Deliverable Report

Deliverable No:	D18. 1		**Delivery Month:**	47
Deliverable Title	Development of the digestive system of wreckfish			
WP No:	18		**WP Lead beneficiary:**	P8. IEO
WP Title:	Larval husbandry - wreckfish			
Task No:	18.1		**Task Lead beneficiary:**	P1. HCMR
Task Title:	Development of feeding methodology (led by HCMR)			
Other beneficiaries:	P8. IEO	P32. MC2		
Status:	Delayed		**Expected month:**	36

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Objective: Description of the digestive system development of wreckfish. Specifically, the present deliverable presents:

- the ontogeny of the digestive system and the eye,
- the variations of lipid deposition at the liver in relation to the feeding items used,
- the visual ability in different developmental stages,
- the identification of critical phases during larval rearing (malnutrition periods), in order to improve the rearing conditions.

INTRODUCTION

Larval rearing of wreckfish *Polyprion americanus* is considered as the major bottleneck for the successful culture of this species, due to the low survival rates observed during this period. One of the main scientific goals for wreckfish larval rearing is the development of protocols according to the specific requirements of the larvae during the early developmental stages. The study of the development of the organs related with larval feeding behavior offers part of the necessary information for the optimization of the larval rearing protocols. During larval stages, the systems that are closely related with the feeding behavior are the vision system, by which the fish perceive the different food items in the rearing environment, and the digestive system, which enables fish larvae to capture, ingest, digest and absorb nutrients from the food. These two systems and the structures of which they are composed are related with the larval rearing feeding protocols. The vision system (*i.e.* the eye) determines the ability of larvae to identify the prey under the light conditions that exist in the rearing environment, whereas the digestive system is also determined by the qualitative and quantitative composition of the feeding protocol that is used during rearing.

During the first 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 included in the feeding protocols, which reflect 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).

Most teleost larvae 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 larval requirements.

The aim of this study was the description of the eye, and the digestive system ontogeny. The information will be used for the improvement of the rearing protocols for the successful wreckfish larval production.

MATERIALS AND METHODS

Larval rearing

The rearing trials were performed at the facilities of the Aquarium Finisterrae (MC2) of La Coruña City Council. A surface egg collector was used to collect eggs from spontaneous spawning of breeders kept in a 33-m^3 tank at the Aquarium Finisterrae of La Coruña. Then, larvae were transferred to 180-l conical incubators. After hatching, newly hatched larvae were transferred to a 10-m^3 tank for rearing.

Larval rearing system

Larvae were released into a 10-m^3 tank where two species of copepods were previously cultured: *Tisbe battagliai* Harpaticoide and *Acartia tonsa* Calanoide. This indoor tank was stocked with 1500 larvae. The water used was natural filtered seawater, salinity 35 ‰. Temperature ranged from 16,5 to 17,5 ˚C (average 17 ± 0,5 °C), while pH fluctuated from 7,7-7,9. Dissolved oxygen varied from 85 to 95 mg l^{-1} during larval rearing. A bottom air stone released 1mm bubles to gently move the water column. The rate of water renewal was kept at 0,5 % /h. The photophase was natural. Light intensity varied, according to weather conditions, between 1000 lux on cloudy days to 3500 lux on sunny days.

Feeding

Larvae were fed with copepods (*Tisbe battagliai* and *Acartia tonsa*) cultured and fed daily with 10 l. 10^6 u/ml *Nannochloropsis gaditana* and, 10 l. 10^6 u/ml *Rhodomona*. In the rearing tank copepod concentration reached 4 individuals ml^{-1}. On 20 days after hatching (DAH) Gemma 0,5 was given to surface larvae.

Sampling procedure

The sambling procedure were performed at the MC2 facilities. Random samples of eggs (n=10) and larvae (n=3) were collected on the following days of rearing: 1 day before hatching, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 22, and 23 dph (day 0 was the day of hatching). Fish were preserved for histology in buffered fixative containing 4% formaldehyde and 1% gluteraldehyde for at least 24 hours (McDowell and Trump, 1976). Before embedding, samples were measured in total length for the description of growth performance.

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, 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 3 larvae that were sampled at 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 22, and 23 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.

Eye ontogeny and histological visual ability (visual acuity)

The study of the eye ontogeny of wreckfish was performed on histological sections using a microscope (Nikon Eclipse 50i, NY, USA). All measurements related to the number of cone and rod 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).

