31**). Therefore the use of the marine phospholipids combined with carotenoids had a beneficial effect on greater amberjack growth, survival and welfare.



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

Figure 31. Whole body cortisol (ng/mg prot), glucose (mg mg protein^{-1}), lactate (mg mg protein^{-1}), Na^+ (mg mg protein^{-1}) and K^+ (mg mg protein^{-1}) levels in 13 dph greater amberjack larvae fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions. Values are mean ±S.D., n=3. ANOVA ($P \leq 0.05$); Tukey's HSD).

From these preliminary experiments, and based on the carotenoid and lipid and fatty acid composition of wild greater amberjack female gonads and eggs, it was demonstrated that rotifers enriched for short periods (3-6 h) with 6g 100 l^{-1} of a marine lecithin (LC60 up to 60 % phospholipids: 40% PC + 20% PE), rich in DHA, and a DHA/EPA ratio of 2.5/1 (PhosphoTech Laboratories, France), with a slight supplementation of AA (2% of total lipids as free AA, Sigma Aldrich, Spain), in combination with 10 ppm of carotenoids (NatuRose ~2% astaxanthin ester, Cyanotech Corporation, Hawaii, USA), display a significant advantage in

larval growth, survival and welfare compared to rotifers enriched with other emulsions including a commercial protocol (see deliverable 9.1 for more details).

4.2. Ontogeny of digestive system

Although knowledge of the optimal feeding regime for rearing larvae is essential for their successful production (Hamasaki *et al*., 2009), the available information on greater amberjack is still incomplete (Papandroulakis *et al*., 2005; Mylonas *et al*., 2017). The digestive system enables the fish to capture, ingest, digest and finally absorb nutrients from the food which are 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. During the first developmental stages until its transformation into a juvenile, numerous changes take place in the digestive system of fish larvae in terms of morphology and functionality (Przybył *et al*., 2006; Gisbert *et al*., 2018) and provided valuable information regarding the assimilation and digestion of consumed food and their contribution to the general nutritional condition of the fish. Therefore, the knowledge of the digestive competence of a fish is essential in order to understand the digestive physiology of larvae and to adjust the feeding protocols to this capacity (Campoverde *et al*., 2017; Gisbert *et al*., 2018). For instance, advancing the early weaning of larvae from its dependence on Artemia onto a dry feed is a priority and one of the major focuses of current larval research on most captive-reared marine species (Campoverde *et al*., 2017). Moreover, this knowledge may contribute to the optimization of diets (Zambonino-Infante *et al*., 2008; Campoverde *et al*., 2017) and to the understanding of functions and limitations in the processing capacity of the digestive system, and consequently, to the delivery of nutrients to the rapidly growing larval tissues (Rønnestad *et al*., 2013).

In order to describe the ontogeny of the digestive system of greater amberjack, larvae were reared under two different rearing conditions, intensive or semi-intensive culture systems. Our objective was to contribute towards an adequate feeding strategy adapted to the digestive capacities and nutritional needs of the greater amberjack during early development while also addressing options to reduce cannibalism and size dispersion, which are of primary importance to improve larval survival and growth.

The rearing trials were performed in the facilities of the HCMR, Crete. Larval rearing of greater amberjack was performed under two different rearing systems, the mesocosm (MES) and the intensive (INT). Eggs from induced spawning of breeders kept in Argosaronikos SA cage farm (ARGO) were used for the rearing. After collection, eggs were shipped by air to the hatchery facilities of the HCMR in polystyrene boxes in ~12 hours, and then were incubated.

For the **MES** rearing method 110 x 10^3 eggs were stocked in a 40-m^3 indoor tank filled with filtered (5 µm) natural seawater (salinity 40 psu) treated with UV which was also the water for subsequent renewal. 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 and the rate of water renewal was increased progressively from the initial 15% to 35% day^{-1} at 17 days post hatching (dph), 100% at 22 dph and 200% at 30 dph. Aeration was provided by means of five tubes of 5mm diameter (without any wooden or stone diffuser), four of them distributed along the perimeter and one in the center of the tank. A surface skimmer was operational during the appropriate period (5 to 13 dph) to keep the surface free from lipids, a requisite for good swim bladder inflation. 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 cylinder-conical tanks, with pairs of tanks connected to a closed water recirculation system coupled to a biological filter. The initial stocking density was 36 x 10^3 eggs $tank^{-1}$. 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}. Water circulation was achieved through a biological filter during embryogenesis, egg hatching and the autotrophic larval stage with aeration

provided at 150–250 ml min^{-1}. After first feeding, water recirculation for each tank was obtained by means of an airlift pump inside the rearing tank, in order to maintain the feed organisms inside the tanks. The water in the biological filter was used only for renewal in the larval rearing tanks at a rate of 3% daily until 15 dph, then increased gradually to 50% at 25 dph. A skimmer was installed at the appropriate period to keep the surface free from lipids. 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.

The growth performance of greater amberjack larvae and the type of feeding in each rearing protocol during the trial are presented in **Fig. 32**. Microalgae (*Chlorella minutissima*) and rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco (INVE S.A., Belgium) were daily added in the rearing tank from 3-4 dph to 23 dph. Rotifer´s concentration was kept at 2-3 individuals ml^{-1} in the MES and at 4-5 individuals ml^{-1} in the INT. Unenriched Artemia AF nauplii (12 to 14 dph) and Artemia EG nauplii (14 to 30 dph) enriched with A1 DHA Selco (INVE S.A.) were offered to the larvae at a starting concentration of 0.05 to 0.35 nauplii ml^{-1}. In both rearing systems, artificial feeds were added progressively according to fish size (NRD 2/4, grain size 200–300 µm; NRD 3/5 grain size 300–500 µm, INVE S.A.) from 16 dph (MES) and 21 dph (INT). Live eggs at the blastula stage, frozen eggs in the embryo stage and 6-8 hours hatched larvae from gilthead sea bream *Sparus aurata* were also introduced in the MES rearing tanks after 20 dph. Mesocosm tanks developed also naturally zooplankton (harpacticoida copepods) which potentially contributed to larval feeding.

