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PROOF

Morphological, Histological, Histochemical and Behavioral Aspects during Early Development of Red Porgy *Pagrus pagrus* L. Reared in Mesocosm

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Abstract

The present study provides a description of the morphological, histological and histochemical development, and the ecological implications of red porgy larvae reared in mesocosm, from hatching to 30 days after hatching (DAH). Four development stages were defined by body total length (TL), in agreement with major patterns of larval organogenesis. The first stage was characterized by the highest TL relative growth rate (RGR=7.23 % day^{-1}) as larvae elongate their body to escape the culture media viscous forces. RGR decreased in the second stage of development (4.34 % day^{-1}), as larvae opened the mouth and initiated exogenous feeding. Protein inclusions at the posterior digestive epithelium were indicative of pinocytosis and intracellular digestion. Yolk reserves were consumed at slower rate (until 5 DAH) than larvae in intensive culture, although the peak of swim bladder inflation occur earlier (8-11 DAH). A significant decrease in RGR (1.55 % day^{-1}) and the notochord flexion characterized the third stage of larval development. Larvae migrated deeper in the tank (14 DAH) and adopted an aggressive behavior (17-19 DAH). Gastric glands in the forthcoming stomach were common at 20 DAH. In the last stage of larval development the RGR was 3.62 % day^{-1}. A sharp increase of *Artemia* consumption from 26 DAH and cannibalism denoted a change in the digestive mode. Results suggest that red porgy larvae growth and development priorities, and behavior patterns at early stages are directed to increase the number of captured prey.

Keywords: red porgy larvae, larval organogenesis, larvae feeding behavior, larvae cannibalism

Introduction

Red porgy *Pagrus pagrus* is a highly prized fish with a wide distribution in the Western and Eastern Atlantic Ocean and Mediterranean Sea, and has been considered a candidate species regarding the diversification of marine finfish aquaculture (Kentouri *et al.*, 1995; Divanach 2002).

Studies on red porgy larvae ontogeny, particularly of the digestive system and associated organs, have been carried out in laboratory conditions, using small size tanks (0.3 to 1.0m^3), "clean water" techniques (absence of microalgae) and high larvae densities, up to 50 larvae *per* L. The first description of histological development of the red porgy digestive system presented by Roo *et al.*. (1999) revealed a closed association with the visual system, necessary for exogenous feeding. According to these authors, 3 days after hatching (DAH), as yolk-sac reserves are almost depleted, the larvae have the mouth and anus opened and initiated the gut differentiation. At 4 DAH, the eye presumptive cone receptors became well developed and pigmentation completed, coinciding with digestive activity detected in the gut brush border cells. Those authors also described the formation of eye rods by 20 DAH at 7 mm, a significant improvement in the visual capacity and photosensitivity of larvae, and coincident with the appearance of the first gastric glands in the digestive tract.

Darias et al. (2007) have recently demonstrated the synthesis of trypsinogen, the trypsin precursor, and bile salt-activated lipase in the exocrine pancreas of newly hatched red porgy larvae. The presence of digestive enzymes involved in protein and lipid digestion indicates that the preparation of larvae for exotrophic feeding starts well in advance of the capture of prey.

On the other hand, red porgy larvae have a late maturation of the digestive system compared to other sparids, evidenced by: a) first signs of gastric glands at 6 mm on 19 DAH; b) their full development and increased number during 26-30 DAH at 7-8 mm; c) pepsinogen gene expression at 30 DAH; and d) the decrease in gastric pH from 35 DAH (about 9 mm) (Darias *et al.*, 2005). Based on the morphological and

functional development of the red porgy digestive tract it was suggested that *Artemia* or inert diets should be supplied to larvae only from 30 DAH onwards (Darias *et al.*, 2007), eventually a disadvantage for the culture of this species at the hatchery level.

Regarding the swim bladder, an important organ for the hydrostatic balance of larvae, Socorro (2006) observed the differentiation from the gut is initiated at 3 DAH, with a period of higher inflation beginning by 8 DAH, at about 3.9 mm.

Fish larvae development has a strong intrinsic adaptable nature depending on genetic and environmental factors (Gisbert and Doroshov 2006). In culture conditions, numerous studies suggested that gut maturation and related enzymatic activities in fish larvae can be influenced by factors such as temperature (Önal *et al.*, 2008; Zambonino Infante and Cahu 2002), salinity (Moutou *et al.*, 2004; Guiffard-Mena *et al.*, 2006), photoperiod (Elbal *et al.*, 2004), presence of microalgae (Reitan *et al.*,1994) and live food (Kolkovsky *et al.*, 1997), food composition and amount of nutrients (Gatesoupe *et al.*, 1997; Zambonino Infante and Cahu 1999; Cahu *et al.*, 2000; Cahu and Zambonino Infante 2001), onset of exogenous feeding (Gisbert *et al.*, 2004; Sarasquete *et al.*, 1995), weaning (Cahu and Zambonino Infante 1994), developmental stage (Péres *et al.*, 1996), larvae nutritional condition and starvation (Kjørsvik *et al.*, 1991).

In the last decade, mesocosm semi-intensive methodologies have been introduced for red porgy larval rearing (Divanach 2002; Andrade *et al.*, 2010; Roo *et al.*, 2010). This technology has characteristics of extensive culture systems such as the use of large rearing enclosures, providing low larval density (5-10 larvae *per* L), low rate of water renewal and the "green water" technique (addition of microalgae), while the input and control of diets are according to intensive larviculture (Divanach and Kentouri 2000).