RESULTS

Growth performance of wreckfish

The growth performance of wreckfish larvae presented an increasing trend during the rearing time, which was described by the linear equations a) y =0.2167x+4.2095, R^2 = 0.7765 and b) y = -0.0111x+5.3662, R^2 = 0.1107 (**Fig. 1**). The duration of the prelarval stage, when feeding of larvae is based exclusively on its lecithotrophic reserves, was 5 days. The mouth opened at 5 dph, initiating the larval stages, which were maintained until 23 dph.

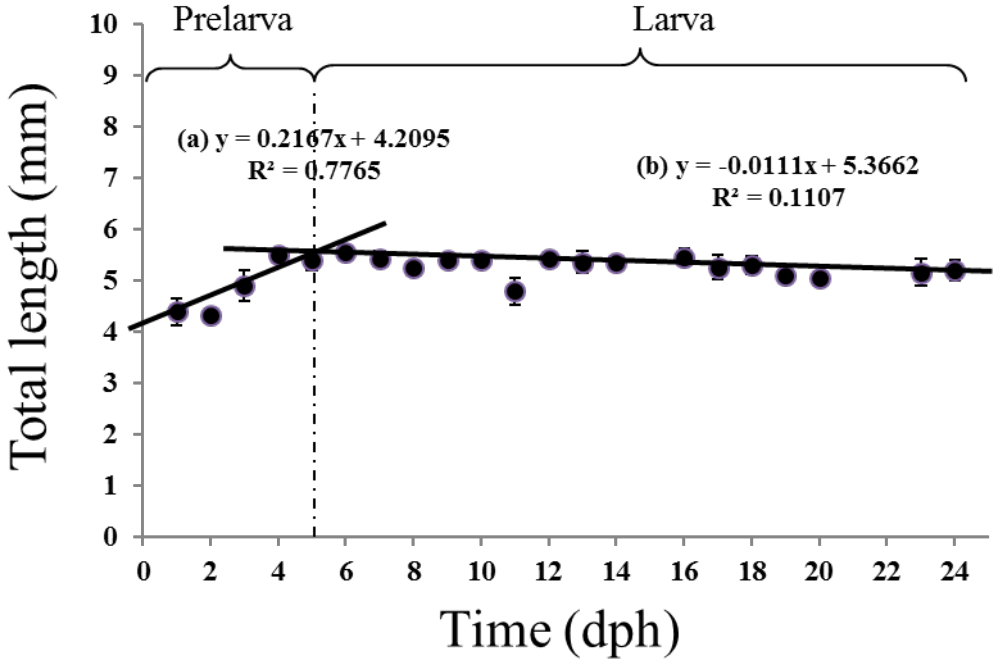

Figure 1. Growth performance of wreckfish larvae (mean ± SD) as a function of days post hatching (dph).

Digestive system ontogeny

As concerning the developmental status of the digestive system, most of the organs (except for the maxillary teeth at the upper jaw that became visible at 19 dph) appeared by 8 dph **(Fig. 2)**. From the ontogenetical point of view, the digestive system ontogeny up to 23 dph can be categorized into two distinct periods.

Period 1: 0-5 dph

This period refers to the the prelarval stages, during which larval feeding was based exclusively on lecithotrophic reserves. During this period, the digestive tract appeared as a closed straight tube located dorsal to the yolk sac and consisted of a single-layer epithelium of simple cuboidal and columnar cells. At 5 dph mouth and annus opening occurred (**Fig. 3a**). At the same time, the liver and the pancreas also appeared. The early hepatic cells appeared at around 5 dph and were located initially behind the yolk sac under the anterior intestine; later they surrounded the anterior part of the intestine (**Fig. 3b**). The pancreas appeared also at 5 dph around the first part of the digestive canal (**Fig. 3c**).

Period 2: 5-23 dph

The mouth opening at 5 dph, initiates the transition from period 1 to period 2 or from the prelarval to the larval stage. Until 23 dph endogenous yolk sack material was visible inside the lecithotrophic sack. The ileorectal valve that separates the midgut from the hindgut appeared at 6 dph (**Fig. 3d**). At 8 dph the

formation of the esophagus folds and the goblet cels on the esophagical epithelium was visible (**Fig. 3e**). The pyloric and cardiac sphincter at the intestine of wreckfish larvae also appeared at 8 dph (**Fig. 3f**) 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 8 dph (**Fig. 3g**). The last structures related with the digestive system ontogeny were the maxillary teeth at the upper jaw that became visible at 19 dph (**Fig. 3h**).