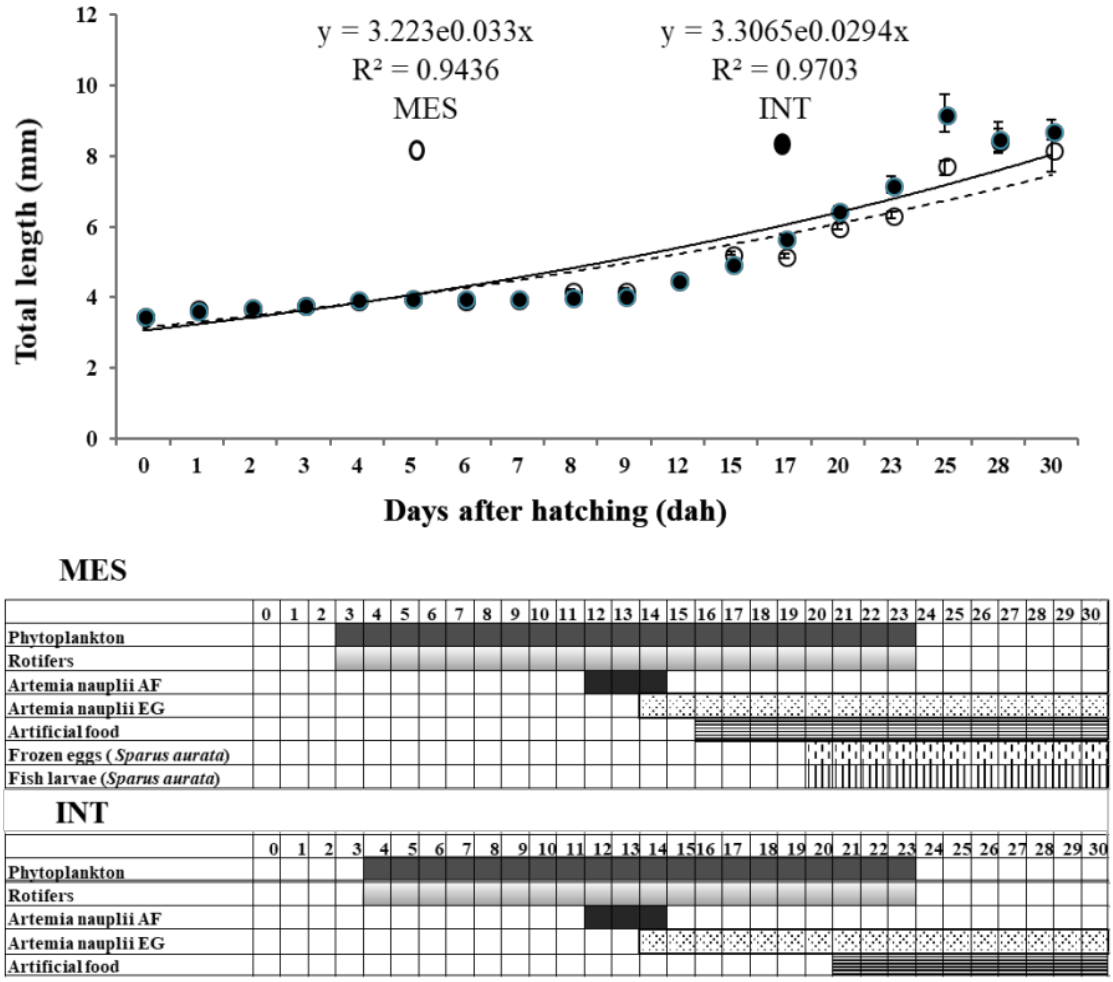
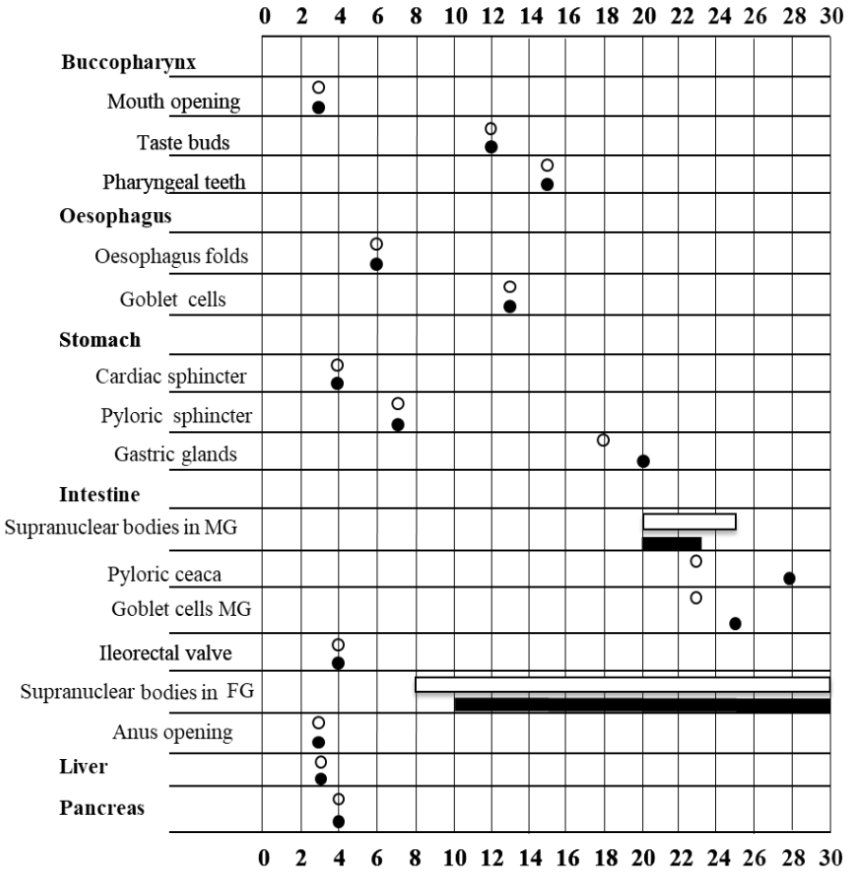


Figure 32. Growth performance of greater amberjack larvae (mean ± SD of total length) reared in MES and INT rearing systems. Below the graph, the rearing protocols used during the rearing procedure are presented, including type and duration of food items provided.

For the histological study, random samples of greater amberjack eggs and larvae (n=10) were collected on the following days of rearing: 1 day before hatching, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 15, 17, 20, 23, 25, 28 and 30 dph (day 0 was the day of hatching). Fish were preserved for in buffered fixative containing 4% formaldehyde and 1% gluteraldehyde for at least 24 hours (McDowell and Trump, 1976). For comparisons of main digestive pancreatic and gastric enzyme activities (Gisbert *et al*., 2009), eggs and larvae were collected and pooled according to their age-size and replicate availability at 1-5, 6-10, 11-15 and 21-30 dph.

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 **Fig. 33.**



	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 33. Schematic representation of the appearance (open solid circles indicate the MES, black solid circles indicate the INT system) of the main developmental structures examined in greater amberjack larval digestive system, as a function of days after hatching (dph, horizontal axis). Horizontal bars (white MES and black INT) indicate the period that supranuclear bodies (vacuoles) were present in the anterior-median intestine (mid gut, MG) and hindgut (HG). Below, mean values of the total length of greater amberjack larvae for each sampling day and rearing system are presented.

