Deliverable Report

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Deliverable Title	Ontogeny of greater amberjack larval visual and digestive system			
WP No:	15		**WP Lead beneficiary:**	P1. HCMR
WP Title:	Larval husbandry –greater amberjack			
Task No:	15.4		**Task Lead beneficiary:**	P1. HCMR
Task Title:	Comparison of semi-intensive and intensive rearing (led by HCMR)			
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Lead Scientist preparing the Deliverable: (HCMR)

Other Scientists participating: Papadakis, I.E., Papandroulakis, N., Cotou, E., Kotsiri, M.

Objective: The objective of the present study was to characterize the ontogeny of the greater amberjack visual and digestive systems, and to study the influence of different rearing conditions (semi-intensive versus intensive rearing) on the ontogeny of the two systems.

Description:

The present deliverable presents:

- the ontogeny of the vision and digestive systems,
- the variations of lipid deposition in the liver in relation to the prey items and rearing system used,
- the larval visual ability at different developmental stages,
- the correlation of amberjack feeding preferences with the feeding protocol,
- the identification of critical malnutrition periods during rearing, and
- the levels of oxidative stress in larvae reared in the different rearing systems, by measuring specific biomarkers..

INTRODUCTION

The larval rearing of greater amberjack *Seriola dumerili* is considered as the major bottleneck for successful culture of this species, due to the low survival rates observed during this period. The knowledge on greater amberjack larval rearing is still considered incomplete. Semi-intensive rearing systems seems to be more effective, but even then the survival rate is rather low, around 3% (Papandroulakis *et al.*, 2005). In order to increase productivity during the larval stages, modifications of the rearing protocols are necessary.

The biological systems that are closely related with the feeding behavior of any species are (a) the vision system, by which the fish perceive the different food items in the rearing environment, and (b) the digestive system that enables fish to capture, ingest, digest and absorb nutrients from the food. These systems and their organs are relevant to the feeding protocols used during the larval rearing. The vision system (*i.e.* the eye) is determines the ability of larvae to identify the prey under the light conditions that exist in the rearing environment, whereas the digestive system is determines with the qualitative and quantitative composition of the feeding protocol that is used during rearing.

During the first larval developmental stages until the transformation into a juvenile, numerous changes appear in the digestive system of fish larvae, in terms of morphology and functionality (Przybył *et al*., 2006). Therefore, the knowledge of the digestive system ontogeny is essential, in order to be able to understand the digestive physiology of larvae. Additionally, studies that are focused on morphological changes in the larval digestive organs, such as the liver, provide the necessary information on the assimilation and digestion of consumed food that are included in the feeding protocols, to the general nutritional status of fish larvae. The liver is considered as an indicator-organ for the nutritional and physiological status of the fish (Caballero *et al*., 1999), because it responds directly and rapidly to the various dietary conditions created by the diet and the rearing protocol (Papadakis *et al*., 2009; Papadakis *et al*., 2013).

The larvae of most teleosts are mainly visual predators. Under rearing conditions, the signals received by the visual system are defined by the lighting conditions (Blaxter, 1986; Miner and Stein, 1993) and these signals are coming from the type and concentration of food items (Hunter, 1981). The visual ability of fish, which is related to the distance that the fish can identify an object, depends on the overall organization of the eye at different developmental stages. Therefore, if the visual ability and the light requirements of the species under commercial rearing conditions are known, the farmers could modify the light conditions in the tank according to the larval requirements.

Furthermore, in order to better understand the efficiency of the applied feeding protocols, the oxidative stress is a reliable indicator. Oxidative stress is a progressive imbalance between reactive oxygen species (ROS) production and antioxidant defense. Reactive oxygen species production is controlled by efficient antioxidant capacity, characterized by a set of antioxidant enzymes, which can together detoxify ROS (Abele and Puntarulo, 2004) and reduce their negative effects on fitness (Malanga *et al*., 2004). The antioxidant defense system, which is comprised of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), is designed to maintain the lowest potential level of ROS in cells and is recognized as an essential component of an organism's response to maintain homeostasis (Castex *et al*., 2010). SOD catalyses the dismutation reaction between superoxide radicals (SOR), yielding hydrogen peroxide (H_2O_2) and oxygen (O_2). The detoxification of H_2O_2 is then catalyzed by GPx. In addition other enzymes such as GR have also a role in protection against free radical damages. Numerous non-enzymatic defenses are also employed to provide protection such as, glutathione (GSH) (Harman, 1972). GSH scavenges ROS directly by oxidizing reduced glutathione to oxidized glutathione (GSSG), which converts oxides (O_2^-) to hydrogen peroxides (H_2O_2) (Dorval *et al*., 2003). Glutathione S-transferase has the ability to detoxify some of the secondary ROS produced when ROS react with cellular constituents (Adeyemi, 2014).

The aim of this study was the description of the eye and the digestive system ontogeny of greater amberjack larvae in relation to the rearing conditions. The above information will be used to evaluate the rearing protocols and suggest ways for their improvement. This comparison will be further elucidated by the oxidative stress study and the consequences of the different rearing systems on the larval antioxidant defense systems by measuring the activity of several antioxidant enzymes in different larval developmental stages of the greater amberjack larvae.

MATERIALS AND METHODS

Larval rearing

The rearing trials were performed in the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture at the Hellenic Center for Marine Research (HCMR). Larval rearing of greater amberjack was performed in three different rearing systems, the mesocosm (MES), the intensive (INT) and the extensive mesocosm (SWMES). Eggs from induced spawning of breeders kept in the 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.

Extensive Mesocosm Larval rearing (SWMES)

An out door tank with a volume of 10-m^3 was stocked with 40 x 10^3 eggs. The tank was filled with unfiltered natural seawater (salinity 40 psu) which was also the water for subsequent renewal. Temperatures reflected natural conditions and ranged from 22 to 26.8˚C (average 25.1 ± 1.3°C) while the pH fluctuated from 7.99 to 8.18. Dissolved oxygen varied from 5.8 to 6.8 mg l^{-1} during the larval rearing. The rate of water renewal increased progressively from 15% to 35% day^{-1} on 17 day post hatching (dph), while reaching 100% on 22 dph and 200% on 30 dph. Aeration was provided in the tanks through 2 pipes (without any wooden or stone diffuser) along the perimeter of the tank. A surface skimmer was operational during the appropriate period (5 to 13 dph) to keep the surface free from lipids, a prerequisite for good swim bladder inflation. The photophase was natural. Light intensity varied, according to weather conditions, between 1000 lux on cloudy days to 2,500 lux on sunny days.

Mesocosm Larval rearing (MES)

For the MES rearing method 110 x 10^3 eggs were stocked in a 40-m^3 indoor tank. Mesocosm tank were 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. The rate of water renewal was increased progressively during the rearing. Starting from 15% to 35% day^{-1} on 17 dph, while reaching 100% on 22 dph and 200% on 30 dph. Aeration was provided in the tanks by means of five pipes (without any wooden or stone diffuser), which were distributed along the perimeter and 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 photophase was constant light from mouth opening until 25 dph and then adjusted to 18L:06D for the remaining 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.

Intensive rearing in closed water recirculation system (INT)

The experimental systems used were 500-l cylindro-conical tanks, with pairs of tanks connected to a closed water system with a biological filter. The initial stocking density of the reared population 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 in two ways according to the stage of rearing. During embryogenesis, egg hatching and the autotrophic larval stage, water circulated in the tanks through a biological filter. Aeration was also provided in the tanks (150–250 ml min^{-1}). After first feeding, water recirculation for each tank was 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% on 25 dph. A skimmer was installed at the appropriate period (5 to 15 dph) 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 period. Light intensity varied between 200 - 800 lux during the day, and was ~200 lux at night.