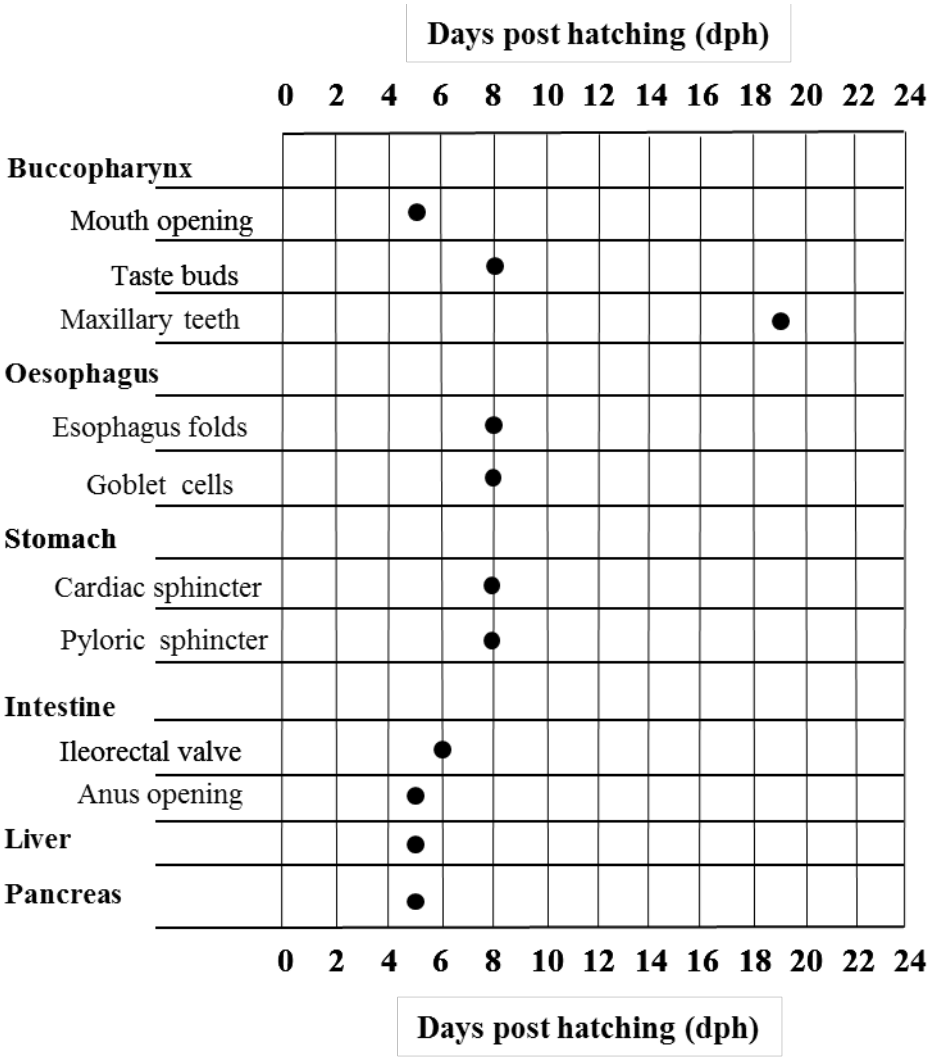

Figure 2. Schematic representation of the main structures of the digestive system that were studied. The time of appearance of each structure is presented with a black solid circle as a function of days post hatching (dph, horizontal axis).

One of the most important events that characterized the first developmental stages in relation to the development of the digestive system was the big period that the endotrophic phase occurred. According to the results from the histological analysis, material from the lecithotrophic sack was visible until 23 dph. Furthermore, due to the fact that no food item was detected in the stomach content during the present study, this means that the yolk sack material was the main nutritional source for the larvae.