Although mesocosm may provide conditions close to field environment (Zouiten *et al.*, 2011) and is considered the most adequate rearing method for red porgy larvae production (Roo *et al.*, 2010), no information is available on the organogenesis of this species under these culture conditions. Therefore, the aim of this work was to describe red porgy larvae morphological, histological and histochemical development (including a first account for lipids) in mesocosm. The major ecological implications, mainly for larvae distribution and feeding, during the first month after hatching are also discussed.

Material and Methods

Egg Incubation and Larval Rearing

The larvae rearing protocol was adapted from Divanach and Kentouri (2000) methods for mesocosm semi-intensive culture. A fiberglass cylinder culture tank of 40 m^3 volume and 2.10 m height was seeded with 320,000 eggs obtained from spontaneous spawnings of wild *P. pagrus* broodstock kept at Centro de Maricultura da Calheta, Madeira Island. Filtered seawater (10 µm) was added daily at a rate of 10 to 150% exchange, starting on 3 DAH. A light regime of 12 hours light, at 2000 lx at the water surface was provided by fluorescent lamps (Roo *et al.*, 2010; Andrade *et al.*, 2013). Phytoplankton (*Nannochloropsis sp.*) was added once daily and the density kept at about $250x10^3$ cell ml^{-1}. The first feeding of larvae was initiated 3 DAH to 30 DAH with enriched rotifers *Brachionus plicatilis* (DHA Protein Selco, INVE Aquaculture, Belgium) and the density kept at 5 rotifers ml^{-1}. From 16 DAH the larvae were fed enriched *Artemia* metanauplii (Protein Selco, INVE Aquaculture, Belgium) three times a day, densities kept from 20 to 300 nauplii l^{-1} and together with dried diets (Lansy, INVE Aquaculture) from 30 DAH to the end of the trial, at 33 DAH. Water parameters were measured once daily. Larvae culture occurred at temperatures and dissolved oxygen of 18.7±0.5 ºC and 7.1±0.6 $mgl^{-}1$, respectively. Salinity was stable at 36±0.5 psu.

Sampling and Methodologies

Fifteen specimens were sampled daily at random from the rearing tank, for morphological, biometrical, histological and histochemical analysis. Deformed or curled larvae were excluded from analysis.

Photographs of the left side of each larva were taken with a SoundVision SV Micro camera mounted on a stereoscopic microscope. Morphological observations and body measurements to the nearest 0.01 mm were performed from the photographs using the software package Zeiss Ks 300. The morphometric characters measured were: larvae total length (TL - the tip of the snout of the lower jaw to the posterior margin of the caudal fin), larvae standard length (SL - from the tip of the snout of the lower jaw to the end of the notochord), larvae height (H - myotome height at anus level), swim bladder maximum length (l_{max}) and maximum height (h_{max}).

A paint mark on the tank wall at every 50cm depth allowed determining the larvae position in the water column.

The inflexion points of the growth curve were determined by iteration procedure according to van Snick *et al.* (1997). Growth coefficients were compared statistically by means of *t*-test ($P< 0.05$) (Sokal and Rohlf 1981). Relative growth rate (RGR, $\%day^{-1}$) between inflexion points was calculated as $(e^{g}-1)x100$, with g=(ln final weight–ln initial weight)/time, following Ricker (1958).

The swim bladder volume (V_{sb}) was calculated by the spheroid equation (Hunter and Sanchez 1976):

$$V_{sb}=4/3\pi(L_{max}/2)(H_{max})^2$$

The ratio of a measurement to the independent variable is often applied to eliminate allometric

effects of body size in morphological analysis (Lleonart *et al.*, 2000). In this study the ratio of the swim bladder volume to larval size (or volume) was used. Larvae volume was estimated as $SLxH^2$ (Hovenkamp 1990), since in red porgy larvae this bivariate measurement has a higher correlation with dry weight than with length (Andrade *et al.*, 2013).

For the histological and histochemical studies the larvae were fixed in Bouin's solution, then dehydrated and embedded in paraffin wax. Sagittal sections of 4-6 µm thickness were stained with haematoxylin-eosin (H&E) for histological study.

Specific histochemical reactions for carbohydrates were: PAS to demonstrate neutral mucosubstances and/or glycoproteins (magenta-stained), and glycogen (Diastasa-PAS), Alcian blue pH 2.5 for acidic (carboxylated and sulphated) mucosubstances, and Alcian blue pH 1.0 and 0.5 for sulphated acid mucosubstances (blue-stained). Bromophenol blue staining was used to detect proteins in general. Histochemical reactions for proteins rich in amino acids were: ninhydrin-Schiff for lysine, Millon's reaction (Hg-sulphate-sulphuric acid-sodium nitrate) for tyrosine, 1,2-Napthoquinone-4-sulphonic acid salt sodium (NQS) for arginine, ferric ferricyanide Fe III for cysteine, and thioglycolatepotassium ferricyanide Fe III for cystine. The staining intensities were evaluated in the sections with a scale of 0 to 3 (0=no staining; 1=weak; 2: moderate; 3=strong). Methods used for carbohydrate, protein and lipid reactions were taken from monographs by Martoja and Martoja-Pierson (1970), Pearse (1985), and Bancroft and Stevens (1990).

