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Larval organogenesis of *Pagrus pagrus* L., 1758 with special attention to the digestive system development

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Summary. Organogenesis of the red porgy (*Pagrus pagrus* L., 1758) was examined from hatching until 63 days post-hatching (dph) using histological and histochemical techniques. At hatching, the heart appeared as a tubular structure which progressively developed into four differentiated regions at 2 dph: bulbus arteriosus, atrium, ventricle and sinus venosus. First ventricle and atrium trabeculae were appreciated at 6 and 26 dph, respectively. Primordial gill arches were evident at 2 dph. Primordial filaments and first lamellae were observed at 6 and 15 dph, respectively. At mouth opening (3dph), larvae exhausted their yolk-sac reserves. The pancreatic zymogen granules appeared at 6 dph. Glycogen granules, proteins and neutral lipids (vacuoles in paraffin sections) were detected in the cytoplasm of the hepatocytes from 4-6 dph. Hepatic sinusoids could be observed from 9 dph. Pharyngeal and buccal teeth were observed at 9 and 15 dph, respectively. Oesophageal goblet cells appeared around 6 dph, containing neutral and acid mucosubstances. An incipient stomach could be distinguished at 2 dph. The first signs of gastric gland development were detected at 26 dph, increasing in number and size by 35-40 dph. Gastric glands were concentrated in the cardiac stomach region and presented a high content of protein rich in tyrosine, arginine and tryptophan. The intestinal mucous cells appeared at 15 dph and contained neutral and acid glycoconjugates, the carboxylated mucins being more abundant than the sulphated ones. Acidophilic supranuclear inclusions in the intestinal cells of the posterior intestine, related to pynocitosis of proteins, were observed at 4-6 dph.

Key words: Gut development, *Pagrus pagrus*, Red porgy, Histology, Histochemistry, Fish larvae

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Introduction

Generally, teleosts present at hatching an undifferentiated digestive tract without any aperture to the exterior which develops in the following days to be ready for digestion at the beginning of the exogenous feeding. Meanwhile, energy needed for the metabolism of maintenance, swimming activity and growth is obtained from the yolk-sac reserves. In carnivorous species, proteins are the main dietary component that is firstly digested by alkaline proteases secreted by the exocrine pancreas (Kurokawa and Suzuki, 1996) and by intracellular proteases of the enterocytes (Zambonino-Infante and Cahu, 2001). A more efficient acidic protein digestion is carried out in the stomach when gastric glands are fully developed and synthesize pepsin, which is the main proteolytic enzyme acting at the end of the larval period and supposes the switch to the adult mode of digestion (Moyano et al., 1996; Douglas et al., 2000; Gawlicka et al., 2001; Zambonino-Infante and Cahu, 2001; Yúfera et al., 2004). It is assumed that fish larvae digestive efficiency is related to the presence or lack of gastric glands and to the proteolytic action of pancreatic juice but, interestingly, it also depends on goblet cell secretion (Kapoor et al., 1975; Sarasquete et al., 2001). It is well known that acid and/or neutral mucosubstances play an important role in several food-processing activities, such as lubricant or protective function, in osmoregulation, and possess a crucial role in regulating the interactions between cells and their microenvironment that are of great importance for the maintenance of differentiated cellular function. Even, for instance, some sulphated mucins have been identified as an important constituent of the mitogenic stimulus controlling cell growth (Gallagher et al., 1986; Parillo et al., 2004). The high mortality percentage obtained during the larval and fry stages of some fish species when they are intensively reared in aquaculture may be due to an incomplete secretory activity of the gut (Domeneghini et al., 1998).

In spite of all this knowledge, there are inter-specific

variations in the timing of organ formation and maturation. Therefore, it becomes necessary to carry out specific organogenesis studies for each species to optimise fish larval rearing techniques.

Red porgy (*Pagrus pagrus*) is a marine teleost, belonging to the Sparidae family, with tropical and subtropical amphiatlantic distribution, specially located in the Mediterranean coasts, where it presents a high commercial value. This species is considered a good candidate to contribute to fish farming expansion. However, few studies have been made on its larval development (Kolios et al., 1997; Hernández-Cruz et al., 1999; Roo et al., 1999; Mihelakakis et al., 2001; Darias et al., 2005, 2006), which is the base of the rearing process to obtain a stable production. The present work aimed to analyse larval organogenesis of the red porgy using histological and histochemical techniques in order to determine the degree of morphological development and functionality of the digestive organs, which is correlated with the digestive enzyme ontogeny (Oozeki and Bailey, 1995; Hidalgo et al., 1999; Martínez et al., 1999; Ribeiro et al., 1999a,b; Gawlicka et al., 2000; Kim et al., 2001; Rojas-García et al., 2001). Thus, an adequate food supply according to their digestive capacity can be established based on this knowledge in order to optimise survival and growth during the larval period.

Materials and methods

Rearing conditions and larval growth

Eggs of red porgy were obtained by natural spawning from a captive brood stock maintained at INIAP/IPIMAR (Olhão, Portugal). Newly hatched larvae were transferred to three 300 L tanks with flow-through water supplied at a constant temperature of 19.5±0.5°C and salinity of 33. Constant illumination was provided during the first two weeks switching to a photoperiod of 12L:12D afterwards. Initial larval density ranged from 30 to 50 larvae L^{-1}. The first 24 h post hatching was considered as day 0. Larvae were initially fed (3 dph) with rotifers (*Brachionus plicatilis*) at 10 mL^{-1} (fed with *Nannochloropsis gaditana*) and then gradually replaced by *Artemia sp*. nauplii, metanauplii and adults at 2 mL^{-1} from 23 dph onwards. A daily dose of microalgae (*N. gaditana*, at $3x10^5$ cells ml-1 initial concentration) was added to the rearing tanks during the first month. Groups of 30 larvae (0-60 dph) were sampled from each tank, euthanized with 200 mg L^{-1} of ethyl-4-amino-benzoate and total length (TL) was measured under stereomicroscope.

Histological and histochemical procedures

Larvae and post-larvae were sampled in triplicate (three tanks) from hatching until 63 dph (0, 1, 2, 3, 5, 6, 9, 12, 15, 16, 19, 22, 23, 26, 30, 40, 50, 63 dph), fixed at 4°C in buffered formaldehyde (pH 7.2) overnight, dehydrated through ethanol series, infiltrated and embedded in paraffin blocks. Each block contained a pool of larvae, the number of which decreased according to the larval size (from 10 larvae at 0 dph to 1 larva at 63 dph). Serial saggital and/or transversal 7 μm thick sections from whole specimens were placed on uncoated glass slides and baked overnight at 40°C. Subsequently, sections were stained with Haematoxylin-Eosin (H-E). Histochemical techniques were performed for identification of carbohydrates (glycogen, mucosubstances and/or glycoconjugates) (Table 1) and proteins rich in different aminoacids (Table 2). All methods and techniques used in this work were taken from Pearse (1985) and Bancroft and Stevens (1990) monographs. Slides were examined and photographed with light microscope (Leitz Etzlar 307).

Results

Larval growth of the red porgy, in terms of total length, during the experimental period is represented in Fig. 1. The main ontogenetic landmarks of the red porgy digestive system are shown in Fig. 2.

Yolk-sac larvae

After hatching, yolk-sac is formed by a squamous epithelium surrounding a homogeneous and acidophilic yolk-matrix (Fig. 3A) which contains neutral glycoconjugates, glycogen and proteins rich in tyrosine, arginine, lysine, cysteine and cystine (Table 3). At 2 dph, a pronounced decrease of the yolk-sac volume was observed, showing a fragmentation of the strongly acidophilic yolk-matrix (Fig. 3B). Yolk-sac reserves

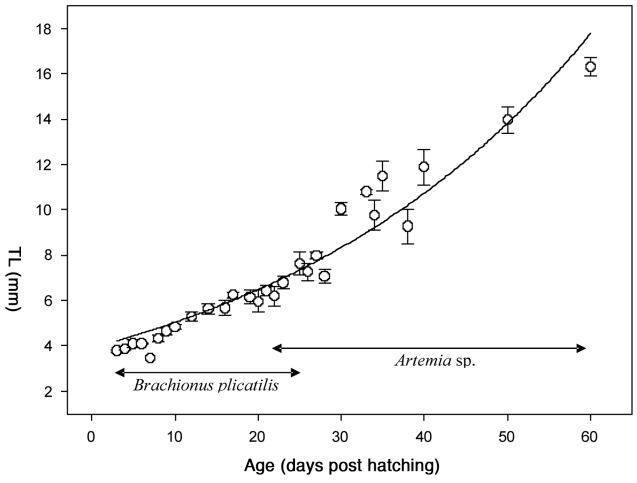

Fig. 1. Red porgy larval growth expressed in terms of total length. The sequence of food supply (*Brachionus plicatilis* and *Artemia sp.*) is indicated by horizontal arrows under the curve. Data were adjusted to an exponential regression ($TL = 3.91\ e^{0.02\ T}$; TL = total length, T = days post hatching; r = 0.96).