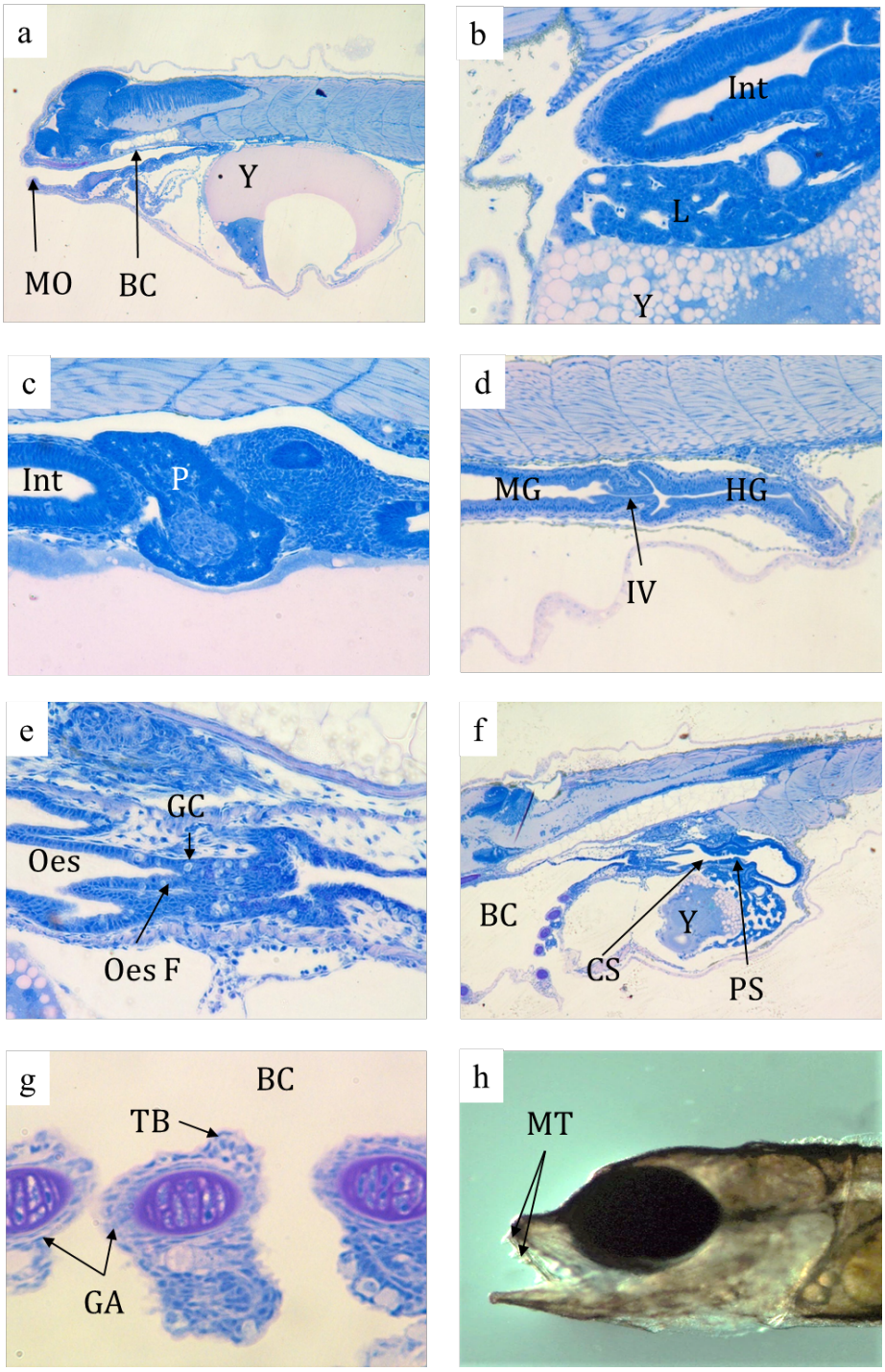

Figure 3. Microphotographs of histological sections from wreckfish larvae at different developmental stages.(a) At 5 dph showing the opened mouth, (b) At 5 dph when the liver appeared. (c) At 5 dph when the pancreas appeared. (d) At 6 dph when the ileo-rectal valve appeared. (e) At 8 dph showing the formation of folds and goblet cells at the oesopagus. (f) At 8 dph showing the formation of the stomach area from the cardiac and pyloric sphincter. (g) At 8 dph when the taste buds appeared. (h) At 19 dph showing the formation of the maxillary teeth at the upper jaw. BC = buccopharynx CS = cardiac sphincter, GA = gill arches, GC = goblet cells, HG = hindgut, Int = intestine, IV = ileo-rectal valve, L = liver, MO = mouth opening, MG = midgut, MT = maxillary teeth, Oes = oesophagus, Oes F = oesophageal folds, P= pancreas, PS= pyloric sphincter, TB = taste buds, Y = yolk.

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

No lipid deposition was detected during the analysis in the liver (ACLV) of wreckfish larvae (**Fig.4a, 4b, 4c**). Furthermore, no food items were detected in any part of the digestive canal (**Fig.4d, 4e, 4f**).