Results

Red porgy presented four growth phases defined by three inflexion points at 3 DAH, 12 DAH and 21 DAH (Figure 1). Larval growth phases and behavior were in agreement with major patterns of morphological, histological and histochemical characterization.

Stage I-From Hatching to 3 DAH, at 3.2-3.6 mm TL

During the yolk-sac period the relative growth rate (RGR) at 7.23 % day^{-1} was the highest of the whole study period. Larvae were all stage static at the top 1 m of the water column.

Immediately after hatching, the larvae presented the yolk-sac consisting of a matrix enclosed by a monostratified layer of cuboid cells. One oil globule with positive reaction to Red O (neutral lipids) was present in the acidophilic yolk, becoming a red point by the end of the yolk sac reabsorption at 5 DAH. .

The yolk matrix was diastase PAS-negative (presence of glycogen), 1,2-naphthoquinone-4-sulphonic acid, sodium salt (NQS) positive (with proteins rich in arginine) and reaction positive with mercury sulphate/sulphuric acid/sodium nitrate (proteins rich in tyrosine). There were also positive but weak reactions to ferric-ferricyanide and reduction thioglycolate (with proteins rich in cystine and cysteine). and to *p*-dimethylaminobenzaldehyde (proteins rich in tryptophan) and ninhydrin-Schiff-positive (with proteins rich in lysine) (Table 1).

The outside layer of yolk-sac had positive

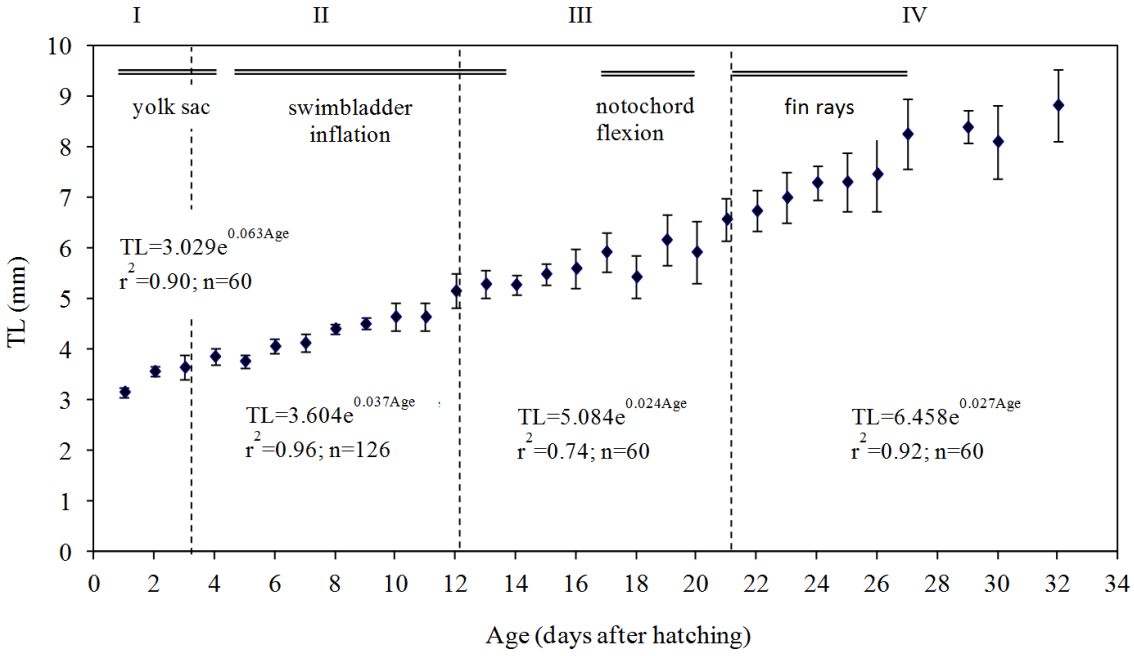

Figure 1. Growth in total length (TL) of *P. pagrus* from hatching to 32 days after hatching (DAH). During this period growth is defined by $TL=3.3983e^{0.0297DAH}$ ($r^2=0.91$; $P<0.05$; n=417). Growth equations presented for different stages of development according to estimated inflexion points, following van Snick *et al.* (1997).

reactions to Nile-blue, PAS-positive concanavallin A, indicating the presence of phospholipids, neutral mucosubstances and glycoproteins, respectively. A granular acidophilic zone was observed between the yolk sac and larval body with affinity to orange G and light green that are precursor cells of liver and a basophilic zone, with precursor cells of pancreas.

Primordial gills are detected at 2 DAH (Figure 2) at the time the posterior part of digestive tube opened, forming the anus. By the third day after hatching the digestive tract presented an increase in length and folding. Simultaneously, the digestive tract becomes differentiated in 4 segments: esophagus, anterior intestine, intermediate and posterior intestine (Figure 3). The primordial swim bladder initiated differentiation from the dorsal wall of the digestive tube (Table 2).

Stage II - From 4 DAH to 12 DAH, at >3.6-5.0 mm TL

Larval growth was almost inexistent during the first 5 DAH. Still, the average value of RGR for stage II was 4.34 % day^{-1}.