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 (Stroband and Kroon,

1981), and it is considered as a defining moment for the physiology of nutrition of the larvae leading to the transition from larval to juvenile function of the digestive system (Kolkovski, 2001; Sarasquete *et al*., 1995; Tanaka, 1971). The gastric glands produce gastric enzymes like pepsin as well as HCL that increase extracellular intestinal digestion of food promoting the digestion procedure (Segner *et al*., 1994). The number of gastric glands in greater amberjack larvae in the present study increased over time, suggesting that the functional capacity was improved too.

The activity of digestive enzymes of greater amberjack larvae in the INT rearing system during the trial are presented in **Fig. 34** and the comparison of the digestive enzymes activities between INT and MES rearing systems in **Fig. 35.**

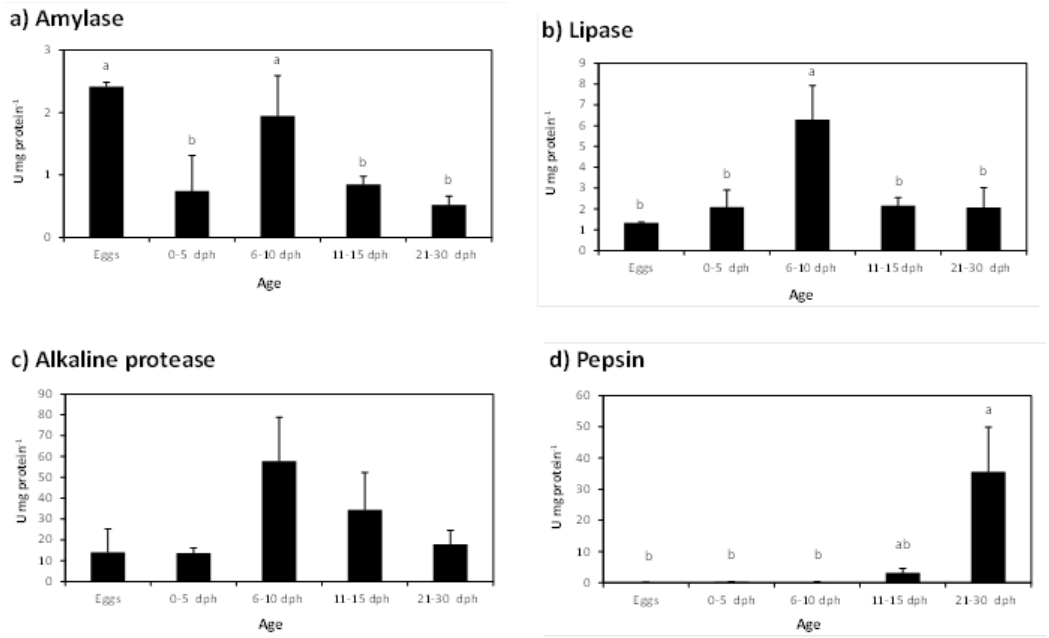


Figure 34. Activity of digestive enzymes during the ontogeny of the digestive tract of greater amberjack cultured under intensive conditions.

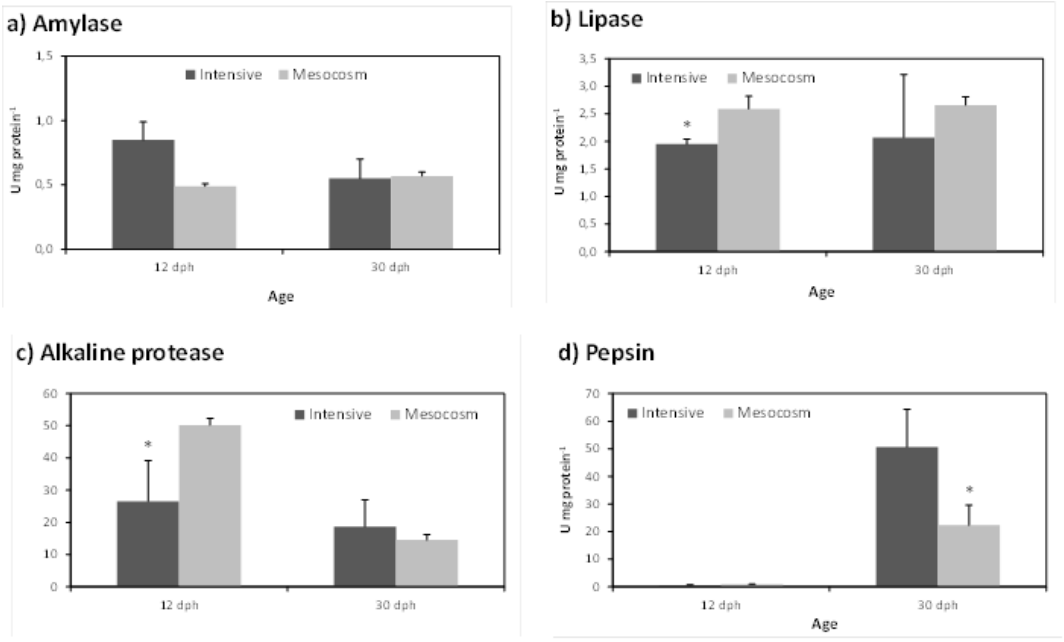


Figure 35. Comparison of digestive enzyme activities of greater amberjack larvae between intensive and mesocosm culture systems at 12 and 30 dph.

The ontogenesis of the digestive system of greater amberjack is considered as a rapid process. The development of the digestive capacity is controlled by endogenous factors and generally, it is genetically programmed. However, the time of appearance of the digestive structures and their functionality can be influenced by a number of factors of which temperature and feeding regime are among the most critical issues (Kamler, 2002). As the ontogenetic rates are correlated with the larval TL, the organization of the rearing protocol has to be synchronized with the larval TL as well. Generally, until the size of 5 mm, the feeding protocol has to include the most digestible food items (rotifers) and Artemia nauplii have to be offered after the larval size of 5 mm (between 5.3 and 5.6 mm; after the appearance of gastric glands, organ necessary for the digestion of proteins, and its complete functionality related to the greater protein content of Artemia with respect to rotifers). The histological description of the main digestive organs, the ontogenetic profile of digestive enzymes, and their dietary adaptation may be used as an indicator of larval development, food acceptance, digestive capacity and of their further survival rate (Ueberschär, 1993).

In the present study, amylase, lipase and alkaline protease activities were found before the onset of the exogenous feeding. At this moment, the high activity of amylase suggests the importance of egg glycogen catabolism as energy supply during the embryonic development and the high specific activity of alkaline protease supports its high importance during embryogenesis of greater amberjack.