Table 1. Histochemical reactions of carbohydrates analysed in red porgy larvae.

Reactions	Functions and/or demonstrated compound
Periodic acid-Schiff/PAS	Aldehydes from the oxidation of glycol or aminool contiguous groups
Diastase-PAS	Glycogen or neutral glycoconjugates
Alcian Blue pH 2.5	Carboxylated mucosubstances or glycoconjugates (sulphated or not)
Alcian Blue pH 1	Carboxylated glycoconjugates sulphated slightly ionized
Alcian Blue pH 0.5	Glycoconjugates sulphated strongly ionized
Esterification / Alcian Blue pH 2.5	Carboxylated glycoconjugates blockage
Esterification / Saponification / Alcian Blue pH 2.5	Carboxylated glycoconjugates reactivation
Esterification / Alcian Blue pH 0.5	Glycoconjugates sulphated loss (sulphatolysis)
Esterification / Saponification / Alcian Blue pH 0.5	Not reactivation of sulphated glycoconjugates (lack of alcianophilia)
Acid hydrolysis-Alcian Blue pH 2.5	Sialic acid extraction (breakdown of glycosides linkages)

Table 2. Histochemical reactions of proteins analysed in red porgy larvae.

Reactions	Functions and/or demonstrated compound
Bromophenol Blue	general proteins
Ninhydrin-Schiff	proteins rich in lysine (-NH2)
Ferric ferricyanide-Fe III	-SH/cysteine protein rich
Thioglycolate reduction	-S-S-/cystine protein rich
1,2 napthoquinone-4-sulphonic acid salt sodium	proteins rich in arginine
Hg-sulphate-sulfuric acid-sodium nitrate	proteins rich in tyrosine
P-dimethylaminobenzaldehyde	proteins rich in tryptophan

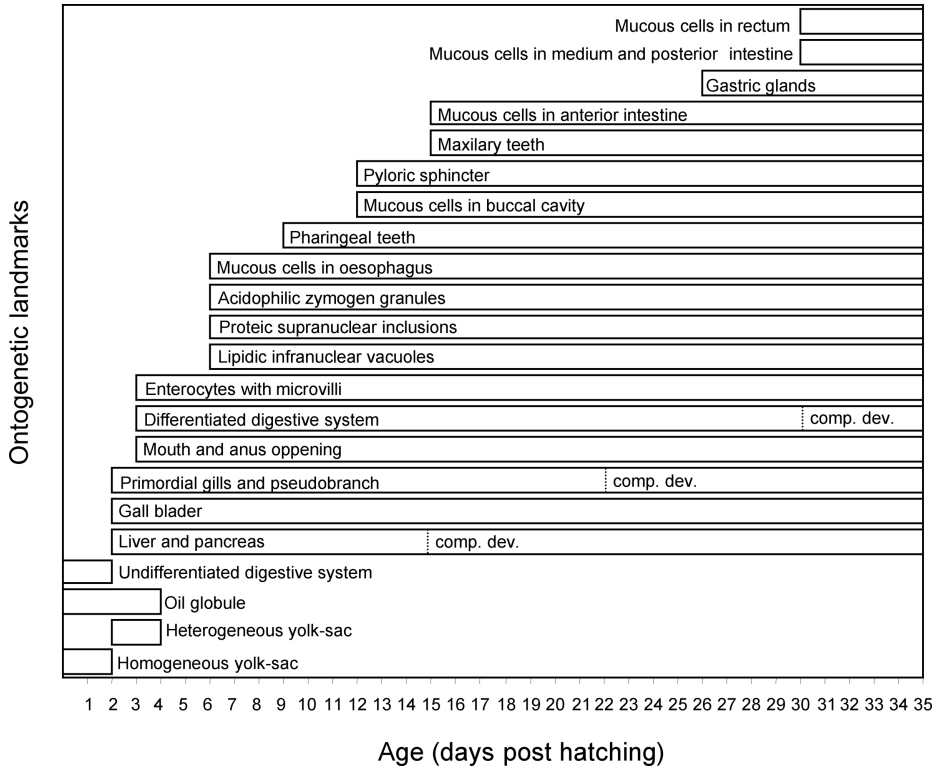

Fig. 2. Diagram showing the main histological ontogenetic landmarks during the larval development of the red porgy. Dotted lines indicate the age at which the corresponding structure is completely developed (comp. dev.) at histological level and only exhibited an increase in number and size of cells and tissues, respectively.

were completely exhausted at 3 dph.

Newly hatched larvae presented a rectilinear duct that curved in the caudal portion, localized dorsally to the yolk-sac, the lumen of which distended in both extremities without aperture to the exterior. The epithelium of the digestive tract was constituted of squamous cells of different height with a basal nucleus. From 2 dph three intestinal portions could be observed: anterior, medium and posterior (Fig. 3B). The enterocytes showed basal nucleus and cytoplasmic projections to the lumen. At 3 dph mouth and anus opening occurred in synchrony with the total absorption of the maternal reserves and the beginning of the exogenous feeding. At the same time, the growing intestine formed a loop to accommodate in the visceral cavity.