Larvae were active and began external feeding. One of the most obvious morphological events at this phase was the swim bladder development and inflation. By 8 DAH at 4.2 mm TL, about 92% of the

Table 1. Histochemical distribution of proteins, carbohydrates and lipids of *Pagrus pagrus* larvae development. The values separated by "-" represent the variation of the color intensity observed in the structures during the first month of larval life.

	Yolk sac/ Oil globule	Liver	Pancreas	Brush border	Esophagus epithelium	Intestine epithelium		Mucous cells	
						Anterior	Posterior	Esophagus	Intestine
Proteins in general	2	2	3	2-3		2-3	2-3	0-1	0-1
Proteins rich in tryptophan	0-1	1	1	1	0-1	1	1	0	0
Proteins rich in tyrosine	2	2	3	1-3	1-2	1-2	1-2	0-2	0
Proteins rich in arginine	2	1	2	1-2	1-2	1-2	1-2	0-2	0
Proteins rich in lysine	0-1	1	2	1-2	1-2	1-2	1-2	1-2	0
Cysteine residues	0-1	1	1	1	1	1	1	0-3	0-3
Cystine residues	0-1	1	1	1	1	1	1	0-1	0-3
Neutral mucosubstances	0-1	0	1	1	0-1	0-1	0-1	1-3	0-2
Carboxylated mucosubstan.	0-1	1	1	2	0-1	0-1	0-1	1-3	0-3
Sulphated mucosubstances	2	1	1	2-3	0-1	0-1	0-1	0-3	0-3
Glycogen	0-1	1	0	0	0	0	0	0	0
Neutral lipids	0-2	2	2	1-2	2-3	2	3	0-3	0-3
Neutral and acid lipids	0-2	2-3	3	3	2-3	2-3	2-3	0-1	0-1

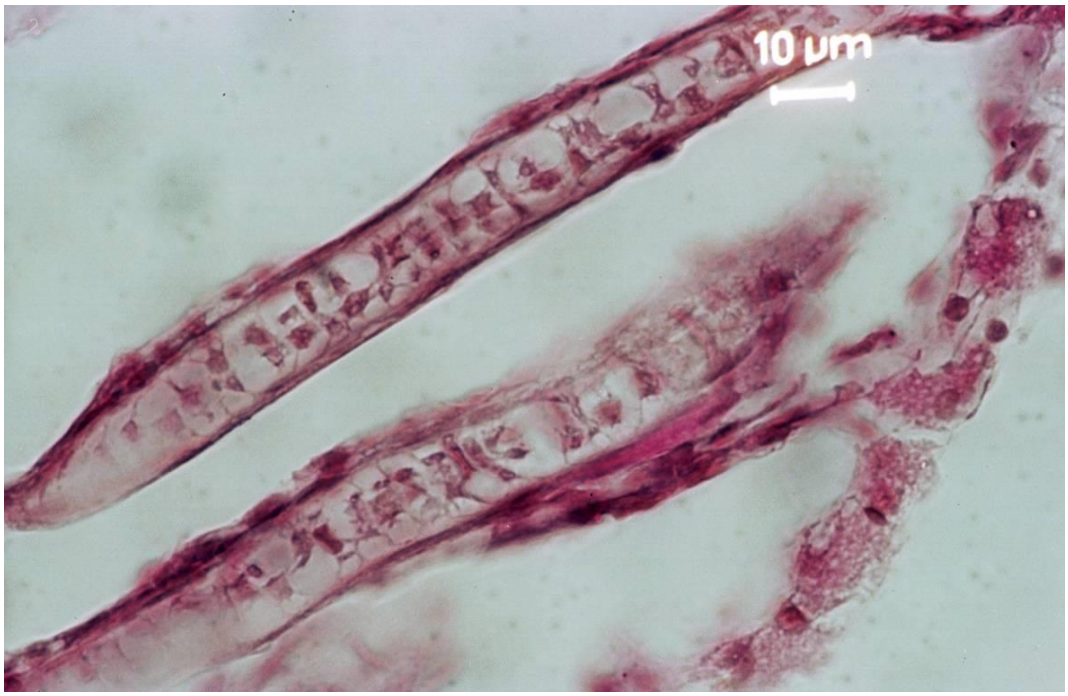

Figure 2. Branchial archs of *Pagrus pagrus* larvae at 3 days after hatching (H&E). Scale bar 10 µm.