From egg to 5 dph, the activity of lipase and alkaline protease remained stable while that of amylase decreased. In addition, the mouth was opened at 3 dph, pancreas began its differentiation in endocrine and exocrine cells at 4-5 dph, and the complete reabsorption of yolk sack took place between days 3 and 4. All this suggests that the enzymatic activity during this stage seems to be more related to the degradation of substrates from the yolk sac (lecithotrophic phase) than to exogenous feeding. Thus, obtaining energy during this stage seems to be related to the degradation of sugar, lipids and proteins. In our study, 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 (area covered with lipid vacuoles, ACLV) 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 on rotifers, which were identified in the larval stomach content. Amylase has been found to be an integral component of the enzymatic equipment in the developing larvae of carnivorous fish. Moreover, enzymatic expression and initial high activity levels of amylase in marine fish larvae do not seem to be induced by food (enriched rotifers and Artemia) and could be better explained as a result of programmed gene expression (Moyano *et al*., 1996; Péres *et al*., 1998; Zambonino Infante and Cahu, 2001). Here, there was a rise in amylase activity after the onset of exogenous feeding and the differentiation of pancreas into endocrine and exocrine cells. Thus, both genetics and feeding issues could be involved in the increment of this activity. Because lipase plays an active role in the degradation of triacylglycerols to diacylglycerols and beyond to monoacylglycerols (Zambonino Infante and Cahu, 2001) it should be expected an increase in the lipase activity during this period of exogenous feeding. However, the increment of protease activity, show the relevance of protein digestion at alkaline pH in this species, just before the onset of acidic digestion.

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. In this stage, after the complete consumption of vitelline storage, larvae not adapted to exogenous feeding die of starvation. The malnourishment in fish generally produces a reduction of lipid content in the liver cells (Power *et al*., 2000) and has also been observed during early life stages of other fish species (Papadakis *et al*., 2009). In seabass larvae, variations in the lipid absorption and its accumulation in

different regions of the intestinal mucosa is also influenced by the type of lipids supplied in the diet (Gisbert *et al*., 2005). The foregut of larvae fed high levels of neutral lipids (11%) showed an important intra and intercellular accumulation of lipids, whereas mucosa of fish fed low or moderate levels of phospholipids (13-15%) and neutral lipids (3-6%) showed normal organization and appearance (Gisbert el al., 2005). The high accumulation of lipids in enterocytes may lead to pathological damages that hamper pancreatic lipase's action. Thus, it would be advisable to evaluate how dietary proportions of polar and neutral lipids may affect the structure of the digestive system and enzymatic activities in fish early life stages. No increase in lipase activity or in the area covered by lipid vacuoles in the liver was evident between 16-30 dph, suggesting a possible state of malnourishment in greater amberjack larvae.

Moreover, acidic digestion rose from 20 dph, which is in agreement with the histological observations where gastric glands started to differentiate at approximately 16 dph and completed its definitive histological organization at 20 dph. The absence of a morphological distinct and functional stomach during the first weeks after hatching is characteristic of most marine fish larvae, and consequently, no acid secretion occurs before stomach development (Govoni *et al*., 1986; Santamaría Rojas *et al*., 2004; Zambonino-Infante *et al*., 2008). The increase in pepsin activity was correlated to a decrease in protease activity, similarly as described to *Dentex dentex* and related to a change in the digestive achievement of an adult-type protein digestion (Gisbert *et al*., 2008). Our present 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.

4.3. Feeding regime and immune-stimulants

At present, limited published information on feeding schedules for greater amberjack is available. Appropriate management of nutrition, feeding regimes and novel strategies may reduce larval mortalities in the commercial production of greater amberjack leading to the improvement of larval fish production efficiency in the commercial production of greater amberjack (Woolley *et al*., 2012).

Particularly at larval stages, an adequate feeding regime can help to mitigate the effects of stress, decrease the susceptibility to diseases, and boost the immune system. Another important factor to ensure that the larvae are consuming sufficient levels of nutrients and minimizing energy expenditure is providing the correct prey concentration. Several studies have shown a direct correlation between rotifer concentration and larval survival (Lubzens *et al*., 1989; Polo *et al*., 1992). However, an extended residence time of prey in the fish tanks can cause a deterioration of their nutritional quality leading to larval starvation (Markridis & Olsen, 1999) and loss of n-3 highly unsaturated fatty acids (Olsen *et al*., 1993).

The onset of diseases in the intensive culture of marine species is generally associated with stressful situations such as environmental instability, deficient management and a sub-optimal diet (Zarza and Padrós, 2008). In this context, the stimulation of the larval immune system is a promising tool to increase survival rates at early stages of fish. Herbal extracts as ginger, green tea, garlic and many others have been shown to modulate of the fish's immune system (Awad *et al*., 2010; Bilen *et al*., 2011; Bairwa *et al*., 2012; Militz *et al*., 2013). For instance, *Echium plantagineum* seeds oil, used in substitution of fish oil, has shown to decrease stress and to improve responses to disease in several fish species (Villalta *et al*., 2008; Díaz López *et al*., 2009; Alhazzaa *et al*., 2013). The black cumin *Nigella sativa* seed is a spice and food preservative with many medicinal properties and its oil has been shown to have beneficial effects in fish growth and immunity. This has been mainly attributed to thymokinone, an essential but volatile component, where the method of administration and the duration of application of this oil are considered as important factors in the success of the treatment (Awad *et al*., 2013).

Since the stimulation of the larval immune system is of high interest for commercial aquaculture and a promising tool to increase survival rates at early stages of fish (García de la Banda *et al*., 2010; Awad *et al*., 2013), *Echium* and black cumin oils were also assayed using rotifers as vectors. These rotifers were previously enriched with the optimum PL/carotenoid/AA preparations mentioned above and supplied under different concentrations and feeding frequency regimes. The efficacy of these dietary supplements, were then evaluated in terms of survival and growth as well as assessing the oxidative stress condition and digestive enzymes activities of amberjack larvae. To this end, several rearing trials of larvae of greater amberjack were designed and conducted in the facilities of IEO and ULL.

A first rotifer enrichment trial was performed in order to select products and period of enrichment. In this assay, rotifers were enriched with a polar lipid rich emulsion containing marine phospholipids and arachidonic acid (AA, 20:4n-6) combined with 10 ppm of carotenoids (esterified astaxanthin). Different concentrations of *Echium* oil were then added as immune-stimulant, given its role as modulator of the stress response in fish. From these preliminary results, the enrichment protocol based on a 6g 100 l^{-1} of the marine lecithin/20:4n-6/10 ppm carotenoids supplemented with 20% *Echium* oil for a short period (3 hours) was selected.

The effect of **prey density (rotifers) and selected enrichment products combined with immune modulators substances** was then assessed on greater amberjack larval performance. In addition to *Echium* oil as immune-stimulant, black cumin oil (*Nigella sativa*) was also tested. The selected protocol was assayed at two different prey densities. To this end, the rotifer enrichment commercial protocol (T1) was compared with three experimental treatments under the same rearing conditions stated previously. T2 consisted of the LC60/20:4n-6/10ppm carotenoids basic emulsion, whereas T3 and T4 consisted of this lipid emulsion combined with 20% *Echium* oil and 20% black cumin oil, respectively.

