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Organogenesis of the digestive tract in the white seabream, *Diplodus sargus*. Histological and histochemical approaches

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Summary. The ontogeny of the digestive tract of the white seabream, *Diplodus sargus* during the larval development up to day 45 post-hatching (dph) has been studied using histological and histochemical techniques. The oesophageal goblet cells appeared around 6 dph and contained neutral and acid mucosubstances (PAS/diastase-PAS and Alcian Blue pH 2.5 positive reactions). An incipient stomach can be distinguished from 2 dph but the first sign of gastric gland development was detected around 13-15 dph, increasing in number and size by 22-23 dph. Gastric glands were concentrated in the cardiac stomach region and they had a high content of protein rich in tyrosine, arginine and tryptophan. Acidophilic supranuclear inclusions related to pynocitosis of proteins, were already observed in the intestinal cells of the posterior intestine around 4-6 dph (exogenous feeding) and they were present until 25 dph. The intestinal mucous cells appeared between 15-18 dph and contained a mixture of neutral and acid mucosubstances/glycoconjugates, carboxylated ones being more abundant than the sulphated ones. The stomach and gastric glands were fully developed by the first month of life marking the beginning of digestive features characteristic of the juvenile stage. Around 4-6 dph, glycogen, proteins and neutral lipids were observed in the granular cytoplasm of hepatocytes. Strongly acidophilic zymogen granules were also present, at this time, in the basophilic cytoplasm of the exocrine pancreatic acinar cells and contained abundant proteins, especially rich in arginine, tyrosine and tryptophan.

Key words: *Diplodus sargus*, organogenesis, histology, histochemistry, larval development, digestive tract.

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Introduction

Aquaculture production of marine fish in the Mediterranean countries and neighbour Atlantic littoral is concentrated on few species: gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*). Diversification of cultured fish species seems then to be a necessary strategy for the future of the aquaculture industry in this region. Research on potential new marine fish is relatively advanced in some species such as Senegal sole *Solea senegalensis* (Dinis et al., 1999, Ribeiro et al., 1999; Yúfera et al., 1999; Sarasquete et al., 1996, 1998, 2001), common dentex, Dentex dentex (Glamuzina et al., 1989; Fernández-Palacios et al., 1994; Crespo et al., 2001; Traveset, 2002) and red porgy, Pagrus pagrus (Hernández-Cruz et al., 1999; Mihelakakis et al., 2001) but there are more species under investigation. The success of gilthead seabream production in Atlantic and Mediterranean aquaculture has stimulated the attempts with other sparids of commercial interest. One of these species is the white seabream, *Diplodus sargus*, inhabiting the Mediterranean Sea and Atlantic coasts of Europe and Africa (Bauchot and Hureau, 1986) and which is regarded as highly promising species for aquaculture considering its good marketing, easy adaptation to captivity and growth performance (Divanach et al., 1982; Abellán et al., 1994).

Growth and survival of larval fish depend primarily on the feeding success and an effective digestion and absorption of nutrients (Tanaka et al., 1995). The sequence of appearance of the digestive enzymes in fish is genetically programmed and enzyme profiles are related with the morpho-functional development of the digestive tract which it seems to reflect the adaptation to specific diets and changes in nutritional requirements (Buddington et al., 1987; Ugolev and Kuz'mina, 1994). The development and functionality of the stomach is mainly related to the gastric glands formation, and the process of metamorphosis presents important inter-specific differences. The success of marine larviculture

depends especially of an adequate knowledge of the functional development of the digestive system and nutritional requirements of the larvae (Yúfera et al., 2000), as well as of the control of potential pathologies occurring during larval life (Sfakianakis et al., 2002). A detailed knowledge of the morphological and histological characteristics of the digestive tract during its development is therefore a prerequisite for determining the functional relationships between feeding and assimilation (Hamlin et al., 2000).

Only few studies have been published on the larval stage of D. sargus concerning morphological and behavioural development (Divanach et al., 1982; Kentouri and Divanach, 1982), skeleton (Koumoundouros et al., 2001) as well as enzymatic activities ontogeny (Cara et al., 2003). The present study aims to advance in the digestive ontogeny by determining the histological and histochemical characteristics of the alimentary tract, liver and pancreas during larval development of this species in order to provide a necessary basis for future nutritional studies.