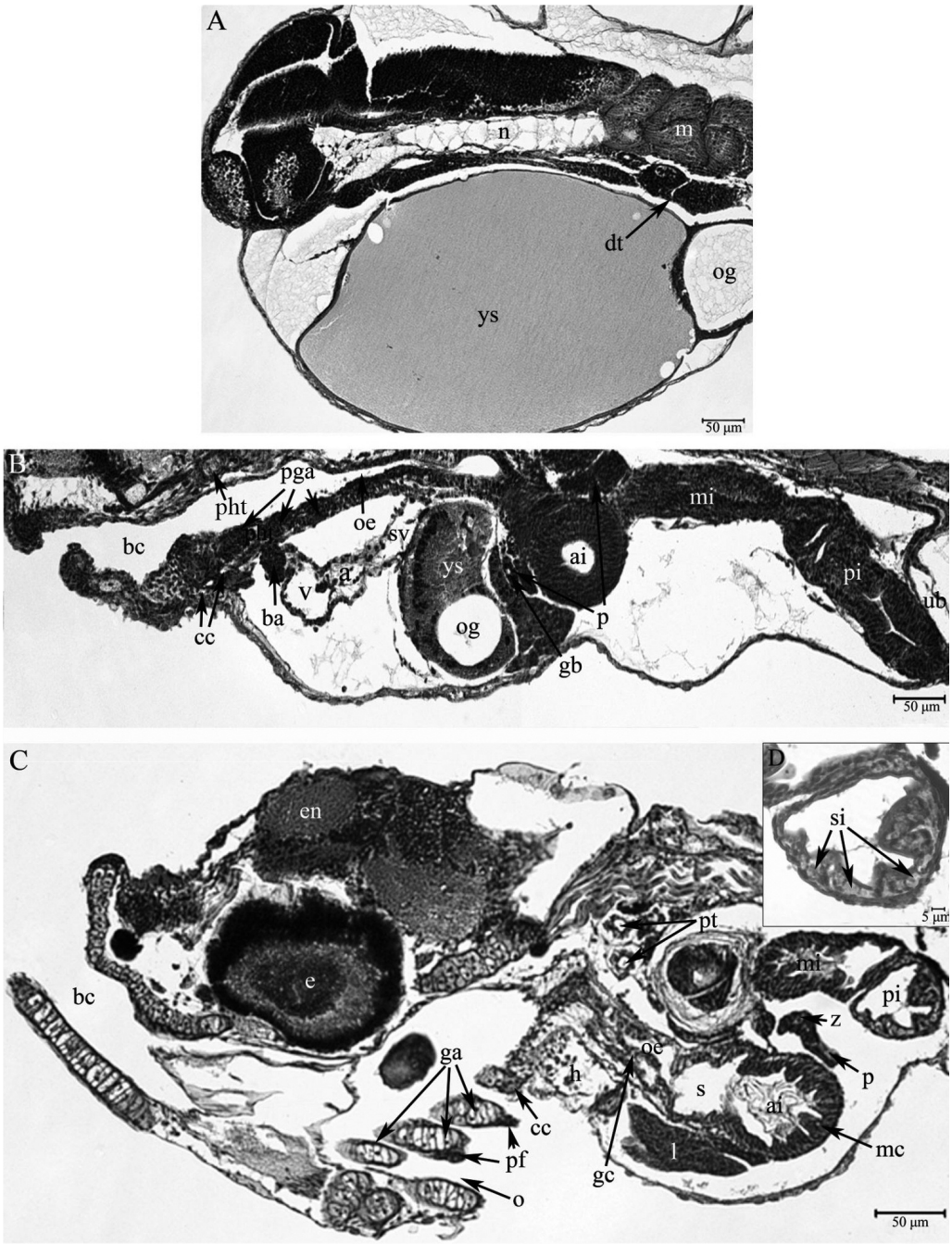

Fig. 3. Light micrographs of sections stained with H-E of red porgy larvae from 0 to 6 dph. **A.** Recently hatched larvae showing a homogeneous yolk-sac matrix and a unique oil globule. The digestive tract is straight and undifferentiated and the mouth is closed. **B.** A 2 day-old larva showing the digestive system differentiated in buccal cavity, oesophagus and intestine in three portions (anterior, medium and posterior). Yolk-sac volume visibly reduced. **C-D**. A 6 day-old larva showing a primordial stomach, the first mucous cells in the anterior intestine and a detail of the posterior intestine with supranuclear inclusions. The first pharyngeal teeth, primordial filaments of the gill arches and pronephros are also evident. a, auricle, ai, anterior intestine; ba, bulbus arteriosus; bc, buccal cavity; cc, chloride cell; dt, digestive tract; e, eye; en, encephalon; ga, gill arch; gb, gall bladder; gc, goblet cell; h, heart; l, liver; m, muscle; mc, mucous cell; mi, medium intestine; n, notochord; o, operculum; oe, oesophagus; og, oil globule; p, pancreas; pf, primordial filament; pga, primordial gill arch; pht, pharyngeal tooth; pi, posterior intestine; pt, pronephric tubule; s, stomach; si, supranuclear inclusions; sv, sinus venosus; ub, urinary bladder; v, ventricle; ys, yolk-sac; z, zymogen granules.

Buccopharyngeal cavity, gills and pseudobranches

Pharyngeal teeth were evident from 9 dph and increased in number and size according to larval development. Maxillary teeth and labial pulps were first observed at 15 dph. Abundant buccal mucous cells were observed at 40 dph. Primordial gill arches were appreciated at 2 dph (Fig. 3B) and four pairs of gill arches were observed at 6 dph, from which cartilaginous tissue (Alcian Blue positive) developed to form primordial filaments (Fig. 3C) in second and third arches firstly. Chloride cells, pillar cells and gill vascular structures were evident at 12 dph (Fig. 4E). Gills were completely formed at 15 dph (Fig. 4H), showing filaments that increased in length and primordial lamellae that increased in number and size throughout larval development (Fig. 4H, 7A, B). Pseudobranches began to differentiate from 2 dph being located in the posterior zone of the gill cavity and behind the eye. Two pairs of filaments were observed at 12 dph. At 22 dph, pseudobranches presented three pairs of filaments that progressively increased in number and length with larval development (Fig. 4D). First lamellae were observed at 26 dph.

Oesophagus

The oesophagus was located caudal to the pharynx and extended from the last gill-arch to the anterior intestine opening (Fig. 3B). Long longitudinal folds and a loose connective tissue were evidenced in larvae from 3-4 dph. The lumen, constituted by an epithelium of squamous cells, was relatively narrow and short. The proliferation of these cells forms a stratified epithelium of cubic cells at 2-3 dph. Goblet cells appeared from 6 dph (Fig. 3C; 4F, I). Around 12 dph, goblet cells contained neutral glycoconjugates and/or acid glycoconjugates, carboxylated and sulphated being equally abundant (Table 4). Some goblet cells were only stained with PAS (neutral glycoconjugates), while others were stained in purple with Alcian Blue pH 2.5 + PAS (neutral and carboxylated mucosubstances). Goblet cells also presented proteins rich in lysine, cysteine, tyrosine, arginine and tryptophan from 12 dph (Table 5). At 12 dph, the oesophageal wall was surrounded by an inner longitudinal thin muscle layer, a circular striated muscle layer and a thin tunica serosa. Abundant goblet cells were detected at 40 dph (Fig. 7E).

Stomach

At 6 dph, the future stomach appeared as a little pocket with a primordial pyloric sphincter (Fig. 3C). Its mucosa was composed of a simple cubic cell epithelium without any signal of secretion and a connective sub-epithelial tissue layer. The epithelial cells presented a

Table 3. Histochemical distribution of carbohydrates and proteins in red porgy from hatching until 6 dph.

	Neutral mucosubstances/ glycoconjugates	Carboxylated mucosubstances/ glycoconjugates	Sulphated mucosubstances/ glycoconjugates	Glycogen	Proteins
Yolk-sac/oilglobule	3/0	0/0	0/0	1/0	3/3
Liver/hepatocytes	2/2	0-1/0-1	0/0	2-3/2-3	2/2
Exocrine pancreas/ acidophilic zymogen granules	2-3/2-3	0-1/0-1	0-1/0-1	0/0	3/3

Reaction intensity: 0, negative; 1, slight; 2, moderate; 3, strong.

Table 4. Histochemical distribution of mucosubstances/glycoconjugates in oesophagus, stomach and intestine of the red porgy at 15 dph.

	Neutral glycoconjugates	Carboxylated glycoconjugates	Sulphated glycoconjugates	Glycogen
Oesophagus				
Epithelium/Enterocytes	0-1/0-1	0-1/0-1	0-1/0-1	0/0
Goblet cells	0-1	3	3	0
Stomach				
Epithelium/Enterocytes	0-1/0-1	1/1	0/0	1/1
Gastric glands (35 dph)	0-1	1	0	1
Intestine				
Epithelium/Enterocytes	0-1/0-1	1/1	0-1/0-1	1/1
Mucous cells	1-2	3	0-1	0
Supranuclear inclusions (posterior intestine)	0	1	0	0

Reaction intensity: 0, negative; 1, slight; 2, moderate; 3, strong.