Feeding

The duration and type of feeding item that was included in the rearing protocols during the experimental procedure are presented in **Figure 1**. The microalgae that was used was *Chlorella minutissima*. Rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco (INVE S.A., Belgium) were added daily in the rearing tank and the concentration was kept at 2-3 individuals ml^{-1} in the MES and in the SWMES and at 4-5 individuals ml^{-1} in the INT. Unenriched *Artemia* AF nauplii and *Artemia* EG nauplii enriched with A1 DHA Selco (INVE S.A., Belgium) were then offered to the larvae at a starting concentration of 0.05 to 0.35 nauplii

ml^{-1}. In all the rearing systems, artificial feeds were added progressively according to fish size (NRD 2/4 grain size which was 200–300 µm, NRD 3/5 grain size which was 300–500 µm, INVE S.A., Belgium). Live eggs of gilthead sea bream *Sparus aurata* at the blastula stage and frozen eggs in the embryo stage were also introduced in the rearing tanks. Gilthead sea bream larvae that were offered had hatched 6-8 hours before their provision. Mesocosm tanks developed also a natural productivity of zooplankton (harpacticoida copepods) after 20 dph, which potentially contributed to larvae feeding.

Sampling procedure

For the MES and INT rearing systems, random samples of 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). For the SWMES, random samples of larvae (n=10) were collected also on the following days of rearing: 0, 1, 2, 3, 5, 7, 9, 12, 15, 17, 20, 23, 25 and 30 dph. Fish were preserved for histology in buffered fixative containing 4% formaldehyde and 1% gluteraldehyde for at least 24 hours (McDowell and Trump, 1976).

Histological analysis

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany), larvae were dehydrated in gradually increasing ethanol solutions (70-96%). Serial sections of 3 µm were obtained with a microtome (Leica, RM 2245, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to (Bennett *et al*., 1976). In order to describe the ontogeny of the digestive system and stomach content analysis all the sections were examined using a compound microscope (Nikon Eclipse 50i, Melville, NY).

Area Covered with Lipid Vacuoles, (ACLV) in the liver

For the estimation of ACLV, sections from three larvae that were sampled at 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, 23, 25, 28, and 30 dph were used for the estimation of liver lipid content according to the methodology of Papadakis *et al*., (2009; 2013). For each larva, 6 microphotographs were obtained at x100 magnification from sections obtained from different areas of the liver. Photographs were converted to gray scale in order to convert the area occupied with lipid vacuoles in white, and the total area covered with lipid vacuoles was calculated using image analysis software (Image J, NIH, USA). Other tissues that could be confused by the software as lipid vacuoles (*e.g.* blood capillaries) were manually excluded from the analysis. Measurement of the lipid vacuole-covered area was performed automatically following manual delineation. The results are presented as the percentage of the total area of the hepatic tissue of the photograph (without other non-hepatic elements) covered with lipid vacuoles.

A. Eye ontogeny and histological visual ability (visual acuity)

The study of the eye ontogeny of greater amberjack was performed from histological sections using a microscope (Nikon Eclipse 50i, NY, USA). All measurements related to the number of cones and rods cells that constitute the retina and the diameter of the lenses were performed using image analysis software (Image J, NIH, USA). Initially the full length of the retina was photographed at x40 scale and selected images were chosen for further measurements using the image analysis system, as described below.

The measurements were performed in two regions of the retina. The first region was from the olfactory area (Nasal, N) and the second region was from the region closer to the torso of the larvae (Temporal, T). The study areas selected were 100 µm long and wide enough to include all tissue layers of the larval retina. Each structure of the retina (cone cells, rod nuclei) was detected visually and was automatically quantified via the image analysis software. The lens diameter (mm), the number of cone cells and the number of rod cells

nuclei were measured, and the minimum separable angle (degrees), which is related to the histological visual acuity, was calculated.

Visual acuity is defined as the minimum angle, which two parallel objects can project at the eye and still be resolved as separate. Histological visual acuity, was expressed as the Minimum Separable Angle (MSA), which was calculated based on the widely accepted methodology by Neave (1984), using the formula: sin (MSA) = 1.11 / (10d x2.55r) where (d) is the number of cone cells and (r) the radius of the lens. As the MSA is reduced, the visual acuity increases, since fish can see objects at a long distance (Neave, 1984). For the estimation of the theoretical maximum distance that greater amberjack larvae are able to locate a prey of the size of a rotifer (0.15 mm) or *Artemia* nauplii (0.35 mm), the equation D = (h / 2) / (tan (MSA / 2)) was used, where D (mm) is the maximum theoretical distance in which larvae can identify an object with a length of h (mm) and "MSA" is the theoretical histological visual acuity, as calculated above (Wanzenbock and Schiemer, 1989).

B. Oxidative stress

Samples from greater amberjack larvae at 7 dph, 18 dph (flexion), 23 dph and 30 dph were collected. The larvae (wet weight of 125 ± 25 mg per stage) were pooled from four tanks (two/system) of the two rearing systems (MES and INT). The samples obtained at 23 dph included big (B) and small (S) size larvae, while the 30 dph sample contained 2 fish per rearing system. All samples were maintained at – 80°C until the analyses. To assess the oxidative stress of the developing greater amberjack larvae, the concentration of total GSH and the enzyme activities of total SOD, GPx, GR and GST were analyzed. Supernatants were prepared and analyzed for enzyme activities in a manner similar to that in Hamre *et al* (2014) with minor modifications. The GSH was analyzed according to Rahman et al (2006) and the GPx, GR and GST according to McFarland *et al* (1999). The SOD activity was analyzed with a commercial kit (706002, Cayman). Total protein concentrations of supernatants for enzymes were measured with a Coomassie brilliant blue reagent (Sigma) according to Bradford (1976).

Statistical analysis

Statistical analyses of the ACLV (%) during the course of the larval rearing period were performed using one-way ANOVA (Sigma Stat statistical package) with ACLV and time as factors, followed by Duncan's New Multiple Range test, at P<0.05. The ACLV values were transformed prior to analysis using the arcsine of the square root transformation, but for presentation the original data were used. Data are presented as mean ± SE. For the description of growth performance of total length as a function of time, exponential equation was used.

For the Statistical analysis of oxidative stress the data was checked for normality before analysis and they followed normal distribution. One-way analysis of variance was performed for all developmental stage following by post-hoc test using SPSS for Windows v. 13.0 program. Student t-test was used for the differences between the two rearing systems at each stage. The accepted level of significance was p<0.05.

RESULTS

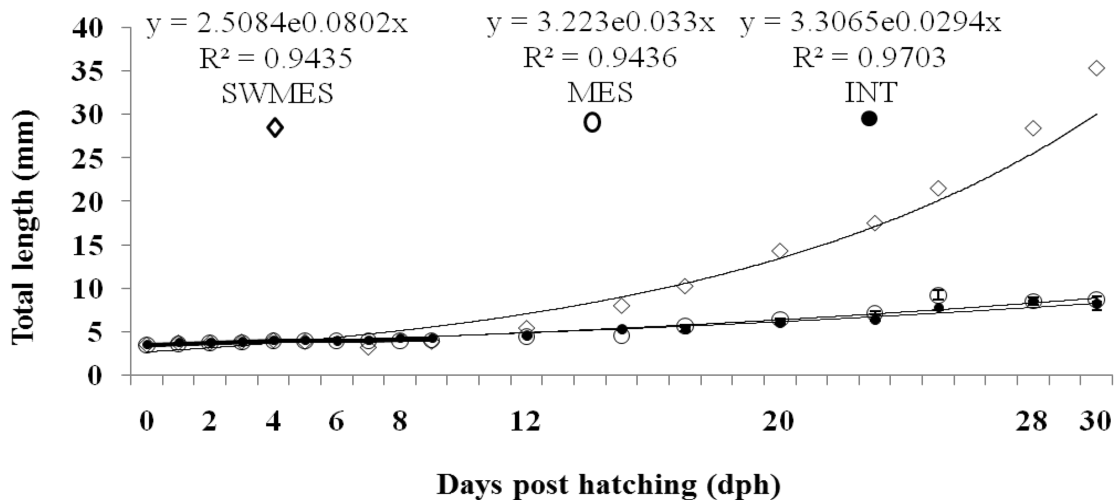
Growth performance of greater amberjack larvae

The growth performance of greater amberjack larvae presented an increasing trend during the rearing time, which was described by the exponential equation y = 2.5084e0.0802x, R^2 = 0.9435 for the SWMES, y = 3.223e0.033x, R^2 = 0.9436 for the MES and y = 3.3065e0.0294x, R^2 = 0.9703, for the INT (**Fig. 1**). The feeding protocols that were followed during the rearing procedure are presented below the growth performance graph.