Two prey concentrations were used, 5 (Low prey density) and 10 (High prey density) rotifers ml^{-1}. Enriched rotifers were added to the larval rearing tanks twice a day. Larvae were randomly sampled at 1, 7 and 12 dph and total length, percentage of larvae with inflated swim bladder and survival were determined.

The growth of greater amberjack was similar in larvae fed rotifers enriched with marine lecithin (T2) supplemented with *Echium* oil (T3) or black cumin oil (T4) at 12 dph. No significant (P>0.05) differences were found in total length and percentage of inflated swim bladder of larvae fed at Low or High prey density for each enrichment treatment at 12 dph (**Fig. 36**). However, the percentage of larvae with inflated swim bladders was significantly (P<0.05) lower in T2 larvae compared to the T3 and T4 cohorts. Fish survival was very low at the end of the feeding period independently of dietary regime and prey density treatment.

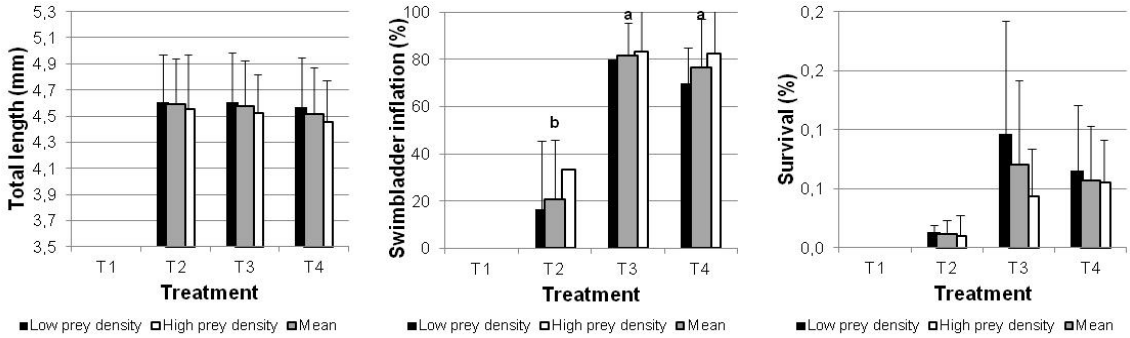


Figure 36. Total length (mm), swim bladder inflation (%) and survival (%) of 12 dph larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, P <0.05).

Regardless of dietary regime, the density of rotifers (5 or 10 rots ml-1) in the larval culture tank did not significantly affect fish growth performance and feeding behavior although larvae receiving the commercial treatment (T1) showed the worst results. There was a positive effect of the experimental emulsions used to enrich the rotifers. In particular, supplementing immune substances suggested that prey density had no effect on larval performance.

To study the combined effect of **feeding frequency and enrichment products containing immune-stimulants** (PUFA-rich lipids, carotenoids and *Echium* oil or black cumin oil, *Nigella sativa*), a set of new experiments were carried out. Rotifers enriched with one of four treatments previously described were added to the larval rearing tanks twice or three times daily. Larval sampling at 7 and 12 dph was carried out randomly and total length, swim bladder inflation percentage and volume, eye diameter, daily prey intakes and survival were determined. Larvae were also examined for oxidative stress, humoral parameters of the immune system and ontogeny of the digestive enzymes.

No significant differences in total length, swim bladder inflation and survival were observed between larvae fed at different feeding frequencies (**Fig. 37**). Dietary regime significantly affected larval growth at 12 dph (P <0.05) where fish supplemented with cumin oil were larger than larvae supplemented with *Echium*.

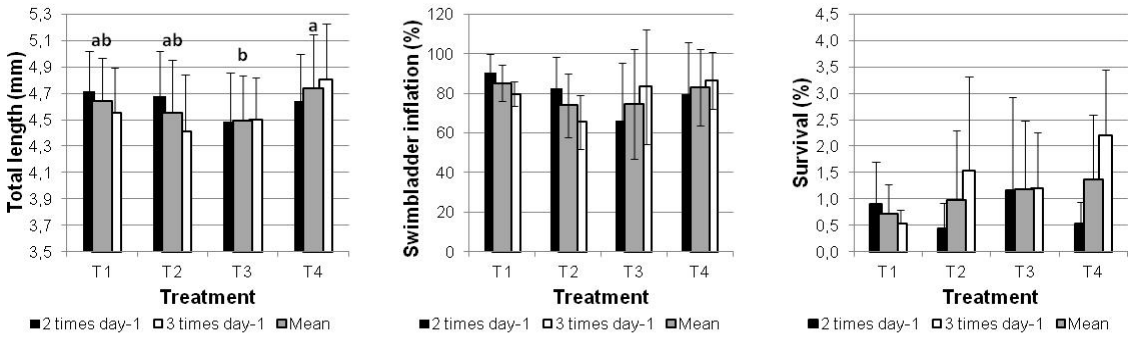


Figure 37. Total length (mm), swim bladder inflation (%) and survival (%) of 12 dph larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, P <0.05).

The digestive enzyme activities were higher in fish feeding on black cumin supplemented rotifers where significantly higher lipase and protease alkaline activities were observed **(Fig 38)**. Presumably the increase in protease and lipase activities resulted 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.

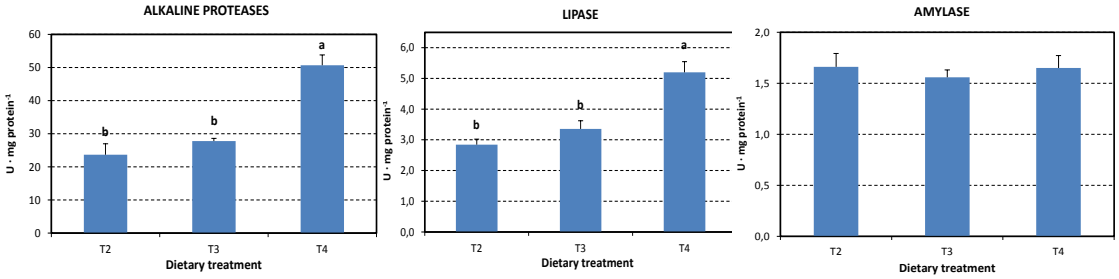


Figure 38. Alkaline proteases, lipase and amylase activities of 12 dph greater amberjack larvae fed rotifers from T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments irrespective of the feeding frequency (ANOVA, P <0.05).

Regarding antioxidant defense enzyme activities and lipid peroxidation products (**Fig. 39**) in response to immune-stimulants, the results showed that age was an important factor to consider determining the biochemical responses to oxidative stress. A general trend to increase GST and TBARS activities with age was evident for all treatments. The activities of the SOD and GST enzymes were reduced by *Echium* oil and cumin oil at 12 dph but only SOD was reduced by cumin oil at 7 dph. The effects of immune-stimulants on the peroxidation status of the larvae were not evident at 7dph although altered levels of lipid peroxidation products, showing higher values of TBARS, were observed in *Echium* supplemented larvae at 12 dph.