Materials and methods

Fish larvae

White seabream eggs were obtained during year 2001 from a captive broodstock held at temperatures ranging from 17 to 20 ºC at the experimental facilities of the CICEM "El Toruño" (Junta de Andalucía, Spain). After hatching, larvae were reared in 250-L tanks at 19.5 ºC temperature with constant illumination and a salinity of 33 g/l. The day of hatching was considered as day 0 (0 dph, day post-hatching). From the opening of the mouth at 3 dph until to 15 dph, the larvae were fed on rotifers *Brachionus rotundiformi* and *B. plicatilis*). From 12 dph to 30 dph the larvae were fed on recently hatched *Artemia nauplii* and from 31 dph onwards commercial fish feed was supplied. At different moments along their development, groups of 40-500 larvae were sampled, rinsed in distilled water and freeze-dried until analysed. Larval growth was determined weighing triplicates of 15-40 individuals for each sample point. The samples for weight determination were dried at 75 ºC until a constant weight was achieved.

Histological and histochemical procedures

Larvae and postlarvae from hatching until 45 dph were fixed in either 10% v/v buffered formaldehyde (pH 7.2) or in Bouin solution and embedded in paraffin blocks. Saggital and/or transversal histological sections of whole specimens of 5-7 μm thickness were stained with Haematoxylin-eosin (H-E) and Haematoxylin-VOF (VOF: light green-orange G – acid fuchsin) according to Gutiérrez (1990). Cytochemical tests were performed for carbohydrates/glycogen and neutral and acidic glycoconjugates respectively (periodic acid-Schiff/PAS, Alcian Blue pH 0.5, 1 and 2.5) utilizing also complementary techniques (diastase, acetylation, saponification and HCl-hydrolysis); general proteins (Bromophenol Blue); proteins rich in lysine (Ninhydrin-Schiff), proteins rich in tyrosine (Hg-sulphate-sulfuric acid-sodium nitrate); proteins rich in tryptophan (P-dimethylaminobenzaldehyde); proteins rich in arginine (1,2 napthoquinone-4-sulphonic acid salt sodium), and those rich in sulphydryl (-SH/cysteine) and disulphide groups (–S-S-/cystine) (Ferric ferricyanide-Fe III and Thioglycolate reduction). Neutral lipids were analyzed by using Oil Red 0 and Sudan Black B stains in unfixed samples processed in a cryomicrotome (2800 Frigocut, Reichert-Jung) and previously treated with a cryo-embedding compound. Neutral lipids were also visualized as vacuoles (lipid disolution) in paraffin sections. All methods and techniques used in this paper were taken from Pearse (1985) and Bancroft and Stevens (1990) monographs.

Results

Dry weight increase of white seabream, *Diplodus sargo* larvae during the experimental period is shown in Fig. 1. The opening of the mouth and the anus occurred at 3 dph, moment at which the yolksac was completely reabsorbed and the exogenous feeding started.

Histological development of the white seabream sargo larvae from hatching until the first month of larval life are shown in Figs. 2-6. A summary of the histochemical results is shown in Tables 1 to 3.

Yolk-Sac Larvae

At hatching (Fig. 2A) the yolksac was surrounded by a squamous epithelium and exhibited a homogeneous acidophilic yolk-matrix (light green or eosin affinity) when sections were stained with H-VOF or H-E

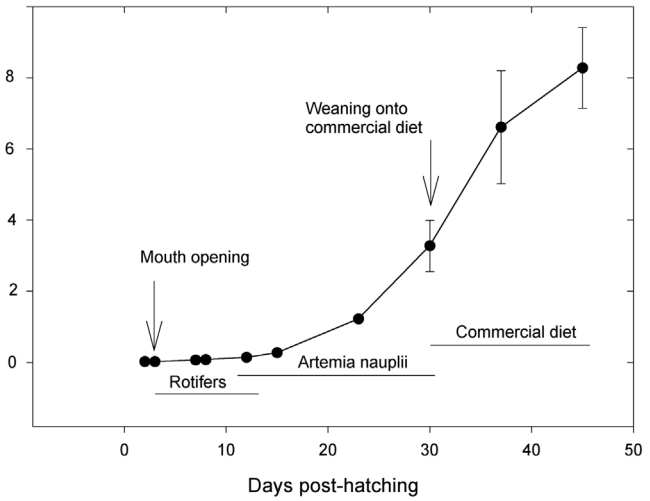

Fig. 1. Dry weight increase of *Diplodus sargus* larvae during the experiment.