Digestive system ontogeny

The comparison of the digestive system ontogenesis between the greater amberjack larvae coming from the MES and the INT rearing systems showed that there were no significant differences in the time of appearance of most of the structures (**Fig. 2**). The most significant differences between these two rearing systems were observed on the time of appearance of the gastric glands, which appeared 2 days later in the INT than in the MES, the pyloric caeca that appeared 5 days later in the INT than in the MES and the goblet cells in the midgut that appeared 2 days later in the INT.

Although there was a synchronization of the ontogenetic events between all three rearing systems until 9 dph, after that day diversifications appeared in the time of appearance of different structures in the SWMES in comparison to the other two systems (**Fig. 2**).



MES

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Phytoplankton																															
Rotifers																															
Artemia nauplii AF																															
Artemia nauplii EG																															
Artificial food																															
Frozen eggs (*Sparus aurata*)																															
Fish larvae (*Sparus aurata*)																															

INT

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Phytoplankton																															
Rotifers																															
Artemia nauplii AF																															
Artemia nauplii EG																															
Artificial food																															

SWMES

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Phytoplankton																															
Rotifers																															
Artemia nauplii AF																															
Artemia nauplii EG																															
Artificial food																															
Frozen eggs (*Sparus aurata*)																															
Live eggs (*Sparus aurata*)																															
Fish larvae (*Sparus aurata*)																															

Figure 1. Growth performance of greater amberjack larvae (mean ± SD of total length) reared in SWMES, MES and INT rearing systems. Below the graph the rearing protocols that were used during the rearing procedure are presented. The rearing protocols include the duration and the type of food items that were provided.

Buccopharynx
Mouth opening
Taste buds
Pharyngeal teeth
Oesophagus
Oesophagus folds
Goblet cells
Stomach
Cardiac sphincter
Pyloric sphincter
Gastric glands
Intestine
Supranuclear bodies in MG
Pyloric ceaca
Goblet cells MG
Ileorectal valve
Supranuclear bodies in HG
Anus opening
Liver
Pancreas

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days post hatching (dph)

Rearing system	0	1	2	3	4	5	6	7	8	9	12	15	17	20	23	25	28	30
INT	3.5	3.7	3.7	3.8	4.0	4.0	3.9	4.0	4.2	4.2	4.6	5.3	5.2	6.0	6.4	7.8	8.5	8.2
MES	3.5	3.7	3.8	3.8	4.0	4.0	4.0	4.0	4.1	4.1	4.5	5.0	5.7	6.5	7.2	9.2	8.5	8.7
SWMES	3.5	3.8	3.8	3.9	4.0	3.9		3.9		3.9	5.3	8.0	10.3	14.3	17.5	21.5	28.4	35.3

Total length (mm)

Figure 2. Schematic representation of the appearance (open solid circles indicate the MES, black solid circles indicate the INT and black crosses indicate the SWMES 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, black INT and grey SWMES) indicate the period that supranuclear bodies (vacuoles) were present in the anterior-median intestine (mid gut, MG) and hind gut (HG). Below, mean values of the total length of greater amberjack larvae for each sampling day are presented.

Period 1: 0-5 dph

During this period no difference was observed regarding the digestive system ontogeny between the rearing systems. From hatching until 2 dph, the digestive tract appeared as a closed straight tube located dorsal to the yolk sac (**Fig. 3a**) and it consisted of a single-layer epithelium of simple cuboidal and columnar cells. The liver developed rapidly. The early hepatic cells appeared between 2-3 dph (3.8 ± 0.1 mm TL) and were located initially behind the yolk sac under the anterior intestine and later they surrounded the anterior part of the intestine (**Fig. 3b**). The pancreas appeared initially as undifferentiated tissue at 2 dph, but at between 4-5 dph (4.0 ± 0.1 mm TL) the differentiation in endocrine and exocrine pancreas begun (**Fig. 3c**).

The first differentiations related with the division of the digestive system canal in different areas appeared at around 3-4 dph in all of the rearing systems. During this period the mouth and the anus opening and the digestive system separations in different characteristic areas occurred (**Fig. 3d**). The ileorectal valve that separates the midgut from the hindgut appeared at the same period (**Fig. 3e**).

Period 2: 5-10 dph

In all of the rearing systems, at 6 dph the formation of the esophagus folds was visible (**Fig. 3f**). The pyloric and cardiac sphincter at the intestine of greater amberjack larvae also appeared at 6 dph (4.0 mm TL) (**Fig. 3g**) indicating the area that the stomach will be formed. This area is defined between the cardiac and the pyloric sphincter. The first taste buds were formed around the buccopharynx at 9 dph (3.9 ± 0.3 mm TL) in the SWMES, but in the INT and the MES they appeared later, at 12 dph (4.1 ± 0.1 and 4.2 ± 0.1mm TL) (**Fig. 3h**).

Period 3: 10-15 dph

Supranuclear vacuoles were present in the larval hindgut 8 dph in the MES (4.1 ± 0.1mm TL), 10 dph (4.1 ± 0.1 mm TL) in the INT and 12 dph (5.3 ± 0.7 mm TL) in the SWMES (**Fig. 4a**). The supranuclear vacuoles in the hindgut were visible until 30 dph in the INT and the MES rearing systems, but in the SWMES they were visible until 28 dph. The first goblet cells appeared at the esophagus at 12 dph (5.3 ± 0.7 mm TL) in the SWMES and three days later at 15 dph in the MES and the INT (5.3 ± 0.2 and 5.0 ± 0.2 mm TL), increasing in number over time (**Fig. 4b**). The first pharyngeal teeth of greater amberjack larvae appeared at the buccopharynx area at 12 dph (5.3 ± 0.7 mm TL) in the SWMES and at 15 dph in the INT (5.3 ± 0.2 mm TL) and the MES (5.0 ± 0.2 mm TL) (**Fig. 4c**). During this period at 12 dph (5.3 ± 0.7 mm TL) the first gastric glands appeared in the SWMES (**Fig. 4d**) at the pyloric portion of the stomach. At 15 dph (8 ± 0.2 mm TL) the number of gastric glands increased significantly.

Period 4: 15-20 dph

The first gastric glands in the MES and in the INT appeared later than in the SWMES, at 17 dph (5.7 ± 0.4 mm TL) for the MES and at 20 dph (6 ± 0.2 mm TL) for the INT, respectively, at the pyloric portion of the stomach. The first goblet cells appeared at the mid gut at 15 dph (8.0 ± 0.2 mm TL) in the SWMES (**Fig. 4e**), at the same time when the first pyloric caeca also appeared in the SWMES (**Fig. 4f**).