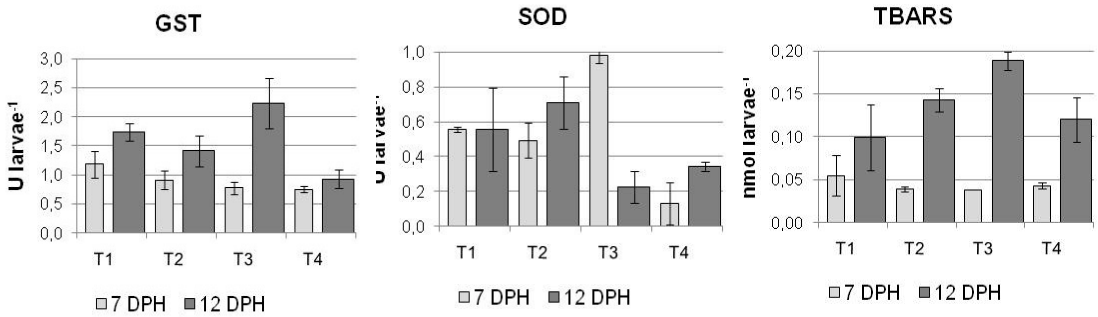


Figure 39. Antioxidant enzymes Catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) of 7 and 12 dph greater amberjack larvae fed rotifers from T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), 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 ± SD (n=2).

Regarding humoral innate activities, larvae fed with the control or T2 diet (PUFA-rich lipids and carotenoids containing diet) had similar levels (**Fig 40**). However the larvae fed with the *Echium* oil containing diet showed lower levels of protease activity at 7dph, although similar levels to the control larvae were observed at 12 dph. In the black cumin oil treatment, a surprising inhibition of peroxidase and bactericidal activities were observed at both 7 and 12 dph, while protease activity was inhibited at 7 dph.

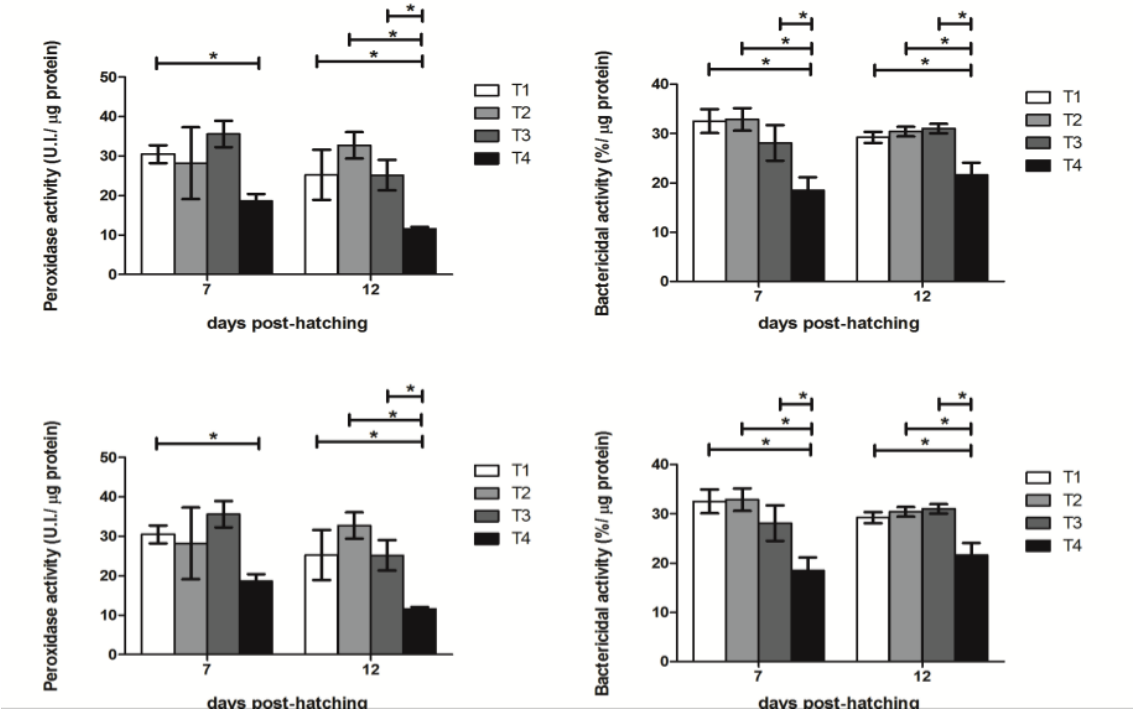


Figure 40. Humoral innate immune activities of 7 dph and 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Asterisks determine differences between groups accordingly with ANOVA (P <0.05) and a Fisher's LSD *post-hoc* test.

In summary, the results of these studies suggest that density of rotifers and rotifer supply frequency do not significantly affect fish growth performance and feeding behavior while a positive effect of experimental enriching emulsions supplemented with immune modulators such as *Echium* oil and black cumin oil was observed compared to commercial emulsions. In the black cumin oil treatment, a surprising inhibition of peroxidase and bactericidal activities were observed at both 7 and 12 dph, while protease activity was inhibited at 7 dph. Dietary intake of black cumin oil has been proposed to be an immune-stimulant in juvenile specimens of several fish species as it increases several innate activities including peroxidase activity (Vallejos-Vidal *et al*., 2016). Decreased bactericidal activity levels was also reported in fish fed for 14 days a diet containing 3% black cumin oil, as also occurs in our study (Awad *et al*., 2013).

Overall, the results suggest the positive effect of experimental live prey enriching emulsions supplemented with PL, carotenoids, AA and immune modulators such as *Echium* oil and black cumin oil compared to commercial emulsions on larval performance of *Seriola dumerili*.

5. Validation of the developed protocol in SME hatcheries.

The evaluation of the developed protocols was tested in two hatcheries: *Galaxidi Marine Farms* and *NIREUS Aquaculture*

Galaxidi Marine Farms (GMF) larval rearing trials

The trials in GMF were performed in the hatchery of the farm at Galaxidi. The hatchery had already performed larval rearing of greater amberjack during 2015 and 2016 unsuccessfully. Therefore, the personnel had experience with this species.

The eggs from induced spawning of breeders kept in GMF and Argosaronikos SA farm were used for the rearing. After collection, eggs were transported to the hatchery facilities in polystyrene boxes in ~ 4 hours, and then were incubated.

Incubation was directly in the larval tanks at a density of around 120 eggs l^{-1}. Following hatching, the density of the larvae was about 75 individuals l^{-1}, indicating a survival rate of 62%. Phytoplankton was added in the larvae tanks from 2-15 dph. Light intensity was 800 lux on 3 dph, and increased to 1200 lux on 6 dph until 12 dph when it was decreased to 1000 lux and gradually to 500 lux until 20 dph. The photophase was continue (24L:00D) from mouth opening to 20 dph when it was decreased to 18L:06D until 30 dph where it was set to natural conditions.