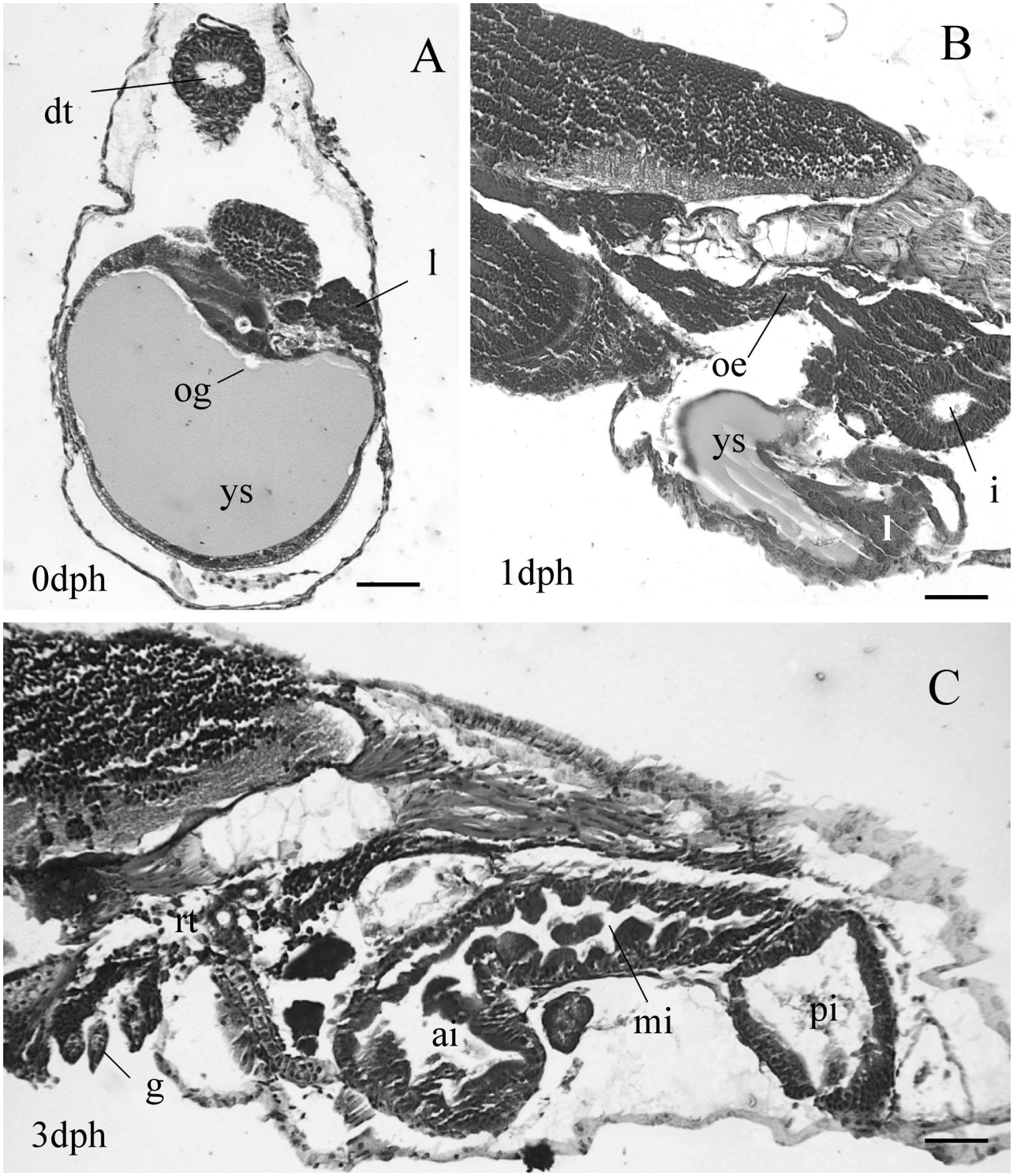

Fig. 2. Histological sections of *Diplodus sargus* larvae. **A.** Larvae at hatching showing the yolksac, oil globules and indifferentiated digestive tract. Primordial basophilic cells correspondant to liver are observed. Haematoxylin-eosin. Scale bar: 75 μm. **B.** Larvae at 1 dph. Progressive resorption of the yolksac and oil globules. The oesophagus becomes differentiated showing a squamuors/stratified epithelium. A columnar epithelium is visible in the rest of digestive portions (future stomach and intestine). Differentiation of the liver (less basophilic) is evident. Haematoxylin-eosin. Scale bar: 75 μm. **C.** Larvae at 3 dph showing the different intestinal portions. Yolksac was reabsorbed at this larval stage. Gills and renal tubules are developping. Haematoxylin-VOF. Scale bar: 100 μm. ai: anterior intestine; dg: digestive tract; g: gills; i: intestine; l: liver; mi: medialan intestine; oe = oesophagus; og: oil globule; p: pancreas; rt: renal tubules; pi: posterior intestine; ys : yolksac.