Period 5: 20-30 dph

In greater amberjack larvae from all of rearing systems supranuclear bodies were present in the midgut. These structures appeared between 15-17 dph in the SWMES and between 20-23 and 20-25 dph at larvae coming from the INT and the MES, respectively (**Fig. 2**). The first goblet cells appeared at the mid gut at 23 dph (7.2 ± 0.7 mm TL) in the MES and at 25 dph (7.8 ± 1.0 mm TL) in the INT. The appearance of pyloric caeca had a 5-day delay in the INT compared to the MES. They were visible at 23 dph (7.2 ± 0.7 mm TL) in the MES and 28 dph (8.5 ± 1.2 mm TL) in the INT respectively (**Fig. 2**).

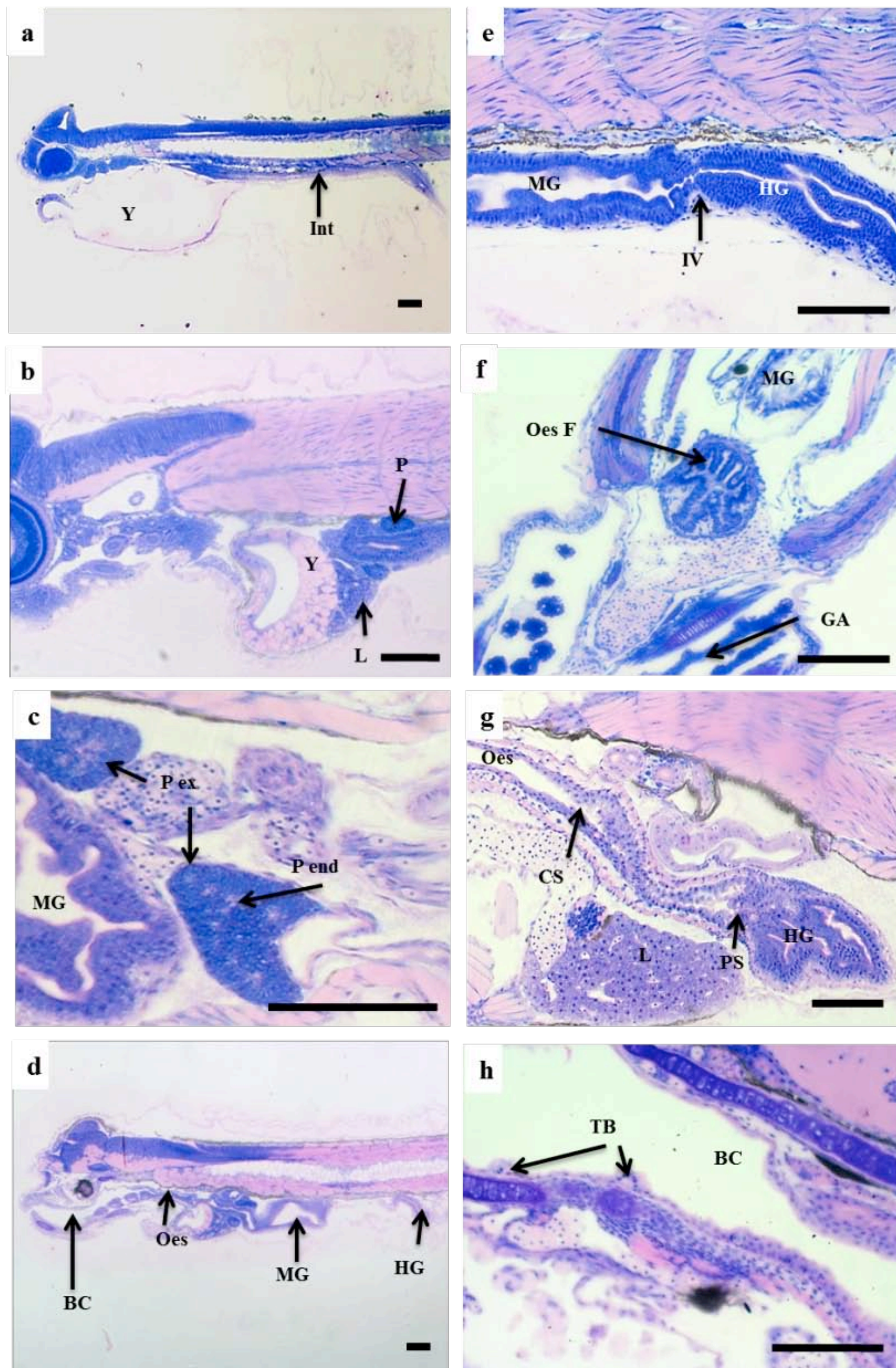


Figure 3. Microphotographs of histological sections from greater amberjack larvae at different developmental stages.(a) At 1dph showing digestive tract as a closed straight tube located dorsal to the yolk sac. (b) At 2 dph when the liver and pancreas appeared.(c) At 4 dph when the pancreas differentiated to endocrine and exocrine parts. (d) Larvae at 4 dph showing the different characteristic areas of the digestive system. (e) At 4 dph when the ileo-rectal valve appeared.f) At 6 dphshowing the formation of folds at the oesopagus, (g) At 6 dph showing the formation of the stomach area. h) At 9 dph when the taste buds appeared (SWMES). BC = buccopharynx, CS = cardiac sphincter, GA = gill arches, HG = hindgut, Int = intestine, IV = ileo-rectal valve, L = liver, MG = midgut, Oes = oesophagus, Oes F = oesophageal folds, P=

pancreas, P end = endocrine pancreas, P ex = exocrine pancreas, PS= pyloric sphincter, TB = taste buds, Y = yolk. Bar represents 100 µm.

Figure 4. Microphotographs of histological sections of greater amberjack larvae at different developmental stages.(a) At 8 dph showing the supranuclear vacuoles at the hindgut (MES). (b) At 12 dph when the goblet cells appeared at the oesophagus (SWMES). (c) At 12 dph showing the pharyngeal teeth at the buccopharynx (SWMES). (d) At 12 dph showing the gastric glands at the stomach (SWMES). (e) At 15 dph showing the goblet cells at the midgut (SWMES) and (f) at 15 dph showing the formation of the pyloric caeca (SWMES). BC = buccopharynx, HG = hind gut, IV = ileo-rectal valve, GC = goblet cells, GG = gastric glands, L =

liver, MG = midgut, Oes = oesophagus, PT = pharyngeal teeth, PC = pyloric caeca, St = stomach SV= supranuclear vacuoles. Bar represents 100 μm.

Lipid deposition in the liver (Area Covered with Lipid Vacuoles, ACLV) and stomach content.

The evolution of lipids deposition in the liver (ACLV) of larvae originating from the INT and the MES rearing protocol presented the same pattern of variation over time. This pattern can be divided into three distinct periods with significant changes in ACLV (**Fig. 5a, b**). On the contrary, in the case of ACLV in larvae coming from SWMES there weren't significant variations in the evolution of ACLV values over time (**Fig. 5c**).

Figure 5. Evolution of ACLV (mean ± SE) in the liver of greater amberjack over time, reared at three different rearing systems, a) INT, b) MES and c) SWMES. Statistically significant differences between means are indicated by different Latin letters (one-way ANOVA, Duncan's New Multiple Range test, $P<0.05$).

Period 1 (5-9 dph)

During period 1, the evolution of ACLV in the liver was characterized by an increasing trend in all of the rearing protocols (**Fig. 5a, b, c** and **Fig. 6a**). Until 9 dph, the feeding protocols were based mainly on rotifers, which were identified in the larval stomach content (**Fig. 6b).** Consumed rotifers with bearing eggs were more visible in histological slides –as eggs stained more intensely than rotifers (**Fig. 7a**). These eggs pass through the digestive canal almost undigested.