Evolution of intestinal villi.

The formation of intestinal villi started between 7-8 dph (Fig.4d). Then their size had an increasing trend until 16 dph. After that day their size was reduced and at 23 dph no more micro villi were found in the intestinal area (Fig. 4e, 4f).

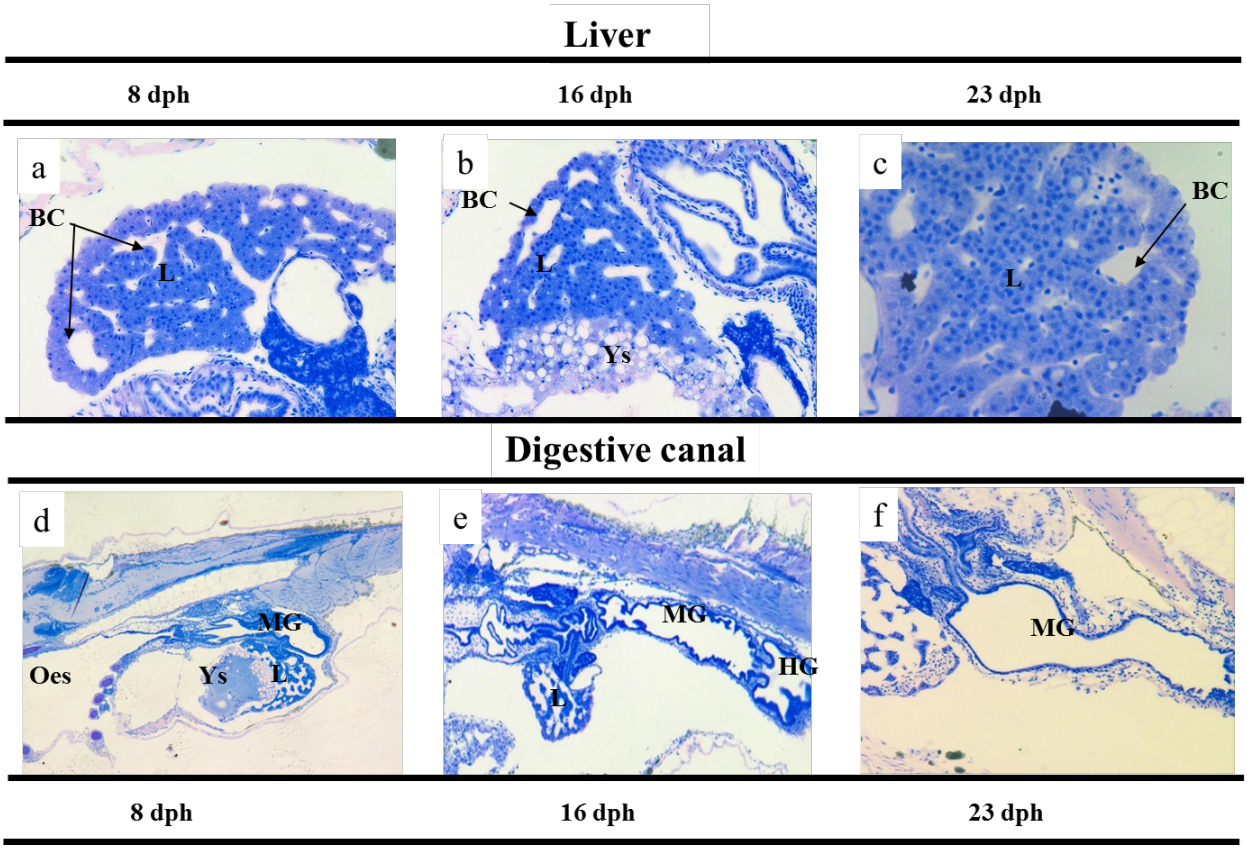

Figure 4. Microphotographs of histological sections of wreckfish larvae at different developmental stages focusing on the absence of lipid vacuoles from the liver and of food items from the digestive canal and on the variation of vili size at 8, 16 and 23 dph. BC = Blood capillaries, HG = hindgut, Int = intestine, L = liver, MG = midgut, Oes = oesophagus, Ys = yolk.

Retina development

During the first day of rearing there were no differences 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.5a**). The first differentiation in the different layers was visible at 3 dph (**Fig. 5b**). The pigment epithelium (PE) was not formed by this day. From 6 dph onwards, the PE appeared on the external area of the retina (**Fig. 5c**). The nucleus of the cone cells appeared at 6 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. Rod cells were not detected until 23 dph (**Fig. 5d**).