Feeding was based on enriched rotifers and subsequently with Artemia and dry feeds. Frozen copepods were added from 10-15 dph while frozen eggs were also added in the tanks after 20dph.

An indicative growth curve of the larvae during 2017 is presented in **Fig. 41 a**.

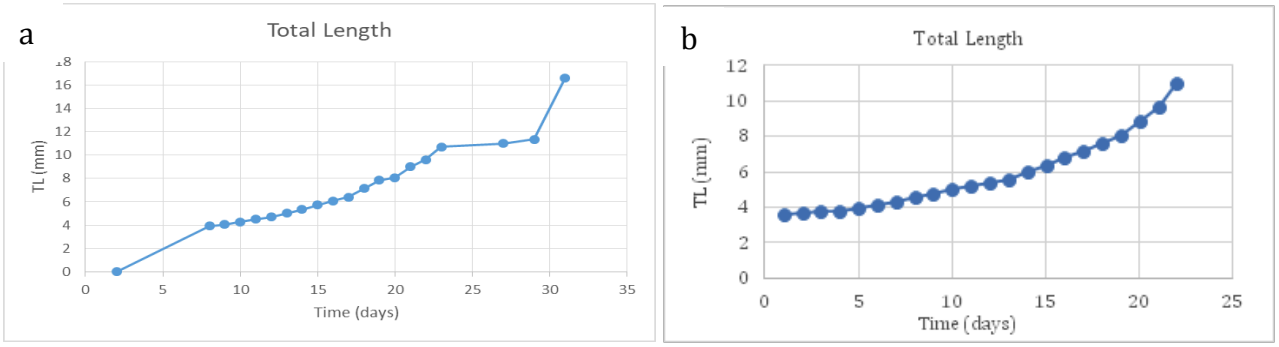


Figure 41 a, b. Total length (mm) of greater amberjack larvae reared in *Galaxidi Marine Farms.* a: 2017 and b: 2018 trials.

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 hatchery repeated the trials in 2018 using eggs from Argosaronikos SA. A Tank was stocked with 40 larvae l^{-1}. The rearing protocol was similar as described above and until 20 dph no significant problems were observed with an estimated survival of about 10%. Fish growth is presented in **Fig. 41 b**. Following this day, significant mortality was observed due to cannibalism. The result of absence of any sorting in less than 10 days was the loss of more than 65% of the population. On day 30 dph 15.000 individuals were transferred to nursery.

NIREUS Larval rearing trials

The trials were implemented in the hatchery of the company at Nafpaktos, where some first trials for the larval rearing of greater amberjack had been performed the previous years. In fact, during 2016 the company

received almost 2.5 million eggs from Argosaronikos SA farm that following transport were incubated following the protocol of the hatchery and were transferred after mouth opening to larval rearing tanks. This procedure proved to be lethal for the larvae and resulted in no actual larval rearing.

During the 2017 period, the hatchery received 4 batches of eggs again from Argosaronikos farm on the June 9, 10, 11 and 25. The received quantities were 1.0, 1.2, 0.65 and 0.5 million eggs respectively. The eggs were directly incubated in the rearing tanks and the rearing temperature was set at 24.5 to 25.0 ºC. 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 the tanks were modified where possible in order to increase the light intensity on the surface of the tanks at >1000 lux. This resulted in significant higher survival in the tanks, which modified light conditions applied.

Furthermore, after 20 dph, fish were selected in size and grouped accordingly. The result of the protocol modifications was a significant improvement in the performance of the larvae and in particular their survival rate.

The hatchery finally transferred in cages 48.300 juveniles of 25-50 g.

During 2018 the hatchery repeated a larval rearing trial starting on June with eggs from the Argosaronikos SA. Eggs were delivered at a temperature of ~24.5 ^{o}C, and pH 7.54-7.8. Eggs were incubated directly in larval rearing tanks (T 24,5 ^{o}C και pH 7.8) without any previous treatments. Since mouth opening light intensity was high and diffused. First feeding was performed with rotifer with live algae supplementation. Artemia AF was delivered when larvae reached TL >5mm (10 dph), and Artemia EG when TL >6mm (14 dph). Since 18 dph artificial diet was provided (Skretting). Swim bladder inflation was >85%.
Density was reduced at 20 dph and larvae were sorted at 35 dph. It is estimated that presently there are several tens of thousands of individuals that are in the on growing phase

6. General recommendations

Fish egg and larvae management

Greater amberjack spawn can be obtained either by natural spawning or inducing it by hormonal treatment. In both cases, the quality of the egg can be very variable and be below the acceptable standards, besides depending on the optimal nutrition of the broodstock.

Egg incubation can take place either in incubation tanks or directly in the larval rearing tank. An early estimate of the egg quality by checking for the presence of dead (opaque) or unfertilized eggs (transparent, but without evidence of cell divisions), the regular rounded shape and homogeneous size of fertilized egg and the regular development of embryo can help to decide the method of incubation to follow.

The incubation in cylindroconical fiberglass tanks of 100 l of volume is a common technical solution. The tank shape, provided with a central aeration source placed near the bottom, gives a better separation of not viable eggs to be regularly decanted. The tank is renewed continuously with the inlet of filtered and sterilized seawater, and the outlet is screened with a removable internal filter (250 µm). Care must be taken in the pre-hatching development as the egg density increases considerably. Decant at that moment would cause the loss of eggs about to hatch. In addition, the aerator must be perfectly located in the bottom of the incubator to avoid the death of the embryos by asphyxia due to the accumulation of eggs in the cone of the incubator.

An excessive egg density (>4000 eggs l^{-1}) is not advisable because it causes an excess of waste that prevents the optimum hatching. To avoid a risky situation, the newly hatched larvae need to be transferred quickly to rearing tanks filled with clean and aerated water.

Advantages of egg incubation in dedicated tank:

- The number of estimated larvae stocked in the rearing tank is more accurate, allowing a better adjustment of the parameters during larval rearing. Abnormal or poor quality larvae die before being transferred to the rearing tank.
- If the hatching is unsuccessful, it is not necessary to empty, wash and refill the rearing tank.
- Avoid the accumulation of organic matter in the rearing tank, and the cleaning of the bottom after hatching using a siphon to remove debris and reduce the bacterial growth is not necessary, preventing also the loss of larvae.

No special operation is needed when eggs are incubated directly in the larval rearing tanks, except that hatching debris must be carefully siphoned out as soon as the hatching ends and the aeration and water exchange are adjusted to the new situation.