Period 2 (10-20 dph)

During this period, the percentage of ACLV in the SWMES rearing protocol continued to increase (**Fig. 5c**). In contrast, at the INT and the MES the ACLV decreased significantly ($P<0.05$) compared to the initial days of rearing and this can be characterized as a period of malnutrition (**Fig. 5a, b**). During this period, based on the provided feeding protocols, AF *Artemia* nauplii were offered for two days (from 9 to 10 dph) in the SWMES, and for three days (from12 to 14 dph) in both the INT and the MES rearing systems. In the stomach contents AF nauplii were not identified. In the MES and the INT rearing protocols, rotifers were detected, similarly to period 1. In contrast, in the SWMES rearing protocol the larvae stopped to consume rotifers from the time that the provision of EG *Artemia* nauplii started. The provision of EG *Artemia* nauplii started at 10 dph in the SWMES rearing protocol and at 14 dph for both the INT and the MES rearing systems and continued until the end of the experimental procedure in all the rearing protocols. EG *Artemia* nauplii were not identified in the stomach content of INT and MES rearing protocols, only rotifers were detected. In the MES during period 2, EG *Artemia* nauplii were identified only at 20 dph. In contrast, in the SWMES the larvae consumed immediately the EG *Artemia* nauplii, as they were identified at 12 dph (at the second day of their application) in the stomach contents (**Fig. 7b**). The provision of artificial food started at 16 dph in MES and at 17 dph in SWMES, but this type of food item was not identified in the stomach content during period 2.

Live eggs and newly hatched larvae of gilthead sea bream were included in the rearing protocol at 13 and 16 dph in the SWMES rearing system, respectively, and at 16 dph newly hatched gilthead sea bream larvae were provided, but none of them were identified in the stomach contents of greater amberjack larvae. Frozen gilthead sea bream eggs were offered at 19 dph and they were identified in the stomach content from the second day (20 dph) of their provision (**Fig. 7c**). It is notable that all the eggs that were identified in the stomach content were at the embryo phase. At 20 dph, frozen eggs and live newly hatched larvae of gilthead sea bream were included in the MES rearing protocol that were not detected in the stomach contents of greater amberjack larvae.

Period 3 (21-30 dph)

During period 3 the percentage of ACLV in the SWMES rearing protocol continued to increase as previously until 25 dph and after that day the amount of ACLV was stabilized. In the INT and the MES the ACLV increased also but its values were significantly lower ($P<0.05$) compared to the values of SWMES. Between the INT and the MES there were no statistical differences in most of the rearing days. The only exception during period 3 was at 25 dph, when the ACLV in the MES showed higher values than the INT ($P<0.05$) (**Fig. 6a**).

During period 3 the protocol in the MES and the INT continued to include rotifers until 23 dph, which were detected in the stomach content. In the SWMES the provision of rotifers had stopped from period 2. The provision of EG *Artemia* nauplii continued in all of the rearing systems but during this period they were detected only in the stomachs of SWMES and MES. There were two exceptions only, at 23 and 28 dph in the MES, in which days EG *Artemia* nauplii were not detected in the stomach contents. In the INT, EG *Artemia* nauplii were detected during the last days of rearing, 28 and 30 dph. In the MES and the SWMES gilthead sea bream frozen eggs and live fish larvae continued to be provided in the rearing protocols. These food items were not detected in the stomach content in the case of the MES rearing system but in contrast in the SWMES from 20 until 25 dph frozen eggs in the embryo stage were detected. The provision of artificial

food was continued until the end of the experimental procedure. Artificial food was identified only in the stomach contents of SWMES at 30 dph (**Fig. 7d**).

MES

	0	1	2	3	4	5	6	7	8	9	10		12			15		17			20			23		25			28		30
Phytoplankton																															
Rotifers						X	X	X	X	X	X		X			X					X			X							
Artemia nauplii AF																															
Artemia nauplii EG																					X					X					X
Artificial food																															
Frozen eggs (*Sparus aurata*)																															
Fish larvae (*Sparus aurata*)																															

INT

	0	1	2	3	4	5	6	7	8	9	10		12			15		17			20			23		25			28		30
Phytoplankton																															
Rotifers									X		X		X			X		X			X			X							
Artemia nauplii AF																															
Artemia nauplii EG																													X		X
Artificial food																															

SWMES

	0	1	2	3	4	5	6	7	8	9	10		12			15		17			20			23		25			28		30
Phytoplankton																															
Rotifers						X	X	X	X	X	X																				
Artemia nauplii AF																															
Artemia nauplii EG													X			X		X			X			X		X			X		X
Artificial food																															X
Frozen eggs (*Sparus aurata*)																					X			X		X					
Live eggs (*Sparus aurata*)																															
Fish larvae (*Sparus aurata*)																															

Figure 6. Evolution of ACLV (mean ± SE) in the liver of greater amberjack over time, reared at three different rearing systems (SWMES, MES and INT). b) The feeding protocols employed showing when a feeding item was identified in the digestive tract (x). Statistically significant differences between means at the different rearing systems for each sampling day are indicated by different Latin letters (one-way ANOVA, Duncan's New Multiple Range test, $P<0.05$).