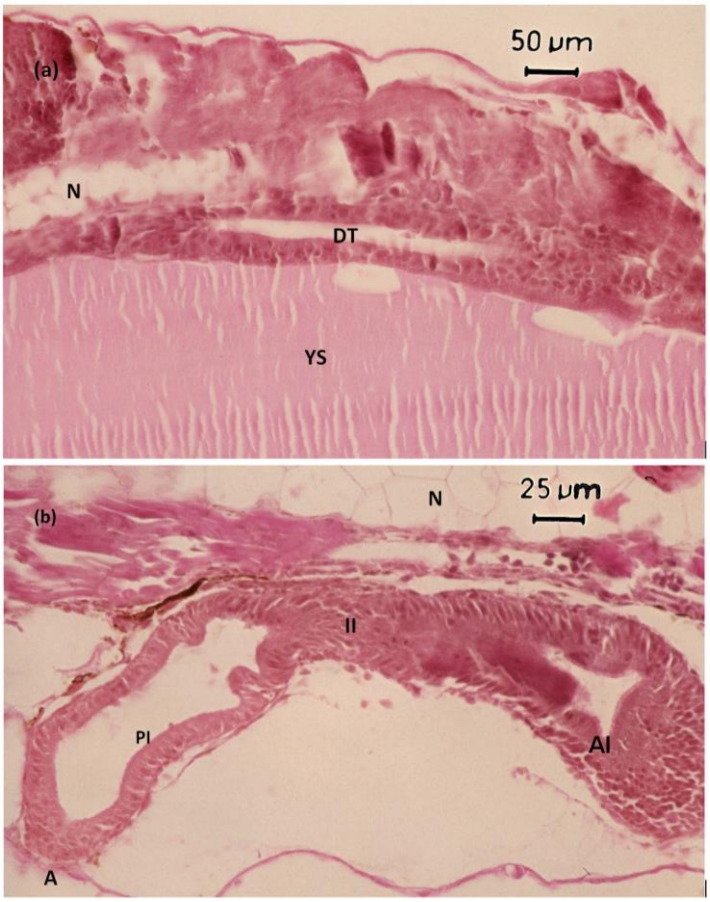

Figure 3. Histological appearance of the digestive tract in red porgy larvae from hatching (a) to 3 days after hatching (b) (H&E). Scale bars 50 µm (a) and 25 µm (b); N = notochord; DT = digestive tract; YS = yolk sac; AI = anterior intestine; II = intermediate intestine; PI = posterior intestine; A = anus.

Table 2. Histochemical distribution of proteins, carbohydrates and lipids of *Pagrus pagrus* swim bladder. The values separated by "-" represent the variation of the color intensity observed in the structures during the first month of larval life

	Gas Gland	*Rete mirabile*	Epithelial layer
Proteins in general	3	1	1
Proteins rich in tryptophan	1	0	1
Proteins rich in tyrosine	2	2	1
Proteins rich in arginine	2	1	1
Proteins rich in lysine	2	1	1
Cysteine residues	2	1	0
Cystine residues	2	1	1
Neutral mucosubstances	1	1	1
Carboxylated mucosubstances	1	1	1
Sulphated mucosubstances	3	2	2
Glycogen	0	0	0
Neutral Lipids	2	1	1

larvae had initiated swim bladder inflation (Table 3). Between 8 and 11 DAH (4.2-4.6 mm TL) the swim bladder volume had the most considerable increment, reaching 3.4 times the previous relative size. During this period, while larval body volume had a considerable geometric increment, TL had a low linear increase.

This was a stage of major changes at the histological and at histochemical level. Although most larvae opened mouth at 3 DAH, the dissection of the digestive tube revealed that only a few had ingested rotifers. A diminished yolk-sac was still visible at 5 DAH. The eyes attained a larger size at 4 DAH, while at the tissue level there were an increasing number of cone cells. The first pharyngeal and mandibular teeth were apparent at 4 DAH.

The first digestive mucous cells were detected containing neutral and/or acid mucosubstances (Table 1). Until 9 DAH (about 4.5 mm TL) stronger reaction was observed at the esophagus. At this larval age the

Table 3. *Pagrus pagrus* larvae size in total length (mm±s.d.) and volume (mm^3±s.d.), swim bladder volume (mm^3±s.d.) and ratio swim bladder to larvae volumes, from 5 to 17 days after hatching (DAH)

	DAH				
	5 (n=12)	8 (n=14)	11 (n=14)	14 (n=14)	17 (n=14)
Larvae size					
TL (mm±s.d.)	3.67±0.14	4.40±0.10	4.64±0.28	5.27±0.20	5.92±0.38
Volume (mm^3±s.d.)	0.81±0.04	1.67±0.50	4.29±0.54	7.86±2.40	10.43±3.38
Swim bladder					
% larvae inflated	33	92	100	100	100
Volume (10^{-3} mm^3±s.d.)	0.26±0.11	0.66±0.06	6.36±3.03	17.22±5.20	29.94±5.48
Swim bladder / larvae size (volumes; 10^{-3})	0.32±0.13	0.42±0.14	1.44±0.59	2.23±0.41	3.02±0.83

epithelium of the anterior intestine contained proteins rich in lisine (stained positive with ninhydrin-Schiff) and there was an increase of proteins rich in tyrosine (stained positive with mercury sulphate/sulphuric acid/sodium nitrate). Reaction to protein rich lysine decreased at 30 DAH, about 8 mm TL.

In regard to the associated organs of the digestive system, by 4 DAH the presence of glycogen (stained with PAS, Diastasa-PAS) and neutral and acidic lipids (stained by Nilo Blue) was evidenced in the liver, whereas proteins, stained by bromophenol blue were more intense in exocrine pancreas. The gall bladder was functional at this age.

Stage III-From 13 to 21 DAH, >5.0-6.4 mm TL

Larval TL growth was the lowest registered during this study resulting in a RGR of 1.55 % day^{-1}.

By 14 DAH (5.4 mm TL) conspicuous larger size larvae moved deeper (2-2.5 m) and stayed along the tank wall, leaving the top water layer (1 m) where the majority of the population resided. Aggressive behavior including fin nipping characterized larvae behavior at 17-19 DAH. With notochord flexion at 17-20 DAH to the end of this developmental stage larvae become lethargic presenting low feeding activity.

Notochord flexion was one the most obvious morphological differentiations at this stage, occurring from 17 to 20 DAH within a TL range of 5.7-6.0 mm. At this stage most organs exhibited an increase in tissue structure and number (Figure 4). At the histological level the occurrence of gastric glands at 19 DAH was the most relevant event.