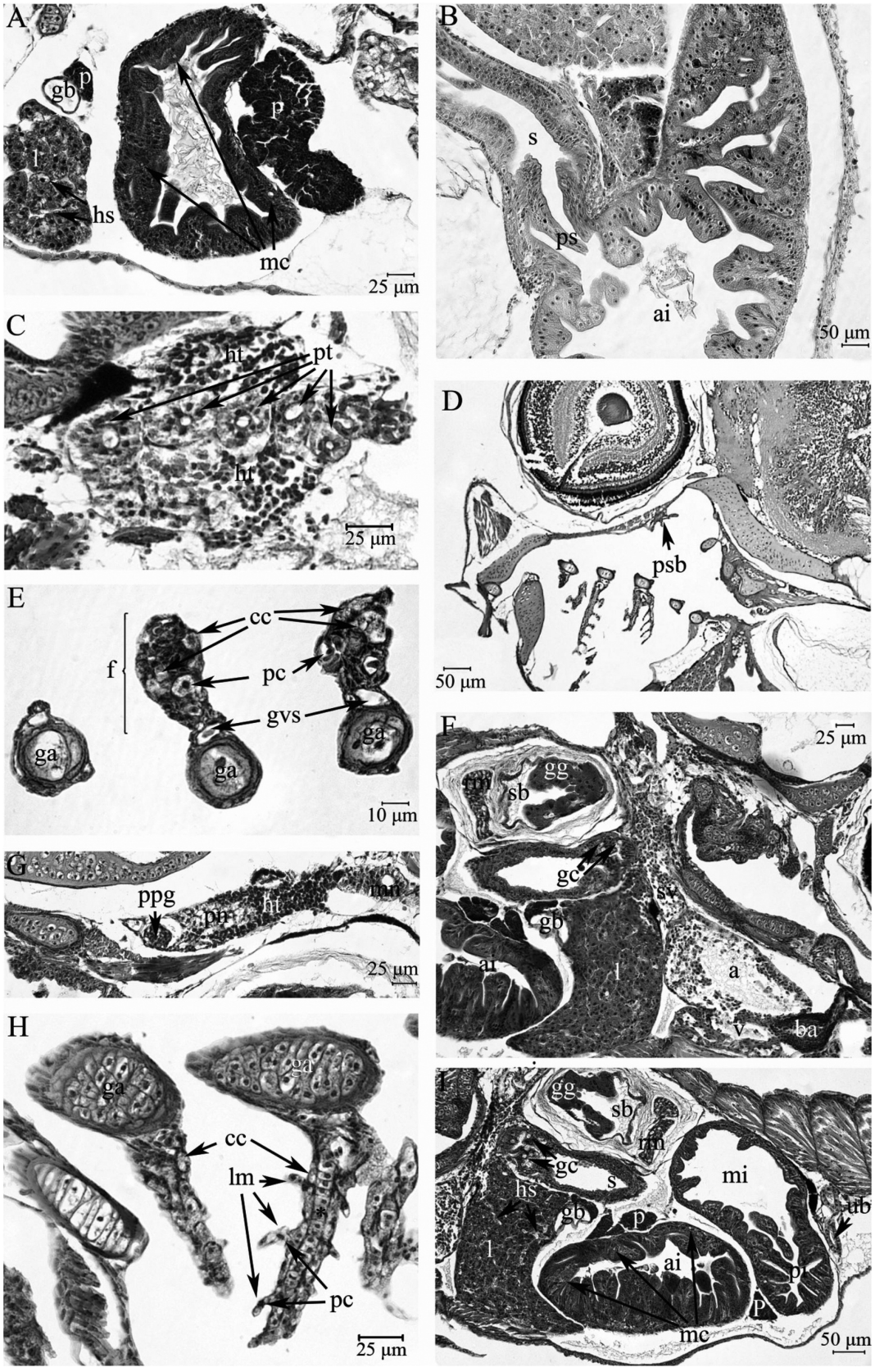

Fig. 4. Light micrographs of sections stained with H-E of red porgy larvae from 9 to 15 dph. **A.** A 9 day-old larva showing mucous cells in the anterior intestine which is surrounded by a diffuse pancreas. **B, C, E.** A 12 day-old larvae showing the pyloric sphincter which connects the stomach with the anterior intestine, the kidney with pronephric tubules and gill arches with filaments. **G-I.** A 15 day-old larva showing a completely developed swim bladder, kidney, and gills with filament and lamella. **D.** A 22 day-old larva showing a pseudobranch with three filaments. a, atrium; ai, anterior intestine; ba, bulbus arteriosus; cc, chloride cell; f, filament; ga, gill arch; gb, gall bladder; gc, goblet cell; gg, gas gland; gvs, gill vascular structures; ht, haematopoietic tissue; hs, hepatic sinusoid; l, liver; lm, lamella; mc, mucous cell; mi, medium intestine; mn, mesonephros; p, pancreas; pc, pillar cell; pi, posterior intestine; pn, pronephros; ppg, primordial pronephros glomerulus; ps, pyloric sphincter; psb, pseudobranch; pt, pronephric tubules; rm, rete mirabile; s, stomach; sb, swim bladder; sv, sinus venosus; ub, urinary bladder; v, ventricle.

granular and narrow cytoplasm with an oval nucleus in basal or central position and short microvilli in their apical border. At 12 dph, the pyloric sphincter connected the stomach with the antero-median intestine (Fig. 4B). The first signs of gastric gland development were observed around 19 dph, being formed by 26-30 dph and the number of which increased significantly at 35 dph (Fig. 6B-D). Gastric glands were composed of a unique type of secretory cell devoid of microvilli in its apical border and forming aggregated cells connected to the lumen and surrounded by a delicate connective tissue layer (Figs. 7F, 8C, D). The stomach wall consisted of mucosa, lamina propria-submucosa, muscularis and serosa layers. Three gastric regions could be distinguished: fundic, cardiac and pyloric. The muscular layer of the cardiac region was thin, and thickened in the pyloric region. Gastric glands were localized in the cardiac region. The stomach presented two layers of smooth muscle: inner circular and external longitudinal ones. Both the stomach epithelium and gastric glands contained glycogen and neutral and acid glycoconjugates, mainly the carboxylated ones. Proteins rich in arginine, tyrosine and tryptophan were especially abundant inside the gastric glands (Table 5).

Intestine

The epithelial intestine of red porgy larvae was formed by a single columnar enterocyte layer with nucleus in basal or medium position and microvilli in their apical border and by an inner circular and outer longitudinal thin muscular tissue layer, separated by a delicate connective tissue layer (Fig. 5A, C). At 2 dph, the intestine became curved to accommodate inside the visceral cavity and three regions could be observed: antero-median, median and posterior (Fig. 3B). The first region presented mucous cells with the same histochemical characteristics as the oesophageal goblet cells: neutral and/or carboxylated and/or sulphated glycoconjugates (Table 4). Such mucous cells first

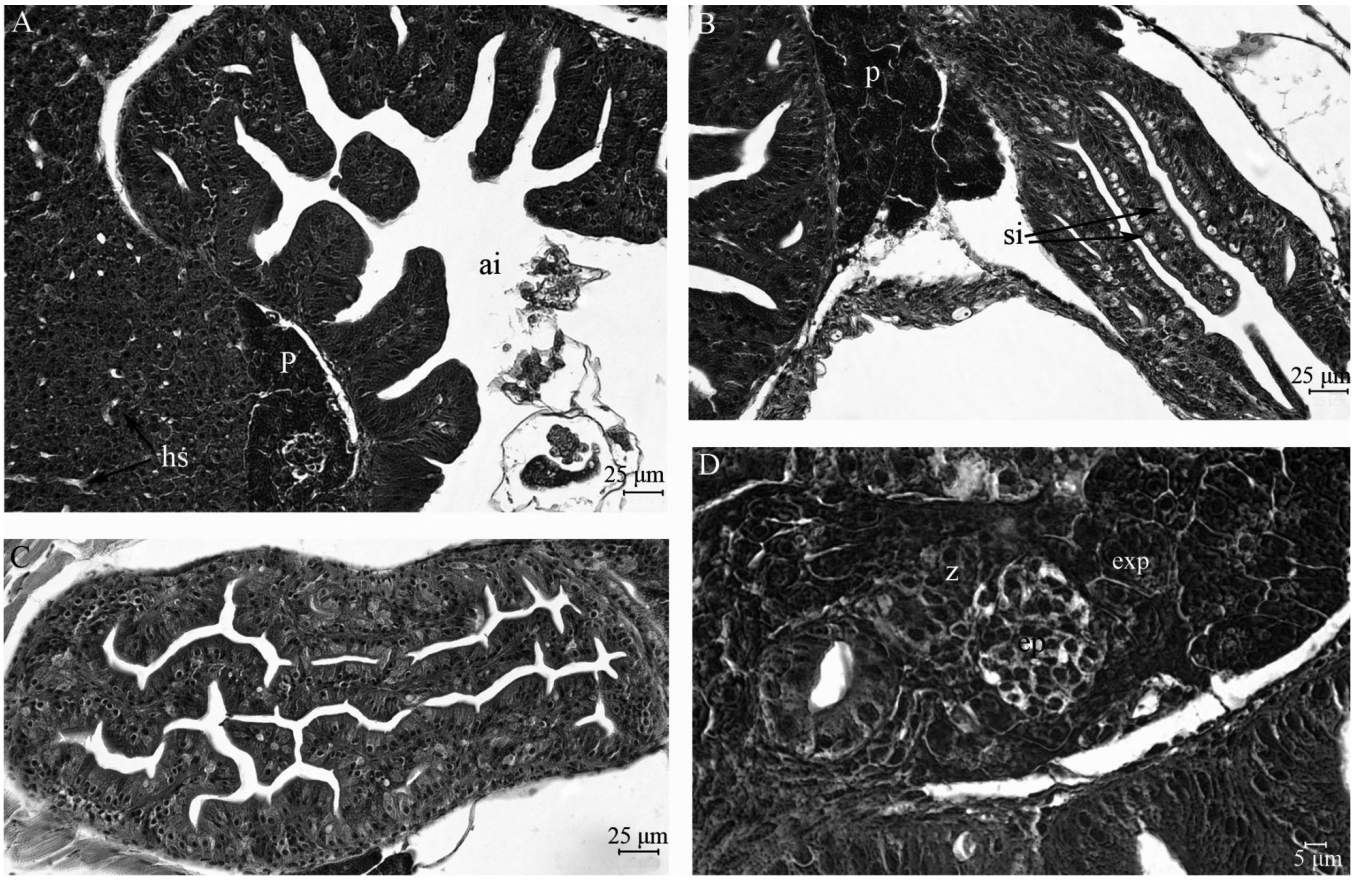

Fig. 5. Light micrographs of sections stained with H-E of 19 day-old red porgy larvae. **A.** Anterior intestine well developed in close contact with the pancreas which secretes enzymes for digestion. **B.** Nutrient absorption by pinocytosis in the posterior intestine is evidenced by the presence of supranuclear inclusions. **C.** Detail of the medium intestine showing numerous intestinal folds that increases the digestive surface. **D.** Detail of the pancreas showing both endocrine and exocrine pancreas and zymogen granules containing digestive enzymes precursors. ai, anterior intestine; ep, endocrine pancreas; exp, exocrine pancreas; hs, hepatic sinusoid; p, pancreas; si, supranuclear inclusion; z, zymogen granules.

Table 5. Histochemical distribution of proteins in oesophagus, stomach and intestine of the red porgy between 6 and 15 dph.