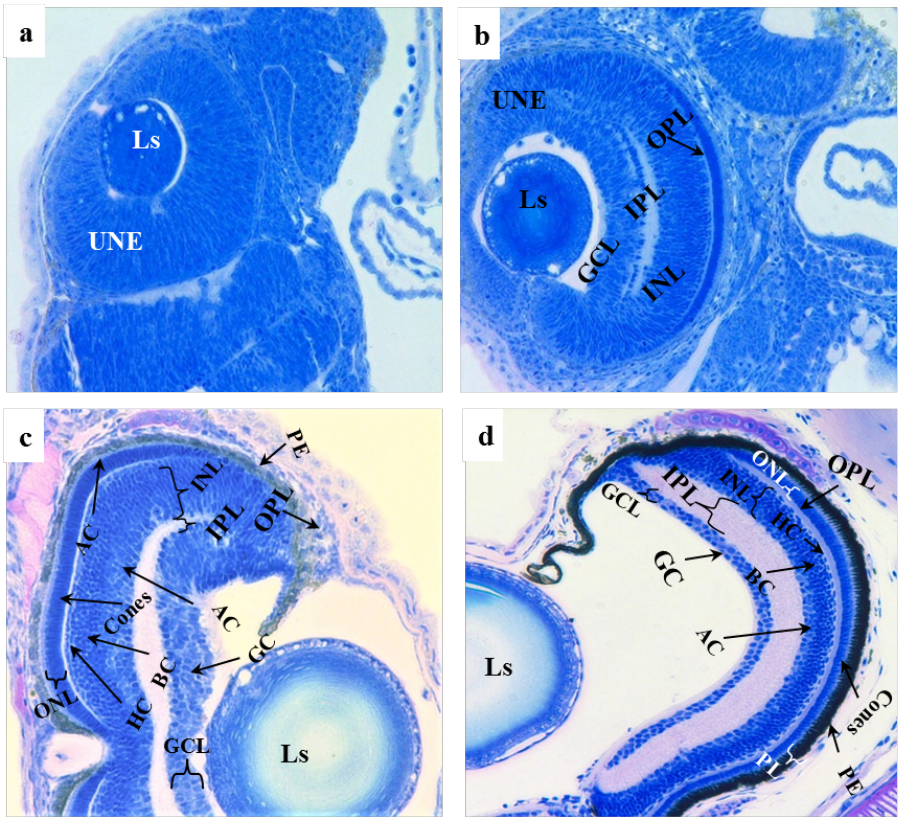

Figure 5. Microphotographs of histological sections of wreckfish larvae (a) at hatching, (b) 3 dph showing the onset of retina differentiation, (c) at 6 dph focused on the appearance of the pigment epithelium of the retina, (d) at 23 dph showing the structure of the retina. AC = amacrine cells, BC = bipolar cells, GCL = ganglia cell layer, GC = ganglia cells, HC = Horizontal Cells, INL = inner nuclear layer, IPL = inner plexiform layer, Ls = lens, OPL = outer plexiform layer, ONL = outer nuclear layer, PL = photoreceptor layer, PE = pigment epithelium, UNE = undifferentiated neural epithelium.