Stage IV-From 22 to 33 DAH, >6.4 mm TL

The RGR for this period was 3.62 %day^{-1} and larvae presented high size heterogeneity.

Larvae behavior was characterized by a sudden increase in swimming activity and food consumption from 25-26 DAH, exhibiting about 2-3 fold the *Artemia* consumed in previous period. Cannibalism with full engulfment of prey occurred from 28 DAH (about 8.2 mm TL) onwards. Smaller larvae tended to aggregate at the margins of the tank. From 21 to 27 DAH, the finfold gives place to pairs of fin rays.

In the forthcoming stomach area the intestinal mucosa evidenced an increase of folding and the number of gastric glands. In the posterior intestine supranuclear vacuoles rich in proteins, carbohydrates (including glycogen) and particularly lipids were observed from an early stage, increasing in number and becoming abundant by 30 DAH (Table 1).

Discussion

Larval Growth in Mesocosm and Development of the Digestive System

Larval growth in TL in this study was comparable to that previously reported for semi-intensive mesocosm and intensive rearing methodologies by Roo *et al.* (2010), despite the warmer (+1°C) rearing water mean temperature. Larvae were 5.5 % and 16.3 % bigger in TL mean sizes, respectively, at 20 DAH and 30 DAH when compared to values for *P. pagrus* reported by Darias *et al.* (2007) at similar temperatures, under intensive rearing (Table 4).

Red porgy growth patterns reflected the differential growth of body parts, which is a function of the organ and tissue development, and the nutrients and energy allocated for growth.

At yolk-sac stage larvae experienced the fastest growth of the several developmental phases. Newly hatched red porgy larvae presented undifferentiated gut and accessory organs. Larvae relied on the yolk sac reserves rich in proteins, neutral and acidic lipids and carbohydrates to fulfill their energy and nutrient requirements.

At the start of the exogenous feeding, 2nd stage of development, growth was almost nil until 5 DAH where larvae were consuming their endogenous reserves. Once a significant part of the population succeeds in catching, ingesting and digesting prey, growth was promoted for the first time (Yúfera and Darias 2007).

The differentiation of the gut occurred at 3 - 4 DAH, being concomitant with the mouth and anus

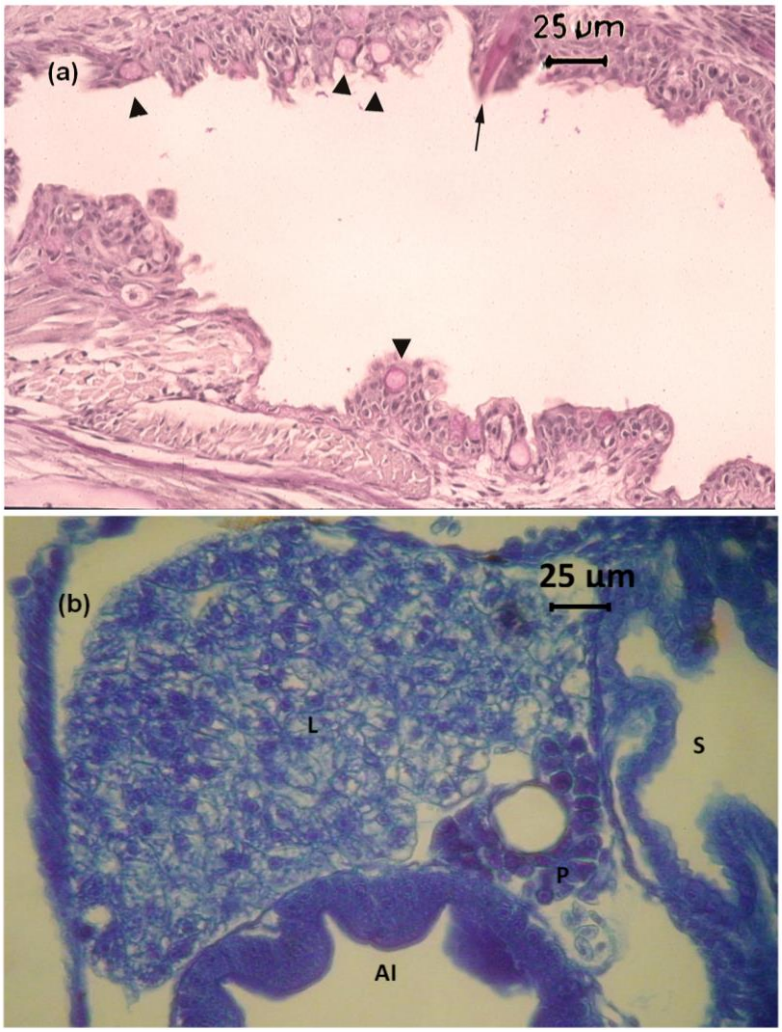

Figure 4. Digestive tract of *Pagrus pagrus* larvae at 22 days after hatching. Tooth (arrow) and mucous cells ( ) at buccal cavity (H&E) (a). Stomach, anterior intestine and accessory digestive organs (Bromophenol Blue) (b). Scale bars 25µm; S = stomach; L = liver; P = pancreas; AI = anterior intestine; E = esophagus.