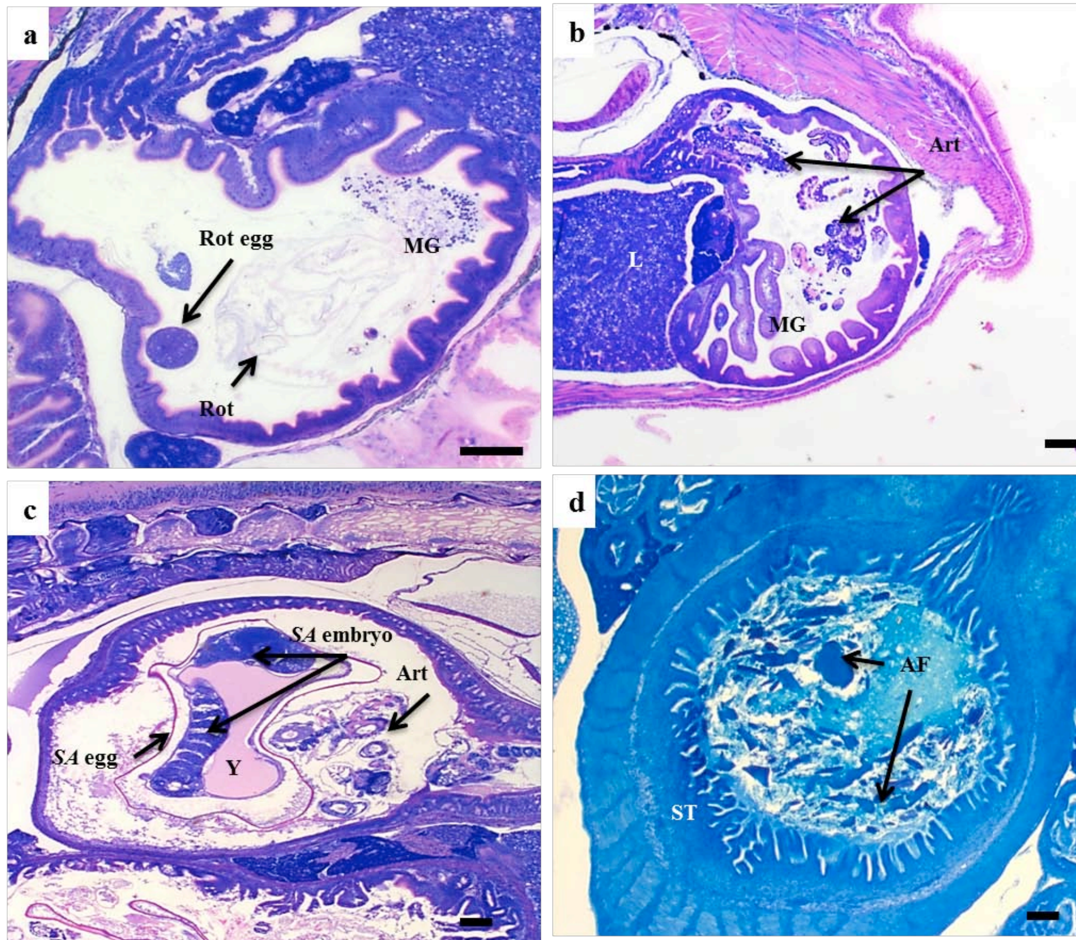


Figure 7. Microphotographs of histological sections of greater amberjack larvae, which show the stomach contents of SWMES larvae. a) At 7 dph showing the rotifers and the eggs of rotifers. b) At 12 dph showing the EG *Artemia* nauplii in the MG. (c) At 20 dph showing the stomach area with the frozen gilthead seabream eggs (*SA* egg), in which the embryo is visible, as well as *Artemia* nauplii and d) at 30 dph showing artificial food in the stomach. Rot egg = rotifer egg, Rot = rotifer, Art = EG *Artemia* nauplii, AF = artificial food, Y = yolk, St = stomach, MG = midgut. Bar represents 100 µm.

Retina development

During the first day of rearing there were no differences between the three rearing systems regarding the ontogenesis of the eye. At the day of hatching (0 dph) the retina appeared as a simple hemispherical sheet of undifferentiated neural epithelium (UNE) enclosing the lens, which comprises of a spiral of unspecialized cells (**Fig.8a**). The first differentiation in the different layers was visible between 1-2 dph (**Fig. 8b**), when the ganglia cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL) and the photoreceptor layer (PL) appeared. The pigment epithelium (PE) was not formed by this day. From 3 dph onwards, the PE appeared on the external area of the retina (**Fig. 8c**). The nucleus of the cone cells appeared at 3 dph in the outer nuclear layer, along with all the other neutral cells in the inner nuclear layer (amacrine, bipolar and horizontal cells), which were now completely distinct.

A second population of darker skinned core cells, the nuclei of rod cells (RC), appeared in the ONL at different times for each rearing system but at the same fish total length, due to different growth rates between

rearing systems. Rods first appeared at 12 dph in the SWMES (5.3 ± 0.7 mm) and then at 15 dph in the INT (5.0 ± 0.2 mm) and the MES (5.3 ± 0.2 mm) rearing system, respectively. The number of rods, which were identified by their nucleus and were placed in the ONL, increased over time (**Fig. 8d**).

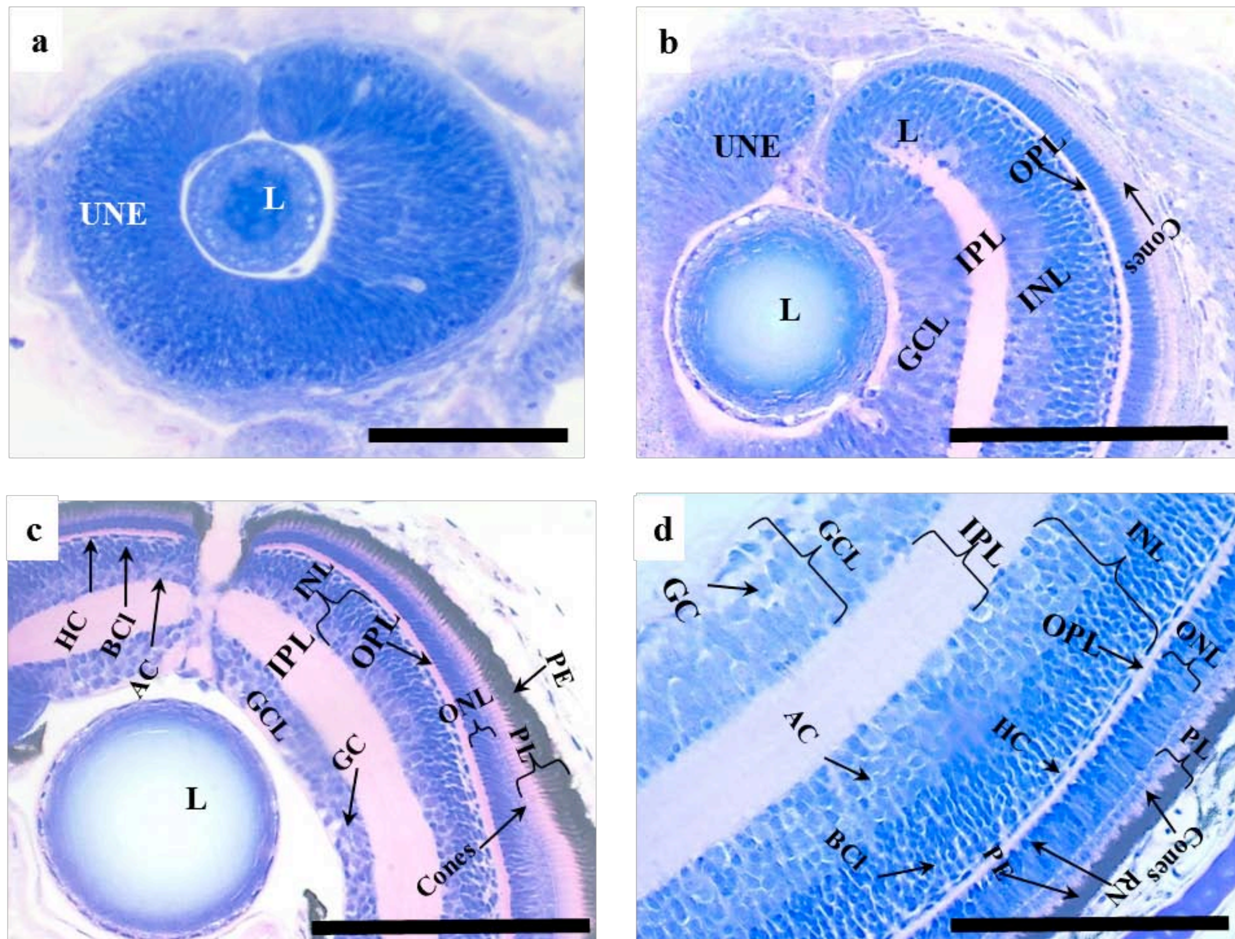


Figure 8. Microphotographs of histological sections of greater amberjack larvae in SWMES(a) at hatching showing the undifferentiated neural epithelium, (b) at 2 dph showing the onset of retina differentiation, (c) at 3 dph showing the pigment epithelium of the retina, (d) at 30 dph showing the structure of the retina with the rod cells placed at the outer nuclear leyer. AC= amacrine cells, BCl= bipolar cells, GCL= ganglia cell layer, GC= ganglia cells, HC= Horizontal Cells, INL= inner nuclear layer, IPL= inner plexiform layer, L= lens, OPL= outer plexiform layer, ONL=outer nuclear layer, PL= photoreceptor layer, PE= pigment epithelium, RN = Rod nuclei, UNE= undifferentiated neural epithelium. Bar represents 100 μm.

Evolution of the number of cone and rod cells on the retina of greater amberjack larvae.