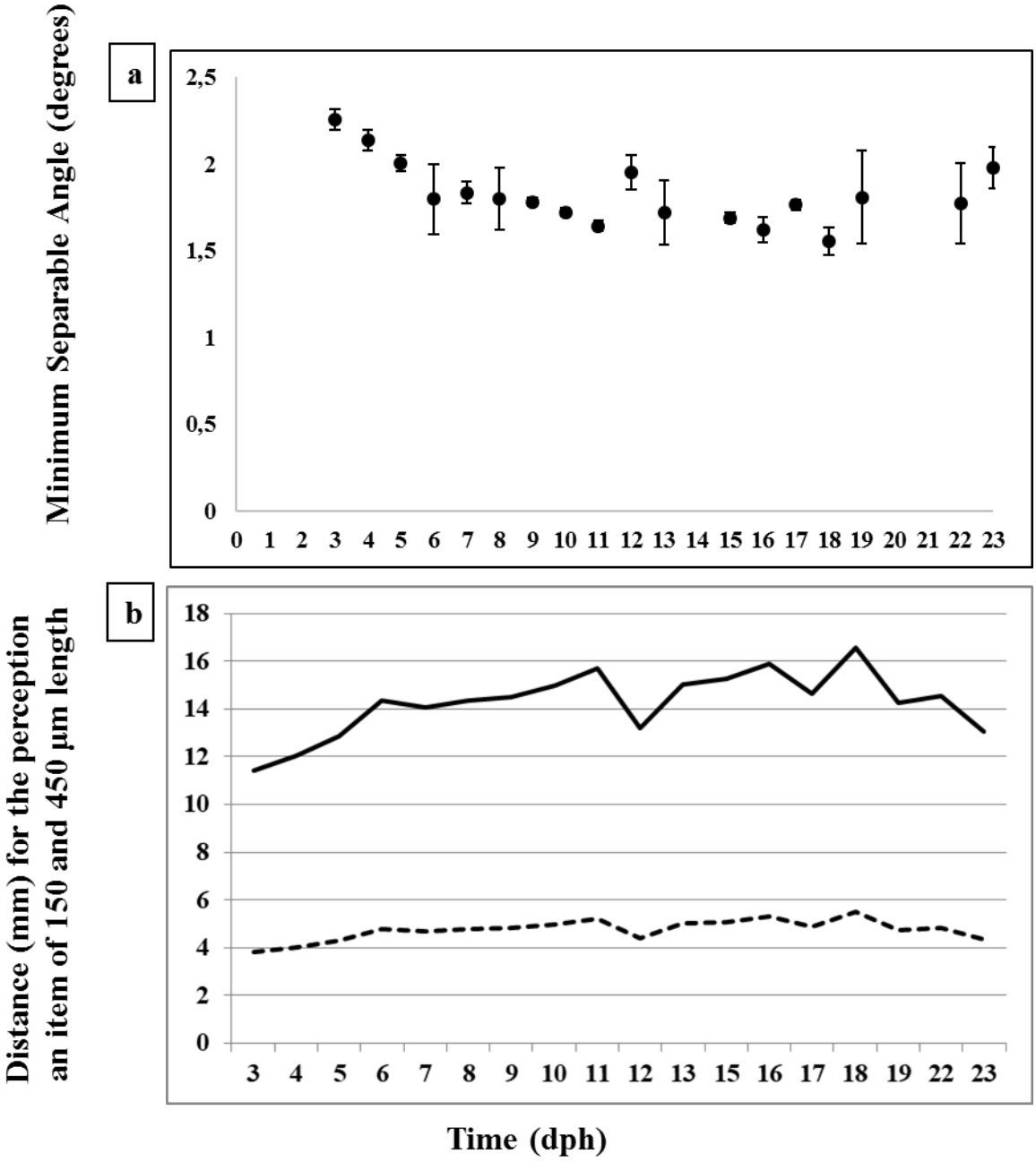

Figure 6. Evolution of: a) MSA (mean ± SE) of wreckfish larvae and b) the distance that wreckfish larvae can see an item of 150 µm (at the size of a rotifer, dashed line) and 350 µm (at the size of *Artemia* nauplii, solid line), in relation to time (dph).

Visual acuity and visual distance

The visual acuity (expressed as minimum separable angle, MSA) decreased until 6 dph and then remained constant with values fluctuating bertween 2 and 1.5 degrees (**Fig. 6a)**. As the MSA decreased the distance that the rotifers and the *Artemia* nauplii could be perceived by the wreckfish larvae, increased (**Fig. 6b**).

DISCUSSION AND CONCLUSIONS

In wreckfish, the ontogenesis of the digestive system is considered as a slow procedure in comparison with other species. 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 exogenous factors, with temperature being one of the most important (Kamler, 2002).

The ontogenesis of the organs related to the digestive and the vision system was not completed until 23 dph. Major structures like the gastric glands or the pyloric caeca, the appearance of which characterizes the time when the development of the digestive system is completed, were not identified in this study. However, the ontogenetic events that occurred in the digestive system, such as the opening of the mouth at 5 dph, the appearance of goblet cells on the esophageal epithelium, the creation of the esophageal folds and the appearance of the pyloric and the cardiac sphincter, suggest that the digestive system of the wreckfish had already developed the ability to manage zooplanktonic organisms at 23 dph. The appearance of the maxillary teeth at 19 dph indicates that the larvae were able to successfully capture a zooplanktonic organism by this age. Moreover, the above indication, that the larvae could be successfully fed, is strengthened by the fact that the visual system of the wreckfish larvae after 6 dph had been developed to such an extent that it could distinguish objects in the rearing environment with the presence of light, since only the cones had developed.