	General proteins	Lysine	Tyrosine	Arginine	Cystine	Cysteine	Tryptophan
Oesophagus							
Epithelium/Enterocytes	2/2	1-2/1-2	2/2	2/2	2/2	2/2	0/0
Goblet cells	0-1	1	0	0	2	3	1
Stomach							
Epithelium/Enterocytes	2/2	1/1	2/2	2/2	3/3	0-1/0-1	2/2
Gastric glands (35 dph)	2-3	1-2	3	3	1	1	3
Intestine							
Epithelium/Enterocytes	3/3	1/1	1/1	2/2	1/1	1/1	1/1
Mucous cells	0	0	0	0	2	0-1	0
Supranuclear inclusions (posterior intestine)	3	2	2	3	2-3	2	2

Reaction intensity: 0, negative; 1, slight; 2, moderate; 3, strong.

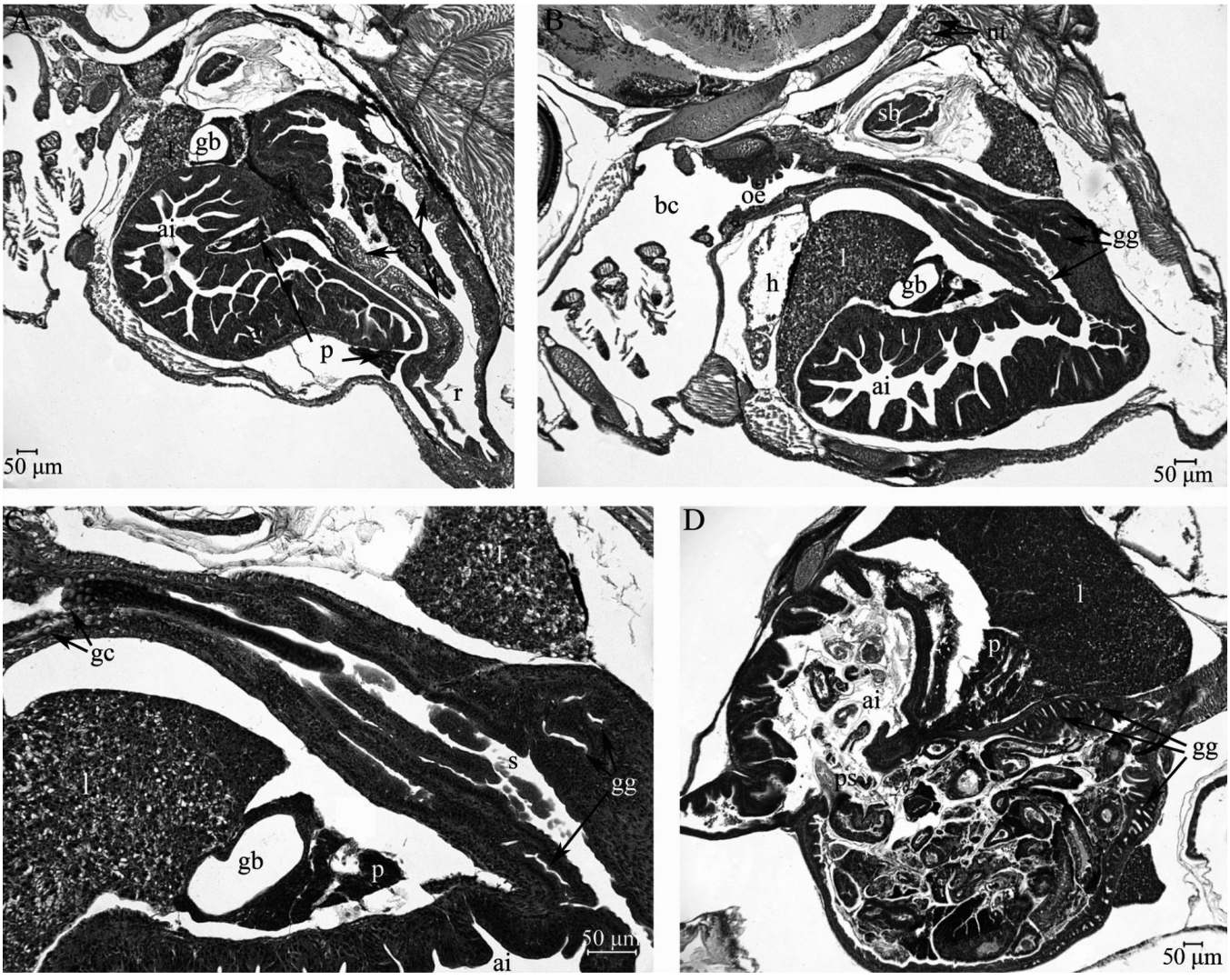

Fig. 6. Light micrographs of sections stained with H-E of the red porgy larvae digestive system. **A-C.** 26 day-old larvae presenting a morphologically developed digestive system showing the first gastric glands in the cardiac region of the stomach. **D.** A 30 day-old larva showing high number of gastric glands responsible for the adult mode of digestion (extracellular acid digestion). ai, anterior intestine; bc, branchial cavity; gb, gall bladder; gc, goblet cell; gg, gastric glands; h, heart; l, liver; nt, nephric tubules; oe, oesophagus; p, pancreas; ps, pyloric sphincter; r, rectum; s, stomach; si, supranuclear inclusions; sb, swim bladder.