The comparison of the evolution of number of cone cells in the retina of greater amberjack, which are responsible for daylight vision, between the three rearing systems (SWMES, INT and MES) showed differentiations until 17 dph (**Fig. 9a**). After that day, no differences appeared as concerning the number of cones per 100 μm of retina. Rod cells, which are responsible for vision under low light intensities, appeared at 12 dph in the SWMES and at 15 dph in the MES and the INT rearing systems. Thereafter, their number increased constantly until 30 dph in all of the rearing systems (**Fig. 9b**). The number of rods was higher in the group of SWMES in comparison with the other two rearing systems from the day that they first appeared ($P<0.05$).

Figure 9. Evolution of (a) cone cell and (b) rod cell density (mean ± SE) at the retina of greater amberjack larvae, in relation to time (days after hatching) for the three rearing systems(SWMES: solid triangle, MES: open square and INT: solid rhombus).

Visual acuity and visual distance

The visual acuity (expressed as minimum separable angle, MSA) decreased over time (**Fig. 10a**). This change implies that the distance that larvae could see and distinguish any item increased many fold. The food particle used in this study to estimate the distance that could be perceived from the larvae in different developmental stages were rotifers (**Fig. 10b)** and *Artemia* nauplii (**Fig. 10c**). As the MSA decreased over time, the distance that the rotifers and the *Artemia* nauplii can be perceived increased.

a

Minimum Separable Angle (MSA) (degrees)

0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8

3 5 7 9 10 12 15 17 20 23 25 28 30

b

Distance (mm) for the perception an item of 150 µm length

0 10 20 30 40 50 60 70

3 5 7 9 10 12 15 17 20 23 25 28 30

c

Distance (mm) for the perception an item of 350 µm length

0 50 100 150 200 250

3 5 7 9 10 12 15 17 20 23 25 28 30

Time (dph)

Figure 10. Evolution of (a) cone cell and (b) rod cell density (mean ± SE) at the retina of greater amberjack larvae, in relation to time (days after hatching) for the three rearing system (SWMES: solid triangle, MES: open square and INT: solid rhombus)

Oxidative stress

It was observed that GPx, SOD, and GST activities were significantly decreased (**Fig. 11)** in all the developmental stages after flexion stage in comparison to 7 dph stage (P<0.05). Significant decreased enzymatic activities were also indicated between intensive and mesocosm rearing systems at all developmental stages for GPX and GST (**Fig. 11)** at 7 dph and flexion stages for SOD (P<0.05). On the other hand, an induction of GSH content was only observed at flexion and 23 dph S stages. However, due to small number of replicates and the high variation observed, it was not possible to establish any significant statistical levels of GSH content among the developmental stages or between the rearing systems (**Fig. 11**). Decreased significant activities were found as well for GR (**Fig. 11)** at all developmental stages except of the 23 dph S stage. However, no significant difference was found between the rearing systems for GR (**Fig. 11**).

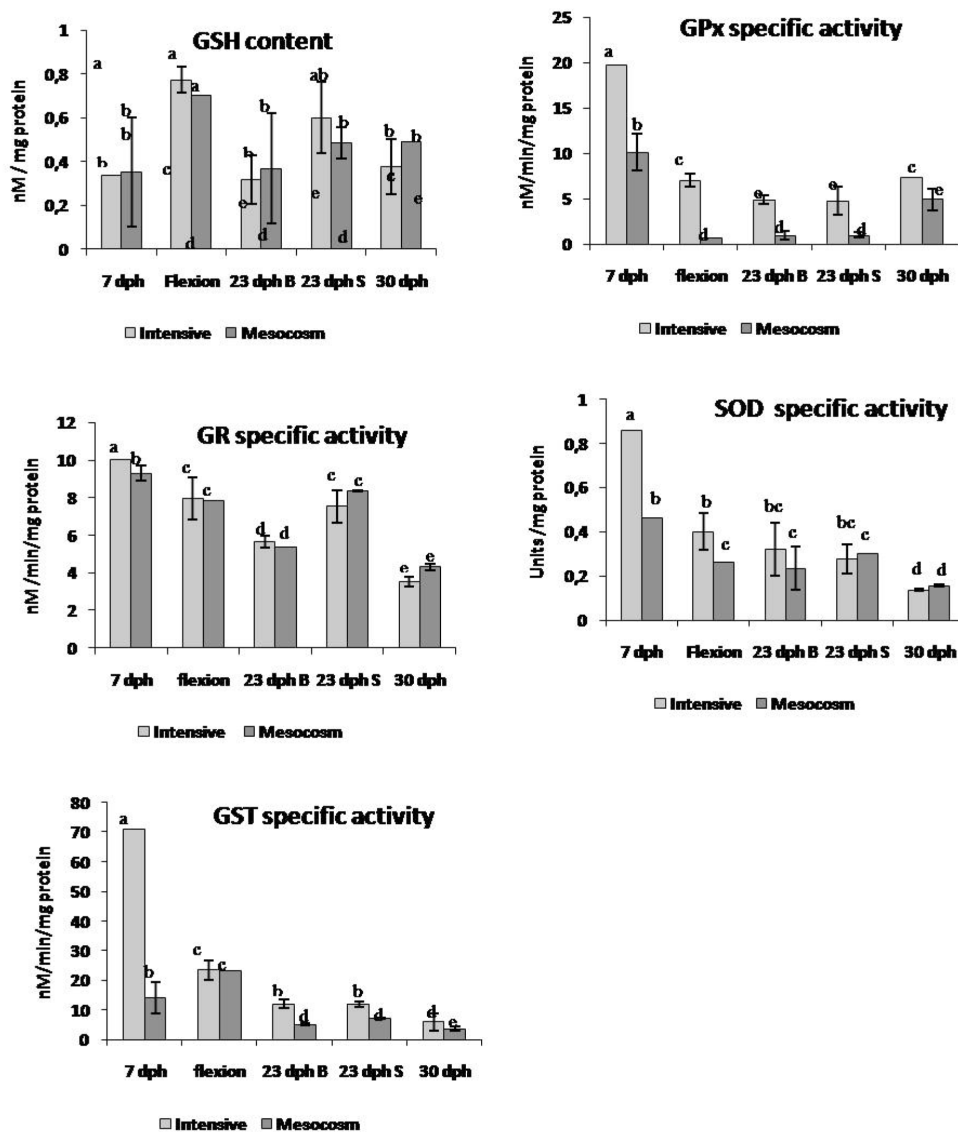


Figure 11. Changes on antioxidant defense systems (GSH content and GPx, GR, SOD and GST specific activities) of developmental larval stages of *Seriola dumerili* reared in intensive and semi-intensive (mesocosm) rearing systems (B and S refer to large and small larvae, respectively, different small letters indicate significant differences at P<0.05).

DISCUSSION AND CONCLUSIONS

Ontogeny of the greater amberjack visual and digestive system

In greater amberjack, the ontogenesis of the digestive system in all the rearing systems is considered as a rapid procedure. The development of the digestive system is controlled by endogenous factors and generally it is genetically programmed, but the time of appearance of the digestive system structures can be influenced by a number of factors, with temperature being one of the most important (Kamler, 2002). Generally, temperature affects the growth performance of fish, as they are poikilothermic organisms, and there is a species-specific range of temperatures, in which each species presents the highest growth rates. When the feeding conditions and the temperature range is the optimum, growth rates are higher. In the present study, during the first days of the rearing procedure the results show that there is a complete synchronization of the ontogenetic events of the digestive system and the eye in all the rearing systems. After 12 dph, a diversification of these events in both the digestive and the vision system appeared. The developmental rates in the SWMES appeared to be faster than in the other two rearing systems after 12 dph. The above is connected with the higher growth rates observed in the SWMES, as a result of the higher rearing temperature used in the larval rearing compared to the other two rearing systems. A good explanation for the higher growth rates observed in the SWMES can also be the initial consumption of zooplankton preys that were introduced in the rearing environment through the unfiltered seawater used in the SWMES. One of the most important structures of the digestive system are the gastric glands that in greater amberjack larvae appeared after 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. As regards the feeding protocol, the introduction of less digestible food items, like *Artemia* nauplii -in comparison with rotifers or artificial food- is suggested to occur in the rearing protocol after the size of 5 mm (after the appearance of gastric glands).