Table 4. Larval growth and development parameters for *Pagrus pagrus* from hatching to 30 days after hatching (DAH), in this study *versus* intensive culture conditions (Darias et al. 2007)

Parameter	Mesocosm	Intensive culture
Growth rate (% TL day^{-1})	2.97	2.59
Length at transformation to juvenile (mm - DAH)	7.13-25	7.12-30
Mouth and anus opening (DAH)	3	3
Yolk-sac resorption	5	3-4
Swimbladder inflation (DAH)	8-14 (8-11 maximum inflation)	12-15
Mucous cells (DAH)	6-11	6-15
Gastric glands (DAH)	19	19
Intestinal folding (DAH)	23	-
Fin differentiation (DAH)	21-27	-

opening, and the establishment of exogenous feeding, similarly to that reported for intensive rearing (Roo *et al.*, 1999; Darias *et al.*, 2007). Larvae still presented a tenuous yolk-sac at 5 DAH, whereas in intensive rearing the yolk-sac was adsorbed 3 DAH, at 21.5±0.5 °C (Roo *et al.*, 1999) and 4 DAH, at 19.5±0.5 °C (Darias *et al.*, 2007). A delayed consumption of yolk-sac reserves for two distinct sparid larvae reared in mesocosm was previously reported by Papandroulakis *et al.* (2004b). Probably, fish larvae require less energy for locomotion in the low hydrodynamics conditions of mesocosm rearing tank compared to intensive culture.

Esophageal epithelium cells developed rapidly

with the transition from endogenous to exogenous feeding, producing neutral mucous from 3 DAH. These secreted mucins were suggested to protect the mucosa against bacteria and physical or chemical damage (Allen 1989).

Larvae had an incipient non-functional stomach at this early stage. They rely mainly on pancreas and intestine for the digestion and absorption of nutrients, with pinocytosis and intracellular digestion as the main mechanisms for protein absorption (Govoni *et al.,* 1986). The high occurrence of protein supranuclear inclusions was indicative of both these processes taking place at the epithelium cells of the posterior intestine. This type of inclusion is also observed in the posterior intestine of *S. aurata* (Sarasquete *et al.,* 1995), of *Pseudosciaena crocea*, a scianid (Mai *et al.,* 2005) and of cod *Gadus morhua* (Kjørsvik *et al.,* 1991). Increased folding of intestinal mucosa will increase absorption area, enhancing the mixing of digestive juices (Grau *et al.,* 1992; Arellano *et al.,* 1999) and nutrient absorption.

Neutral lipid vacuoles observed in the epithelial cells of the mucosa of red porgy from 3 DAH onwards, suggest that the anterior intestine was involved in the absorption and storage of lipids, as previously reported for cod fish larvae by Kjørsvik et al. (1991). Live prey are normally enriched with lipid emulsions to fulfill fish larvae requirements of essential fatty acids, one of most important factors affecting growth, survival, neural tissue development (Izquierdo 1996) and vision (Benítez-Santana *et al.*, 2007). Previous studies have demonstrated that red porgy larvae in particular have high requirements of docosahexaenoic acid at early stages and shortages of this essential fatty acid may originate skeletal anomalies (Roo *et al.*, 2009). Lipid vacuoles were present in hepatocytes from 6-7 DAH, reflecting fish larvae ability to store energy, and their number increased with larval growth/development.

Most larvae initiated swim bladder inflation within 5 and 8 DAH achieving 100 % inflation, similarly to previous reports for this species using intensive rearing methods (Mihelakakis *et al.,* 2001). A peak of swim bladder relative volume increase occurred between 8 and 11 DAH (maximum), four days in advance of that observed by Darias *et al.,* (2007). This allows mesocosm reared larvae at earlier stages to improve locomotion capacity and to increase their ability to capture live feed.

A significant decrease in growth rate and the notochord flexion characterize the 3rd phase of larval development. Larvae lethargic behavior towards the end of this phase translates the priority mobilization of energy to the dramatic increase in size and complexity of most body tissues and organs.

The presence of neutral mucosubstances within cells and brush border of anterior and posterior digestive epithelium preceded the development of gastric glands at 20 DAH, Both events occurred about the same larval age reported in intensive culture by Darias *et al.* 2007 (Table 4). The mucosubstances' most important role is to protect the digestive mucosa from the hydrochloric acid and enzyme secretions to be produced by gastric glands (Hachero-Cruzado *et al.,* 2009).

A slight inflection in larvae TL growth characterizes the final (4th) phase of larval development. Larvae evolve towards the external morphology of a juvenile fish, particularly with fin ray differentiation occurring from 21 to 27 DAH, 6.5 to 8.2 mm. In intensive culture conditions at mean temperature 1-2ºC higher than in this study, the transformation is reported to occur between 23 to 32 DAH, about 8.57 to 10.28 mm TL (Mihelakakis *et al.,* 2001).

Stomach gastric glands increased in number until the end of this study (30 DAH). Based on the morphological development of gastric glands and the detection of pepsinogen expression at this age, Darias et al. (2007) considered that larvae acquire the acid digestion mode and suggested the introduction of *Artemia* in larval diet.

In our trial, 25-26 DAH (about 7.3 mm TL) larvae presented a sharp rise in *Artemia* consumption, followed by cannibalism of conspecific larvae from 28 DAH (8.2 mm TL). Although the choice for larger prey may be related to larval size, the apparent full digestion of both *Artemia* and larvae suggest the onset of acidic digestion.