Larvae rearing

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

- **Rearing structures.** Before stocking larvae, rearing tank have to be ready in order to avoid pathology, stress and escape of newly hatched larvae. It is recommended that the water inlet of the rearing tank be at the periphery and opposite to the outlet and placed carefully in order to avoid strong and tangential currents. The outlet is screened with a removable internal filter of 330 µm from 1 to 17 dph, to discard uneaten rotifers, and then changed to 500 µm mesh net. The air inlets slightly separated from the bottom of the tank. The situation of the outlet screen and any other internal structure should not prevent free circulation in the tank, to avoid accidental mortalities due to accumulation of larvae in funnel zones.
- **Larval transfer from incubation tank to larva rearing tank.** The newly hatched larvae are easily transferred to rearing tank. For it, the aeration and sea water inlet of incubation tank are stopped for a few minutes. Dead eggs and waste decanted and discarded through the bottom valve. Afterwards, the aeration is restored and the larvae are harvested slowly avoiding any mechanical stress by using plastic jars that allow to concentrate them into partially filled buckets with 250 µm mesh size net at the bottom.

When enough larvae have been caught, slowly empty out the bucket around the perimeter in order to achieve a homogeneous distribution of the larvae in the rearing tank. It is completely inadvisable to transfer larvae to the rearing tank after opening the mouth.

Egg stocking densities greater than 25 eggs l^{-1} affects negatively the results during the larva rearing of greater amberjack.

Environmental parameters for larval rearing

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 high quality rearing environment during larvae rearing:

Dissolved oxygen ranged between 4.9 and 8.2 mg l^{-1} but it is preferable to reach 6.0 mg l^{-1}, salinity between 35 and 40 psu, pH between 7.8 and 8.5, and a temperature range from 22 to 27ºC, preferably between 23.5 and 25.0ºC.

Regarding the light and photo phase conditions, the recommended parameters are:

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

Feeding

In general;

1. Live microalgae must be added daily at 150-300 x 10^3 cell ml^{-1} from 1 to 15 dph. The addition of algae should be with caution because excessive amounts of microalgae could affect the light intensity and cause the oxygen depletion at nights.
2. Enriched rotifers (*Brachionus plicatilis*) are fed at least two times a day, from 3 to 15 dph, at densities between 3 and 6 rot ml^{-1}. It is recommended to estimate every day the density of rotifers in the larval rearing tank, at least in two sampling points, in order to manage properly the number of fresh rotifers addition per day. Large fluctuations of prey density are expected due to the larvae faster and greater ingestion rate.

The addition of enriched rotifers should be done gently around the tank using a floating system or funnel that allows concentrating most of the enrichment residues inside, while the rotifer is distributed in the water column. Once the rotifer has been distributed, the bottom of the funnel is closed and the rest of enrichment remains inside.

Some hatcheries have a production of live copepod together with rotifer production that can be used to feed fish larvae, although it is very difficult the daily estimation of live copepods density in the rearing tank due to heterogeneous distribution. However, larvae can also be fed with frozen copepods.

3. Artemia AF nauplii are fed when larvae reach 5.5 mm in TL for a period of 3-5 days, while enriched Artemia EG (24 h post hatching) are fed after larvae reach TL of 6.5 mm, and weaning diet (200-800 µm) from 18 dph.

- **Storage.** Due to the very rapid development of Artemia at 28-30°C of temperature, their nutritional quality for larvae decreases rapidly (10 h). To avoid this, store them with smooth aeration at low temperature (5-10°C) for the shortest time possible and never more than 12-h.

It is advisable to add the Artemia nauplii and metanaupli at least 4 times a day around the tank.

4. Prey must be supplemented with phospholipids (PL), carotenoids, arachidonic acid (AA) and immune modulators such as *Echium* oil and black cumin oil in order to improve the larval rearing of greater amberjack. The duration of enrichment of the prey and the times of addition to the tank have to be correctly established.

5. An appropriate protein rich diet and size of particles are required for successful transition from live feed to artificial food. Particle diameter should be well adapted to the mouth and avoiding sudden changes in diameter.

- It is advisable to start the distribution of inert feed in the morning before live feed is offered.
- The best way to feed larvae is to distribute artificial diet ad libitum. However, be careful, because sometimes an over-distribution can reduce water quality, resulting in disease or fish mortality. The number of daily meals decreases with the age of the larvae and the amount of each intake increases.

Hygiene and cleaning

Although the rotifers are added using the aforementioned funnel system, it is advisable to clean the surface film caused by enrichments products after each addition of enriched rotifers using a "surface skimmer". In this way we can also reduce larval losses during cleaning.

The frequency in cleaning the bottom of the tank by siphoning to eliminate more waste and keep the associated bacteria to a minimum should be increased with the start of artificial feeding. It is strongly recommended that this cleaning operation should be done before the first feeding in the morning. The outlet of the siphon should be placed in a collector, with mesh size lower than the size of the larvae at that moment, located in a bucket in order to recover the live larvae that are sucked during the operation.

Monitoring and controls

As soon as the larval population develops an active behaviour and starts feeding, the following controls should be performed to monitor its health status:

- **Feeding**. A strict control of feeding behaviour is necessary to prevent both under and overfeeding. For proper management of the feeding, it is recommended to estimate the percentage of larvae that have prey in the stomach and the number of these by larva. To check the ingestion rate of Artemia, it is sufficient to visually estimate the percentage of larvae with reddish-coloured digestive tracts.
- **Morphology.** The control of swim bladder development an early and correct determination of the percentage of swim bladder inflation as well as look for body deformities is vital to decide how to proceed with the rearing tank.
- **Stress**. The recognition of normal behavioral patterns and healthy appearance of larvae is also very useful for early detection in order to improve culture conditions or to replace as soon as possible a poor larval batch. Starvation (stop larval feeding), abnormally passive behavior and low prey attacks frequency, absence of "schooling" and high concentration of larvae in the surface are some of the most direct symptoms. However, there are some warning behavior signs of stress and onset of diseases less obvious but of great importance as gape the operculum, increase of ventilation rate, apathetic swim of the larvae near the water inlets, hang over an air source, fecal color, fecal thread hanging, sinking, etc. If measures cannot be taken to counter the stress inducing factors, it should be consider eliminating the rearing tank.

Cannibalism and fish transfer from larval rearing tank

Cannibalism becomes a big problem in greater amberjack especially when there is heterogeneity of sizes. In order to reduce its effect, the fish should be size-graded and transfer from rearing tank to new tanks in order to increase the survival. Greater amberjack can be moved to the weaning section at about 20 dph. Depending on the design of the larval tank, fish can be harvested in different ways (e.g. by netting them or by draining the tank). The use of floating cages structures with mesh size according to larvae size help to handling and grading the larvae. The larvae trapped inside the floating structures are transferred to weaning tanks. This fish grading improve the survival and reduce the risk of pathologies in larval rearing tank.

All recommendations proposed in this document or some of them should be valued according to the facilities and the scale of greater amberjack production.

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