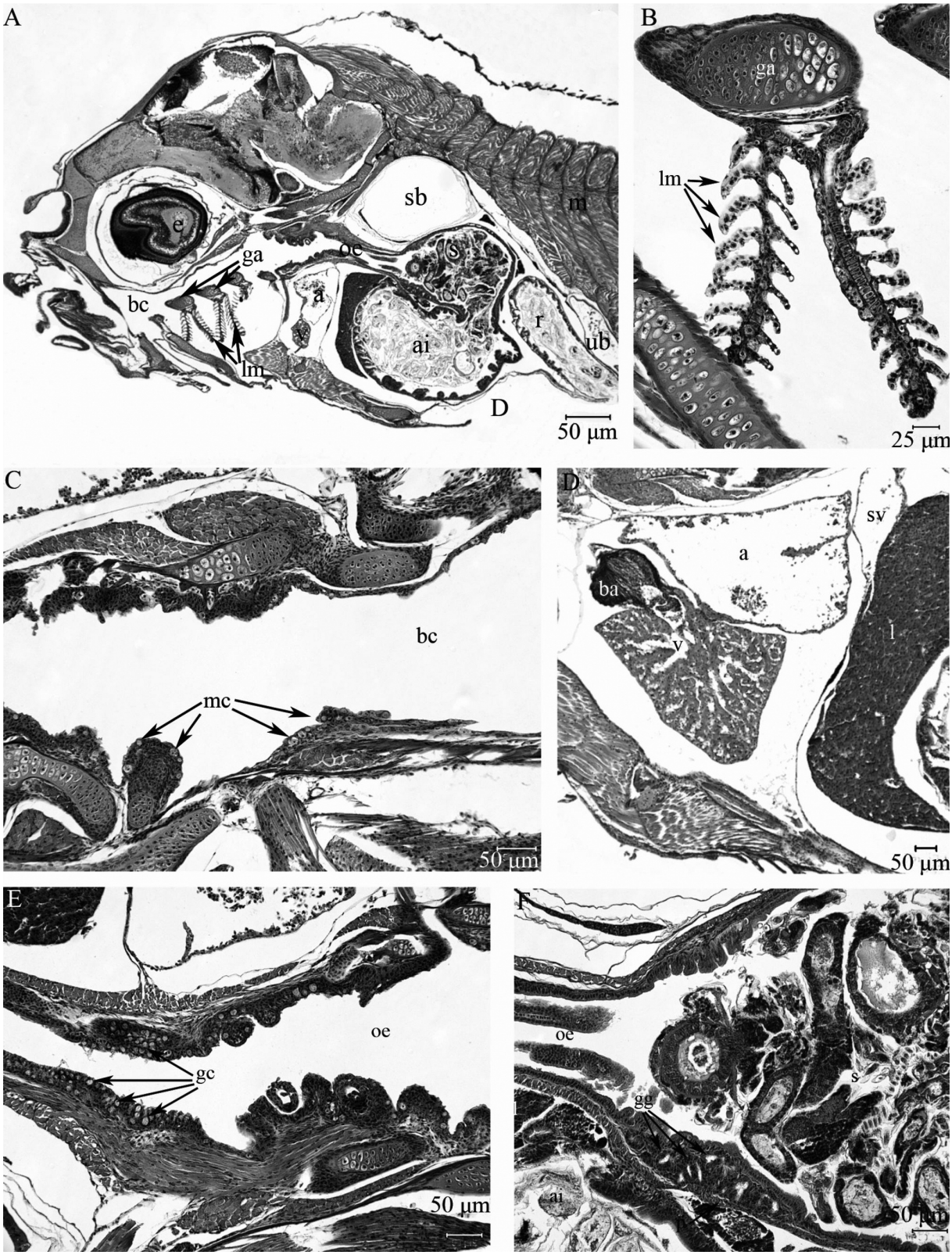

Fig. 7. Light micrographs of sections stained with H-E of 40 day-old red porgy larvae. **A.** General view of the mature digestive system. **B.** Detail of the gill arch showing filaments with numerous lamellas that improve the oxygen uptake, among other functions. **C.** Detail of the buccal cavity showing the mucous cells that lubricate the food to protect the mucosa. **D.** Detail of the heart showing the bulbus arteriosus, ventricle with trabeculae, atrium and sinus venosus directly connected to the liver. **E.** Detail of the oesophagus showing numerous goblet cells that secrete mucous to lubricate the food. **F.** Detail of the cardiac region of the stomach showing gastric glands. a, atrium; ai, anterior intestine; ba, bulbus arteriosus; bc, buccal cavity; e, eye; ga, gill arch; gc, goblet cell; l, liver; lm, lamella; m, muscle; mc, mucous cell; oe, oesophagus; r, rectum; s, stomach; sb, swim bladder; sv, sinus venosus; ub, urinary bladder; v, ventricle.

appeared at 6 dph although they increased in number from 15 dph onwards (Figs. 3C, 4A,I, 8C). In the medium intestine, the intestinal folds were deeper and more abundant (Figs. 5C, 8A,B). Finally, acidophilic supranuclear inclusions in the posterior region of the intestine appeared at 6 dph (Fig. 3C,D), containing abundant proteins, especially rich in arginine, tryptophan, tyrosine, cysteine and cystine (Table 5). Only Alcian Blue pH 2.5 showed a positive staining in such inclusions. Active pinocytosis was observed in the base of the microvilli of the enterocytes, except for those placed near the anus. The rectum was short and formed by a simple cubic epithelium. The enterocytes of this region presented supranuclear inclusions during the studied period (Fig. 5B).

Swim bladder

The swim bladder differentiated from the gut, being initially composed of columnar cells identical to the enterocytes. Subsequently, the swim bladder developed into a simple cubic cell epithelium, surrounded by fibroblast, which inflated from 12-15 dph (Figs. 4F,I, 6A,B, 7A). The rete mirabile, placed on the right side of the swim bladder, was well defined at this time (Fig. 4F).

Liver

After hatching, the liver was situated dorsally to the yolk-sac and ventrally to the developing digestive tract. Around 2 dph, the liver started to elongate and to adapt to the body cavity (Fig. 3B). Approximately at 4-6 dph, the hepatocytes organised in cords and showed an evident granular cytoplasm, eccentric nucleus and prominent nucleolus. Glycogen granules (PAS positive and diastase-PAS negative), proteins (Table 3), as well as neutral lipids (vacuoles in paraffin) were easily detected in the cytoplasm of the hepatocytes from 4-6 dph. Hepatic sinusoids containing blood cells could be observed at 9 dph (Fig. 4A), which ended into the sinus

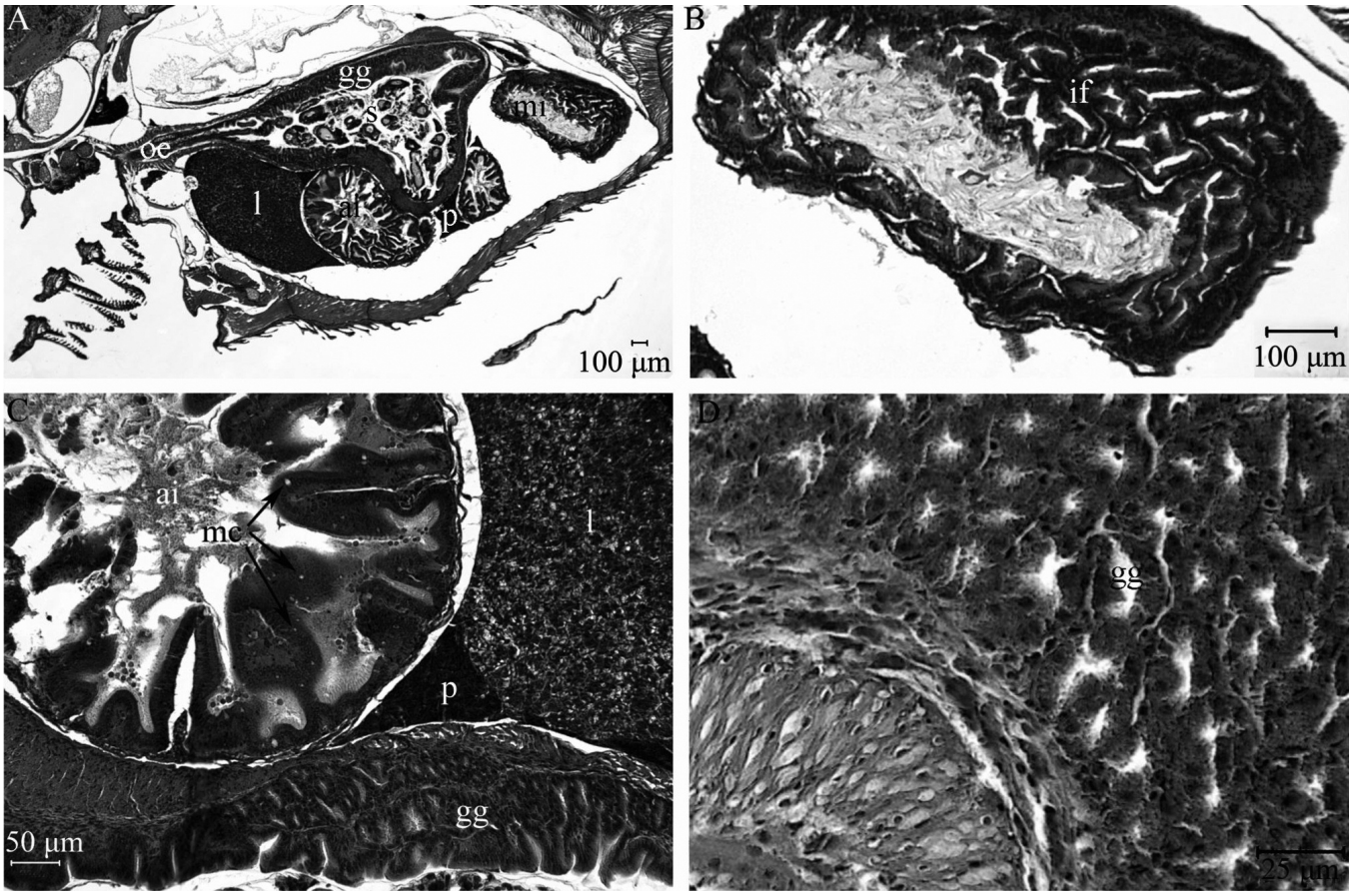

Fig. 8. Light micrographs of sections stained with H-E of the red porgy larvae digestive system. **A-C.** 50 day-old larva. **A.** General view of the digestive system showing a large stomach. **B.** Detail of the medium intestine showing numerous intestinal folds which help in breaking down the food in the intestinal lumen and increase the digestive surface. **C.** Mucous cells in the anterior intestine, liver, pancreas and stomach with gastric glands. **D.** Detail of the gastric glands in the stomach of 63 day-old larvae. ai, anterior intestine; gg, gastric glands; if, intestinal folds; l, liver; mc, mucous cells; mi, medium intestine; oe; oesophagus; p, pancreas.

Table 6. Gastric gland appearance in the stomach of several fish species under different culture conditions.

Species	Age(dph)	Rearing temperature (ºC)	Authors
Acipenser baeri	6-7	18	Gisbert et al., 1998
Dentex dentex	22	16-20	Santamaría et al., 2004
Dicentrarchus labrax	55	18-19	García-Hernández et al., 2001
Diplodus sargas	13-15	19-20	Ortiz-Delgado et al., 2003
Gadus morhua	25	11	Pérez-Casanova et al., 2006
Hippoglossus hippoglossus	40	11	Luizi et al., 1999
Melanogrammus aeglefinus	30-33	8	Hamlin et al., 2000
Pagrus auriga	16	19-22	Sánchez-Amaya et al., 2006
Pagrus pagrus	19	19-20	Darias et al., 2005 and present study
Paralichthys californicus	23	18	Gisbert et al., 2003
Pleuronectes dentatus	30-33	20	Bisbal & Bengtson, 1995
Pleuronectes ferruginea	29-36	10	Baglole et al., 1997
Psetta maxima	17-20	8	Cousin & Baudin Laurencin, 1985; Sala, 1993; Segner, 1994
Seriola lalandi	15	24	Chen et al., 2006
Solea senegalensis	33	19-20	Sarasquete et al., 1996, 2001; Ribeiro et al., 1999; Vieira, 2000
Sparus aurata	*60*	*18-19*	*Elbal et al., 2004*

venosus (Fig. 4F, I). The gall bladder was evident from 2 dph composed of a simple epithelium surrounded by a delicate connective tissue layer (Figs. 3B, 4A,F,I, 6A-C).