Supranuclear vacuoles were abundant in the enterocytes of posterior intestine until the end of this larval stage. Lipids seemed to be absorbed mainly by the posterior intestine, similarly as described for turbot *Scophthalmus maximus* (Koven *et al.,* 1994), for Senegal sole *Solea senegalensis* (Morais *et al.,* 2006) and brill *Scophtalmus rhombus* (Hachero-Cruzado *et al.,* 2009). In *S. aurata* larvae the supranuclear vacuoles observed are of protein nature (González de Canales García *et al.,* 1997). The above-mentioned physiological changes occurred with a concomitant increment of body reserves (glycogen and neutral lipids) at liver level.

Setting of Development Priorities in Red Porgy Larvae

The acquisition of an acidic mode of digestion by 30 DAH red porgy larvae marks the transformation to juvenile stage (Darias *et al.,* 2007). This enhances fish larvae digestive capacity and broadens the type of food larvae are able to digest. This might explain the increasing consumption and full digestion of *Artemia*, as well as the occurrence of cannibalism by larger and juvenile-like fish at late 4th developmental phase.

In red porgy larvae the digestive system maturation is a long transitional period (from 19-20 to about 30 DAH) and arguably occurring later in their ontogeny than in most other sparids (review by Yúfera *et al.,* 2011). Considering that fish larvae growth and developmental patterns are set according

to priority functions and prepare them for future needs (Osse *et al.*, 1997ab), red porgy larvae will have to establish a sequence of developmental and behavior patterns to cope with the high energy demands from metamorphosis, evidenced by a slow growth rate (stage 4 of development).

At yolk-sac stage, red porgy larvae registered the fastest growth rate in length from the larval period. An elongated body increases the Reynolds number (a ratio between inertial and viscosity forces), diminishing the effects of friction (Müller and Videler 1996; Osse and van den Boogaart 1999). Consequently, energy losses for larvae locomotion will be minimized and swimming performance improved. In fact, Andrade et al. (2011) reported that red porgy larvae at early stages of development, from 3 DAH to 4 DAH, were able to increase by 55% their swimming speed. According to these authors, the simultaneous 20% increase in mouth gape over the same period of time prepares larvae to prey capture.

The most striking feature in the second stage of development is the swim bladder significant increase in volume, to about 1.4 times that of *S. aurata* larvae of same size (Soares, 1994). Improvements in larvae displacement in the water column are expected with ecological relevance, particularly for larvae reared in mesocosm. Larvae will be able to explore the variety of habitats and new foraging areas of the large mesocosm tanks, lowering conspecific competition for food.

Simultaneously, larvae were strengthening their musculature with a noticeable increment of myotomal height, followed by notochord flexion at 17 DAH, towards the end of the 3rd stage of development. Both morphological transformations are referred by Blaxter (1988) to increase fish larva swimming performance and provide energy savings. Consequent improvement in prey capture performance may explain cultured red porgy larvae sharp increase of rotifer consumption rates, up to 4 times those of *S. aurata* larvae, from about 14 DAH to 20 DAH (Papandroulakis *et al.*, 2004a).

Finally, regarding the locomotion organs, finfold differentiation to unpaired fins occurred from 21 to 27 DAH (about 6.5-8.2 mm TL). According to Fukuara (1985) fins and musculature raise exponentially the swimming speed of *Pagrus major* larvae. The enhanced swimming capabilities provided by fins will enable larvae to cover a wider area and to improve prey capture efficiencies (Gisbert *et al.*, 2002; Sala *et al.*, 2005). Moreover, from 20 to 30 DAH there is a significant improvement in red porgy larval vision with the rod photoreceptors development (Roo *et al.*, 1999), which is likely to increase prey detection under low light intensity, in concordance with the observation of larvae migration from the surface to the bottom of the tanks.

Red porgy larval digestive system has to adapt to the higher levels of ingested food. Pancreatic enzymes lipase and trypsin, involved in lipid and protein digestion respectively, are reported to have a sharp increase of specific activity between 5-7 mm TL (Suzer *et al.*, 2007), about 12-23 DAH in our study. In addition, the anterior intestine ought to add area for food-enzyme mixing and uptake of nutrients, as it was observed with increasing intestine folding and length by 23 DAH. An increasing number of supranuclear vacuoles rich in carbohydrates, proteins and particularly lipids were observed at the posterior intestine, suggesting a nutrient shift to provide energy for growth.

From early 4th stage of development, the liver presented increasing body reserves denoting that larvae have assembled the development steps to succeed in the detection, capture and ingestion of prey, and stored the energy needed for transformation to juvenile.

Conclusions

In advance of a demanding transformation to juvenile stage, red porgy larvae seem to have set a sequence of developmental events to favor the number of captured prey and their assimilation. The larvae only switch to a novel and more efficient digestion after fin development. This is in agreement with a previous suggestion that fish larvae have priorities established in their life history in order to develop swimming and feeding organs to escape predation and starvation (Osse *et al.*, 1997a).

Larval TL growth and ontogenetic development in red porgy reared under mesocosm of semi-intensive methodologies were faster compared to those of larvae from intensive rearing methods. Mesocosm-rearing methodologies promoted the delay in the consumption of larval yolk-sac reserves and a precocious swim bladder inflation that may broaden the energy conservation and ecological strategies, and improve larvae performance.

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