The ontogeny of the retina of the wreckfish 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). Cone cells were the first photoreceptors that appeared. This fact indicates that at this developmental stage wreckfish 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 wreckfish after 5 dph.

Wreckfish visual acuity - the distance the eye can differentiate between two points - 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, the density of the rotifers which are considered the smallest food particle provided in the rearing tank, could be theoretically calculated according the visual abilities of the larvae of wreckfish.

Although both the digestive and the visual system had developed to such an extent that would allow the larvae to feed, we did not detect any food item in the digestive channel, which means that their diet during that period was based exclusively on the reserves of the lecithotrophic sack. The inability of larvae to feed on exogenous food sources resulted in limited growth performance as the endotrophic reserves were not enough to allow further growth.

This resulted in a continuous period of larval malnutrition, which was confirmed by the lipids analysis in the liver. According to the analysis, no lipid depositions were detected in the liver and the total liver tissue showed an additional significant shrinkage, as well. The lipids analysis in the liver is considered as an accurate indicator for the nutritional status of the fish. The malnourishment in fish that generally appears as a reduction of lipid content in the liver cells (Power *et al*., 2000) has been observed to occur during early life stages in other species as well (Papadakis *et al*., 2009). Another indicator which is directly connected with malnutrition conditions is the reduction of intestinal villi (Hall and Bellwood 1995, McLeese, and Moon, 1989), which in the case of the wreckfish larvae appeared after 13 days of rearing and at 23 dph they were not visible any more.

Summarizing the results of this study, it appears that after the first 23 days of rearing, the digestive system and the eye of the larvae was developed to such a degree that by that time fish were, in principle, able to detect, capture and utilize the different types of zooplanktonic organisms. However, the fact that no food items were detected inside the digestive canal leads to the conclusion that the use exclusively of copepods in

feeding protocols is considered inadequate. As concerning the total length of wreckfish larvae, they were found larger in size at hatching, in comparison with common species reared in the Mediterranean area. Wreckfish larvae were also characterized by the large size of the yolk sac, the absortption of which lasts until 20 dph at 17 ± 0.5 ^{0}C. The presence of the large yolk sac and the large oil droplet, indicates the presence of a long autotrophic larval stage. From a hydrodynamic point of view, the large yolk sac and the large oil droplet increase the buoyancy of the wreckfish larvae. This phenomenon is inverted over time as the volume of the yolk sac is reduced by the procedure of yolk sac absorption by the larvae. The high buoyancy may affect negatively the velocity of the larval horizontal movement. The above explains the reason why wreckfish larvae are observed close to the surface of the rearing water during the early stages exhibitibg a relatively small swimming performance (personal opresvation in previous larvar rearing experiments). However, during the autotrophic stage the digestive system and the vision system of wreck fish larvae were developed to such an extent that larvae were able to identify, capture and assimilate zooplanktonic organisms and this should be included to the feeding rearing protocol. The exclusive diet with the copepods harpacticoid *Tisbe battagliai*, which are mainly benthic (Støttrup, 2006), that are away from the hunting habitat of the larvae and the calanoid *Acartia tonsa*, which due to the high speed they develop have a particular ability to escape from larvae (Buskey, et al., 2012), are considered unsuitable for the wreckfish larval rearing feeding protocol. The study of ontogenesis and the formation of the basic systems of the rearing organism, as has been also proved in other cases, seems to be the basis on which the optimization of the protocol of wreckfish larval rearing should be carried out. As the main organs like the gastric glands did not appear until the length of 5.5 mm, a combination of easily captured and more digestible preys as rotifers or different types in different developmental stages of copepods, have to be included in the larval rearing feeding protocol of wreckfish. The above, in combination with the optimization of the rearing conditions, such as the tank hydrodynamics, the temperature protocol during the rearing procedure and the photic conditions in the rearing water, is considered necessary for the development of the wreckfish larval rearing protocol.

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Deviations: The delay in submitting the deliverable is due to the fact that the results of the rearing period of 2017 were anticipated, in order to be introduced to the final report. Eventually, the present report is based only on the results of the 2016 rearing period, since no samples were provided from this year's rearing. Also, on eof the objectives of this deliverable was the description of the changes of the main digestive enzyme activities (proteases, carbohydrases and lipases) during the larval to juvenile digestive system ontogeny. Unfortuntely due to the very limited number of larvae available, as well as the fact than no larvae survived past 28 days post hatching, this task was not accomplished.

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