The stomach contents are considered as good indicators of the larval food preferences. In general, the fish can consume the item that they can see and are attracted to. The attractiveness is affected by the visibility (size and color) and the moving ability of the food item. The above is considered as the most important criterion for the fish food selection activity but it isn't a good criterion for capturing, digestibility and assimilation of food and especially during the first developmental stages when the digestive system is under development. Capturing is affected by the combination of the larval and the prey size. In the present study, the higher growth rate of the larvae in SWMES increased their ability to consume and swallow immediately all the types of food, in contrast to the larvae of the other two rearing systems. The digestibility and the assimilation of food are affected by the ontogenetic status or the organization and the functionality of the digestive system. For this, the larvae from the INT and the MES, where the ontogenetic clock was slower than the larvae from the SWMES, either didn't consume or didn't digest the low digestible food items that were included in the rearing protocols. The above affected the nutritional status of the larvae and this became more obvious by the lipids analysis in the liver that is considered as an accurate indicator for the nutritional status of the fish. The malnourishment in fish that generally appeared as a reduction of lipid content in the liver cells (Power *et al*., 2000) has been observed to occur also during early life stages in other species as well (Papadakis *et al*., 2009). Significant changes in lipid content in the liver of greater amberjack were observed in the present study as the larvae advanced through development and between the rearing systems. No alterations in values of lipid content appeared in the SWMES rearing system in comparison to the other rearing systems. In the MES and INT rearing systems the malnutrition periods appeared during the transition from rotifers to *Artemia* nauplii and during the transition to artificial food, a fact that is connected with the low ability of the larvae to assimilate these types of foods during this period for the reason that the

digestive system was not appropriately developed neither from a structural nor from a functional point of view.

The ontogeny of the retina in greater amberjack was found to be similar to the general pattern shown in most fish species. At hatching, the retina was an undifferentiated and non-functional tissue, as occurs in most marine fishes with pelagic early life stages (Pankhurst and Eagar, 1996; Pankhurst and Hilder, 1998; Pankhurst *et al*., 1993; Pankhurst *et al*., 2002; Roo *et al*., 1999; Shand *et al*., 1999). The first differentiation of the retina of greater amberjack occurred during the first days of rearing and it was connected with the photopic (i.e. high light intensity) vision. Cone cells were the first photoreceptors that appeared. This fact indicates that at this developmental stage greater amberjack larvae were able to see different items in the rearing environment only during daylight hours. Thus, it is necessary to provide light in the rearing tanks of greater amberjack from 3 dph.

Visual acuity – the distance the eye can differentiate between two points - of greater amberjack improved over time, as shown by the histological assessment. Although the density of cones (number per 100 μm length) decreased over time in the retina, the radius of the eye lens increased, which contributes to an overall increase in the distance that the fish are able to see food items like rotifers and *Artemia* nauplii. Therefore, larvae could theoretically identify the rotifers, which are considered the smallest food particle that was provided, in the lowest density that was used (2-3 individuals/ml).

Regarding scotopic vision (*i.e.* low light intensity), it has been shown that it is affected by the density of rods, which is the number of rods per surface unit in the retina (Fernald, 1991; Higgs and Fuiman, 1996). There was a differentiation in time of appearance and increase rates of rods in time between rearing systems. Generally, as described by other authors, after their first appearance rods spread very quickly across the surface of the retina and their density increases rapidly (Higgs and Fuiman, 1998; Pankhurst *et al*., 1993; Poling and Fuiman, 1998; Shand, 1997). In the SWMES, the earlier appearance of rod cells (12 dph), in comparison with the MES and the INT (15 dph), is connected with the highest growth rates of the SWMES larvae. The above fact indicates that greater amberjack larvae have the ability to see the feeding items under lower light intensities and for this, the light intensity on the rearing tank may be decreased gradually after their appearance in such a way that it can coincide with the increase in the rod cells.

The combination of the rearing temperature and the feeding protocol is the key factor in order to achieve better rearing results in the greater amberjack larval rearing. 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). The variety of zooplanktonic items in the unfiltered seawater may increase the possibility of the larvae to consume more digestible and nutritional food particles. Although these items were not identified in the stomach contents (maybe due to the small size and/or color transparency), this hypothesis has to be taken in serious consideration for the further optimization of the greater amberjack rearing protocol.

Oxidative stress

There have been many studies on the endogenous antioxidant enzyme system in fish particularly in relation to specific oxidative stresses and also in relation to age and development. Generally, the enzymatic antioxidant system includes some enzymes which catalyze the reaction of ROS degradation. SOD protects against oxidative damage by catalyzing the reaction of dismutation of the superoxide anion to H_2O_2, GRx reduce both hydrogen peroxide and organic hydroperoxides, GR catalyses the reaction to form GSH and maintain a ratio GSH/GSSG under oxidative stress and GST detoxify some of the secondary ROS produced when ROS react with cellular constituents (Rudneva, 2013). However, there have not been relevant studies specifically investigating the activity of the antioxidant enzyme systems in developmental larval stages of greater amberjack reared in intensive or mesocosm systems.

The present study has shown that readily measurable activities of most important antioxidant enzymes were present in the developmental stages of greater amberjack larvae (from 7 dph to 30 dph) in both rearing

systems. Generally, the activities of GPx, SOD, GR and GST were decreasing after flexion stage, while the decrease was significantly lower in the mesocosm than in the intensive rearing system. According to literature similar reduction of antioxidant enzyme activities during fish larvae development have been found in Atlantic cod for GPx but not for SOD (Hamre *et al,* 2014).

The activity profile and the overall patterns of all antioxidant enzymes observed during the developmental stages of greater amberjack larvae might suggest specific compensatory mechanism of antioxidant defense to compensate ROS production/removal (neutralize ROS) and to eliminate the damage of oxidative stress. Oxidative stress appears to be more severe in intensive compared to mesocosm rearing system. Decreased antioxidant enzyme activities in mesocosm could have been related to a lower oxidative stress and more stable rearing conditions than in intensive system.

Various aspects of intensive aquaculture stimulate stress responses in fish larvae because artificial systems are frequently exposed to a range of 'unnatural' stressors which are related to rearing practices. Antioxidant enzymes play an important role in inactivation of ROS and thereby control oxidative stress as well as redox signaling. Both processes change across the life span of the organism and thus modulate its sensitivity and resistance against free radical damage. Due to the lack of any information related to antioxidant defense systems in greater amberjack and for a better evaluation of the consequences of these outcomes, an association of our results with other parameters and performance indicators such as population density, mortality, survival, growth during the examined developmental stages might be crucial.

Additionally, and according to the DOW, the ontogeny of the digestive system of greater amberjack larvae was also addressed focusing on proteases, lipases and amylases activities (Sub-task 15.2.3 (ULL), through a combined Action of Subtask 15.2.1"Comparison between intensive (RAS with 500 l tanks) and semi-intensive (Mesocosm with 40,000 l) tanks" (HCMR) and Subtask 15.2.2 "The effect of stocking density on larval performance" (FCPCT).

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Deviations: The delay of the above deliverable (it should have been delivered in month 27of the project) is because, except from INT and MES rearing conditions, an additional rearing condition (SWMES) was analyzed (for the a, b, c, d, deliverables) which was not in the original design of this deliverable. The above analysis was necessary because it was considered (and then it was proved) it would offer more useful results that are necessary for the optimization of the greater amberjack rearing protocols.

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