Pancreas

The exocrine pancreas initially appeared placed along the right side of the stomach to subsequently extend to the liver and to the dorsally and ventrally mesenteries of the gastrointestinal region (Figs. 4I, 6A,D). Between 2-3 dph, the basophilic cytoplasm of the exocrine pancreas was homogeneous and its cells were similar to the hepatocytes in shape and possessed spherical nucleus (Fig. 3B). After yolk-sac exhaustion, the acinar pancreatic cells were grouped to form ducts. Around 6 dph, acidophilic zymogen granules containing proteins rich in tryptophan, tyrosine, cysteine, cystine, lysine and arginine were observed in the exocrine pancreas (Table 3; Figs. 3C, 5D).

The morphology of liver and pancreas remained invariable from 15 dph onwards, only showing a progressive increase in size.

Heart

The heart was present at hatching as a tubular structure and subsequently divided into four regions (bulbus arteriosus, ventricle, atrium and sinus venosus) at 2 dph, which connected directly to the perivitelline space (Fig. 3B). First ventricle trabeculae appeared at 6 dph, increasing progressively with larval development, while first atrium trabeculae were observed from 26 dph. Valves between bulbus arteriosus and ventricle and between atrium and sinus venosus were also detected at that time (Figs. 6B, 7D).

Kidney

The kidney was already present from hatching as a straight duct located just behind the notochord. At 2 dph, pronephric tubules as well as pronephric ducts were observed. The urinary bladder connected with the posterior intestine close to the anus that was not yet opened (Fig. 3B). At 6 dph pronephros developed, showing tubules more convoluted, and the haematopoietic tissue increased (Fig. 3C). At 15 dph the urinary bladder opened directly to the exterior and two regions could be differentiated: haematopoietic tissue with pronephric glomeruli zone and mesonephric zone (Fig. 4C,G).

Discussion

Overall, red porgy presented a similar pattern of histo-physiological digestive system ontogeny to other teleosts, either belonging to the same family (Sarasquete et al., 1995, 2001; Roo et al., 1999; Ortiz-Delgado et al., 2003, Elbal et al., 2004; Santamaría et al., 2004; Sánchez-Amaya et al., 2006) or not (Blaxter et al., 1983; Cousin and Baudin-Laurencin, 1985; Kjørsvik et al., 1991; Boulhic and Gabaudan, 1992; Segner et al., 1994; Tanaka et al., 1996; Luizi et al., 1999; Ribeiro et al., 1999b; Hamlin et al., 2000). They presented a common organ location, as well as similar cellular characteristics. The main inter-specific variability encountered resides in the timing of tissue and organ development. The quality of spawners determines in the first place the quality and amount of larval energetic reserves and the environmental conditions directly affect the rate of larval development. Thus, it is possible to find differences in larval development when species inhabiting temperate water of the same geographic area are compared. For example, Senegal sole (*Solea senegalensis*), white sea bream (*Diplodus sargus*), common dentex (*Dentex dentex*), redbanded seabream (*Pagrus auriga*) and red porgy exhibited higher speed of tissue development than gilthead sea bream (*Sparus aurata*). Indeed, gastric glands were not observed in the last species before two months of life (Domeneghini et al., 1998; Sarasquete et al., 2001; Elbal et al., 2004), while these glands were

completely developed within the first month in the other above mentioned species (Ribeiro et al., 1999; Roo et al., 1999; Ortiz-Delgado et al., 2003; Santamaría et al., 2004; Sánchez-Amaya et al., 2006 and red porgy present study). It is worth paying attention to the variations found in tissue development between red porgy and white sea bream (Ortiz-Delgado et al., 2003) since they were reared in the same installations under the same conditions of water temperature and salinity. Such differences could be attributed to feeding habits of each species as well as in ecological strategies.

As in many other teleosts, red porgy larvae presented at hatching an undifferentiated straight gut without mouth and anus opening (Govoni et al., 1986; Segner et al., 1994; Sarasquete et al., 1996; Ribeiro et al., 1999b; García-Hernández et al., 2001; Ortiz-Delgado et al., 2003; Elbal et al., 2004; Sánchez-Amaya et al., 2006) which developed during the following days into an anatomically and functionally formed digestive tract at the beginning of the exogenous feeding period, with the exception of the stomach. During the first days of larval life, gills and pseudobranches are not developed yet and, therefore, osmorregulation and respiration functions are carried out by chloride cells of the buccopharyngeal epithelium and trough skin, respectively, as was pointed out in other fish species (Sarasquete et al., 2001; Santamaria et al., 2004).

The primordial gill arches could be observed in 2 days-old red porgy larvae. Cartilaginous structure of gill arches was detected on 6 dph and primordial filaments of the second and third arches began to develop. Primordial lamellae were first observed at 15 dph, indicating the beginning of gills functionality. An incipient pseudobranch was detected in 2 day-old larvae that developed into a paired structure with two filaments at 12 dph and three filaments at 22 dph. At 26 dph, first lamellae could be appreciated, as well as a notable increase of filament size. Similar results were reported by Santamaría et al. (2004) and Sánchez-Amaya et al. (2006) for common dentex and redbanded seabream, respectively. The function of this structure is not yet well known, although several roles have been attributed, such as osmoregulation, regulation of hydrostatic pressure and sensorial, respiratory and endocrine functions, among others.

According to Tanaka (1971), pharyngeal teeth appeared before mandible and maxillary teeth. However, the opposite has been observed in other fish species such as summer flounder (*Paralichthys dentatus*) (Bisbal and Bengtson, 1995), common pandora (*Pagellus erythrinus*) (Micale et al., 2006) and redbanded seabream (Sánchez-Amaya et al., 2006).

The oesophageal goblet cells of red porgy , white sea bream (Ortiz-Delgado et al., 2003) and redbanded seabream (Sánchez-Amaya et al., 2006) appeared a few days after first feeding (5-6 dph), as well as in turbot (*Psetta maxima*) (Cousin and Baudin-Laurencin, 1985), gilthead sea bream (Sarasquete et al., 1995) and sea bass (*Dicentrarchus labrax*) (García-Hernández et al., 2001). In haddock (Melanogrammus aeglefinus), goblet cells were observed at 10 dph at 8°C (Hamlin et al., 2000). However, the mucin secretion was detected earlier in other species, coinciding with the mouth opening, such as in Dover sole (*Solea solea*) (Boulhic and Gabaudan, 1992) and *Senegal sole* (Sarasquete et al., 1996; Vieira, 2000). Goblet cells are components of the postgastric mucose layer of fish larvae and adults (Sarasquete et al., 1995, 2001; Ribeiro et al., 1999a; Arellano et al., 2001), being implied in absorption of easily digested substances (disaccharides and short-chain fatty acids) and transport processes, as well as in digestive tract protection (Rhodes et al., 1985; Anderson, 1986; Pajak and Danguy, 1993; Baglole et al., 1997; Ribeiro et al., 1999b). Grau et al. (1992) attributed the microelongations of the oesophageal epithelium to a protective function against mechanical impacts of the ingested food as well as to facilitate the anchorage of the mucous produced by the goblet cells. García Hernández et al. (2001) found oesophageal cells with similar ultrastructural features of chloride cells of gill epithelium in sea bass larvae, suggesting that oesophagus plays an osmoregulatory role in this species.

Acidic mucosubstances were only found in goblet cells of Dover sole (Boulhic and Gabaudan, 1992), Senegal sole (Ribeiro et al., 1999b) and sea bass (García Hernández et al., 2001). In red porgy and white sea bream larvae (Ortiz-Delgado et al., 2003), as well as in other species (Sarasquete et al., 1995, 2001; Sánchez-Amaya et al., 2006) some goblet cells reacted exclusively to PAS (neutral mucosubstances). However, other cells were stained blue or purple when a double Alcian Blue 2.5-PAS staining was carried out, which indicates either the presence of a mixture of neutral (magenta) to acidic mucins (purple) or a secretion of an unique type of acidic glycoproteins (blue), especially the carboxylated ones (Ortiz-Delgado et al., 2003; Sánchez-Amaya et al. 2006). Harrison et al. (1987) considered this variability of staining of a determined goblet cell as a result of a temporal sequence of glycoprotein mucous synthesis and secretion. According to Elbal and Agulleiro (1986) and Sarasquete et al. (2001), the PAS-positive reaction in the goblet cells of different fish species could represent an early stage of development, when cells are mainly producing neutral glycoproteins. Goblet cells are stained with Alcian blue (pH 2.5) when glycoproteins are being carboxylated. The presence of sulphated glycoproteins (Alcian blue, pH 0.5) coincides with the stage in which sulphated groups are conjugated with glycoproteins. Contrary to this, the acid type of mucins appeared before the neutral ones in the goblet cells of the oesophagus of sea bass larvae (Hernández-Cruz et al., 2001).

As observed in other fish species (Boulhic and Gabaudan, 1992; Grau et al., 1992; Murray et al., 1994a,b; Bisbal and Bengtson, 1995; Arellano et al., 1999; Hamlin et al., 2000; Ortiz-Delgado et al., 2003; Elbal et al., 2004), in red porgy, the transition from oesophagus to stomach is evidenced by goblet cell

disappearance and by the replacement of a stratified epithelium by a columnar epithelium in the stomach. The incipient stomach could be observed in red porgy larvae by 6 dph, as well as in gilthead sea bream (Sarasquete et al., 1995), while in Senegal sole, white sea bream and redbanded seabass it was distinguished at 2-3 dph (Ribeiro et al., 1999; Ortiz-Delgado et al., 2003; Sánchez-Amaya et al., 2006). The columnar epithelium of the stomach could be the precursor of gastric glands (Douglas et al., 1999). Once again, red porgy showed a slight delay in tissue development with respect to the white sea bream, the first signs of gastric glands being detected around 19 dph, while in the white sea bream this occurred by 13-15 dph (Ortiz-Delgado et al., 2003) and by 60 dph in gilthead sea bream (Elbal et al., 2004). An inter-specific variability of gastric gland formation was observed in reared species (Table 6). The complete maturation of gastric glands takes place when these are able to secret pepsin and hydrochloric acid, which supposes the adult mode of acid digestion and is considered by many authors as an indicator of the larval to juvenile transition (Baglole et al., 1997; Douglas et al., 1999; Hamlin et al., 2000; Gawlicka et al., 2001; Elbal et al., 2004). Gastric glands were morphologically developed in red porgy at 26 dph and pepsinogen expression was detected at 30 dph (Darias et al., 2005), while gastric glands were histologically developed in the white sea bream 10 days earlier (Ortiz-Delgado et al., 2003).

Neutral glycoconjugates, cysteine and especially cystine residues were detected in the columnar epithelium of the stomach of the red porgy. It is interesting to note that proteins rich in tyrosine, arginine and tryptophan were very abundant in red porgy at 35 dph, while in white sea bream at 23 dph (Ortiz-Delgado et al., 2003) and in Senegal sole between 50-80 dph (Vieira, 2000; Sarasquete et al., 2001). These substances could be related to the synthesis and secretion of enzymatic precursors such as pepsinogen (Gutiérrez et al., 1986; Grau et al., 1992; Gisbert et al., 1999; Vieira, 2000). Neutral glycoconjugates detected in the surface of epithelial gastric cells of red porgy larvae could indicate the existence of nutrient absorption processes in the stomach (Reifel and Travill, 1977; Elbal and Agulleiro, 1986; Grau et al., 1992). In addition, neutral mucins could serve as a protection against auto-digestion processes caused by HCl and enzymes secreted by gastric glands (Ferraris et al., 1987). Sulphated glycoconjugates were not present in red porgy larvae or in other species either (Sarasquete et al., 2001; Ortiz-Delgado et al., 2003). However, such mucosubstances have been found in other fish species and are considered to be pepsin stabilizers by forming complexes for buffering or neutralizing the enzymatic activity (Spicer and Schulte, 1992, Arellano et al., 2001). This could indicate that red porgy gastric glands do not secrete pepsin at this time.

Gastric glands of red porgy were specifically located in the cardiac region of the stomach, the same as were observed in gilthead sea bream (Elbal and Agulleiro, 1986), European eel (*Anguilla anguilla*) (Ostos-Garrido et al., 1996) and white sea bream (Ortiz-Delgado et al., 2003). However, in other fish species gastric glands were found in the fundic region of the stomach, such as in yellowtail flounder (*Pleuronectes ferruginea*) (Baglole et al., 1997), summer flounder (Bisbal and Bengtson, 1995) and turbot (Segner et al., 1994). In redbanded seabream (Sánchez-Amaya et al., 2006) gastric glands were localized in the cardiac and fundic regions of the stomach, and in Senegal sole juveniles and adults those were concentrated in the fundic and pyloric zones (Vieira, 2000; Arellano et al., 2001), while the stomach of Atlantic halibut (*Hipposglossus hippoglossus*) adults was completely glandular, probably because this species consumes large preys (Murray et al., 1994a). Such inter-specific differences in the gastric glands localization in the stomach suggest different strategies for feeding and digestion.

The intestinal mucosa of red porgy larvae presents primary and secondary folds that increases the digestive surface and helps in breaking down food and mixture with the aid of enzymes secreted by the exocrine pancreas. Intestinal mucous cells of red porgy larvae were rich in acidic mucosubstances, although they also contained neutral mucins. This result agrees with those obtained in gilthead sea bream, white sea bream and redbanded seabream (Sarasquete et al., 1995; Ortiz-Delgado et al., 2003; Sánchez-Amaya et al., 2006). However, Hernández-Cruz et al. (2001) and Ribeiro et al. (1999b) only observed neutral mucosubstances in sea bass and Senegal sole, respectively. Such differences in the content of the intestinal mucous cells could be due to variations in feeding conditions (Sarasquete et al., 1995, 2001). The final portion of the intestine is actively implied in the absorption of the resultant products of digestion during the larval stage. The existence of acidophilic supranuclear inclusions in the posterior intestine of fish larvae has frequently been observed (Govoni et al., 1986; Kjørsvik et al., 1991; Sarasquete et al., 1993, 1995; Gisbert et al., 1999; Ribeiro et al., 1999b; Hamlin et al., 2000, Ortiz-Delgado et al., 2003; Sánchez-Amaya et al., 2006) which normally disappear when the stomach is completely functional. While gastric glands are still developing, pynocitosis of proteins in the posterior intestine is responsible for the intracellular digestion of proteins (Govoni et al., 1986). Waldford and Lam (1993) suggested that, in absence of stomach, the anterior intestine takes charge of the food digestion through alkaline trypsin activity (Moyano et al., 1996). In white sea bream (Ortiz-Delgado et al., 2003) and Atlantic halibut (Luizi et al., 1999), pinocytotic supranuclear vesicles disappeared when gastric glands were developed and an elevated level of protease activity was measured (Cara et al., 2003). These inclusions were absent in starved larvae of other sparids (Yúfera et al., 1993; Crespo et al., 2001). However, in red porgy they did not disappear during the studied period, as was observed in sea bass (García Hernández et

al., 2001). This result suggests that intracellular protein digestion occurs independently of the degree of stomach development.

Acidophilic zymogen granules were detected at 6 dph in the exocrine pancreas of red porgy larvae, revealing the presence of enzymatic precursors (basophilic cytoplasm pancreocytes or RNA-proteins synthesis), as suggested by Sarasquete and Gutiérrez (2005). Yolk-sac fish larvae confer priority to the synthesis and accumulation of pancreatic digestive enzymes to be ready for food digestion at the beginning of the exogenous feeding.

In summary, red porgy larval organogenesis was concentrated within the first three weeks of development and essentially exhibited an increase in tissue structure number and size from that date onwards. Generally, red porgy displayed a similar pattern of digestive system ontogeny to other teleosts, although showing some differences in the timing of organ development which should be taken into account for designing diets adequate to the degree of maturation of their digestive tract. In this sense, this study showed that red porgy larvae were able to digest food from first feeding as demonstrated by the good larval growth rate obtained (Fig. 1). However, the progressive development, both morphologic and functional, of the digestive tract and associated organs, implies a replacement of the less efficient intracellular and alkaline digestion by a more efficient acidic digestion inside the gastric glands, which occurred at 30 dph, allowing larvae to improve food assimilation. Considering this, red porgy larvae probably would grow better if Artemia sp. was supplied from 30 dph onwards. For the aquaculture potential of this species it would be interesting to test the effects of an easily digestible proteins-based inert diet until such critical date on larval assimilation efficiency. Studies based on the recently known digestive capacity of red porgy larvae to improve their nutritional needs will optimise survival and growth during the larval period.

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