respectively. Several peripheral oil globules (vacuoles in paraffin sections) were present in the yolksac matrix which were positive to Oil Red O and Sudan Black B reactions (neutral lipids). The yolksac matrix also contained neutral glycoconjugates, glycogen (PAS and diastase-PAS reactions) and proteins (Table 1), and especially proteins rich in tyrosine, arginine, lysine, cysteine and cystine; being those proteins rich in tryptophan scarcely observed.

Digestive tract

At hatching, the digestive tract appears as a straight tubular segment laying dorsally to the yolksac. There were no anterior or posterior openings. The digestive tract lumen was narrow with a tendency to widen at both extremities. The digestive tract epithelium consisted of an epithelium whose cells varied in height, lined by a layer of squamous cells and, later on, by numerous mesenchymal cells. The epithelial cells had a basal nucleus with one or two nucleoli. Between the digestive tract and the yolksac (Fig. 2A,B), two groups of round cells with a spherical nucleus, prominent nucleolus and basophilic cytoplasm were observed, which corresponded to incipient liver and exocrine pancreas. At 1 dph, the caudal portion of the digestive tube bent slightly and the yolksac volume decreased, being this completely absorbed by the end of 3 dph (Fig. 2B)

By 1-2 dph, the digestive tract was histologically differentiated in two portions (Fig. 2B). The anterior portion was lined by a squamuous epithelium (oesophagus), and the external pavement of this portion had cell groups with a circular disposition, which whould develop later into the gill arches. The following digestive tract portion was lined by a simple columnar epithelium (stomach and intestine) whose cells showed basal nuclei and cytoplasmic projections to the lumen. An anterior, median and posterior intestinal portions were identified progressivelly from 3dph. Few differences exist between the enterocytes of the anterior and posterior intestine, and no mucous cells are present in any of the gut regions at the earliest stages. Enterocytes of the anterior and median intestinal portions shown small and scarce vacuolar inclusions (neutral lipids) near of the brush border. Supranuclear inclusions were only observed in the enterocytes of the porterior intestinal portion, which first (4-5 dph), appeared as empty vacuoles, and gradually became filled with an acidophilic substance (Figs. 2C, 3A-C, 4A-D) and containing abundant proteins.

Oesophagus

The larval oesophagus differentiated on 2 dph. The oesophagus lumen was relatively narrow and short, around which squamuous epithelial cells are dividing to form a stratified epithelium of cubic cells evident in larvae at 2-3 dph. The oesophagus it was located caudal to the pharynx and extended from the last gill-arch to the anterior intestine opening. Long longitudinal folds and a

Table 1. Histochemical distribution of glycoconjugates and proteins in *Diplodus sargus* larvae from hatching until 6 dph.

	NEUTRAL GLYCOCONJUGATES	CARBOxYLATED GLYCOCONJUGATES	SULPHATED GLYCOCONJUGATES	GLYCOGEN	PROTEINS//NEUTRAL LIPIDS
Yolksac/Oil globules	2/0	0/0	0/0	1/0	3//3
Liver/Hepatocytes	1-2	0-1	0	2-3	1-3//1-3
Exocrine Pancreas/Zymogen Granules	0-1	0-1	0-1	0	3//0

Intensity of reaction: 0, negative; 1, weak; 2, moderated; 3, strong.

Table 2. Histochemical distribution of glycoconjugates in oesophagus, stomach and intestine in *Diplodus sargus* larvae at 15 dph.

	NEUTRAL GLYCOPROTEINS	CARBOXYLATED GLYCOPROTEINS	SULPHATED GLYCOPROTEINS	GLYCOGEN
Oesophagus				
Epitelium/Enterocytes	0-1	0-1	1	0
Mucous cells	0-1	2-3	1-2	0
Stomach				
Epithelium/Enterocytes	1-0	2	0	1
Gastric Glands/Larvae at 25 dph	1-0	2	0	1
Intestine				
Epithelium/Enterocytes	1	1	0	1
Mucous Cells	2	3	2	0
Supranuclear Inclusions/Posterior Intestine from 6dph	0	0	0	0

Intensity of reaction: 0, negative; 1, weak; 2, moderated; 3, strong.

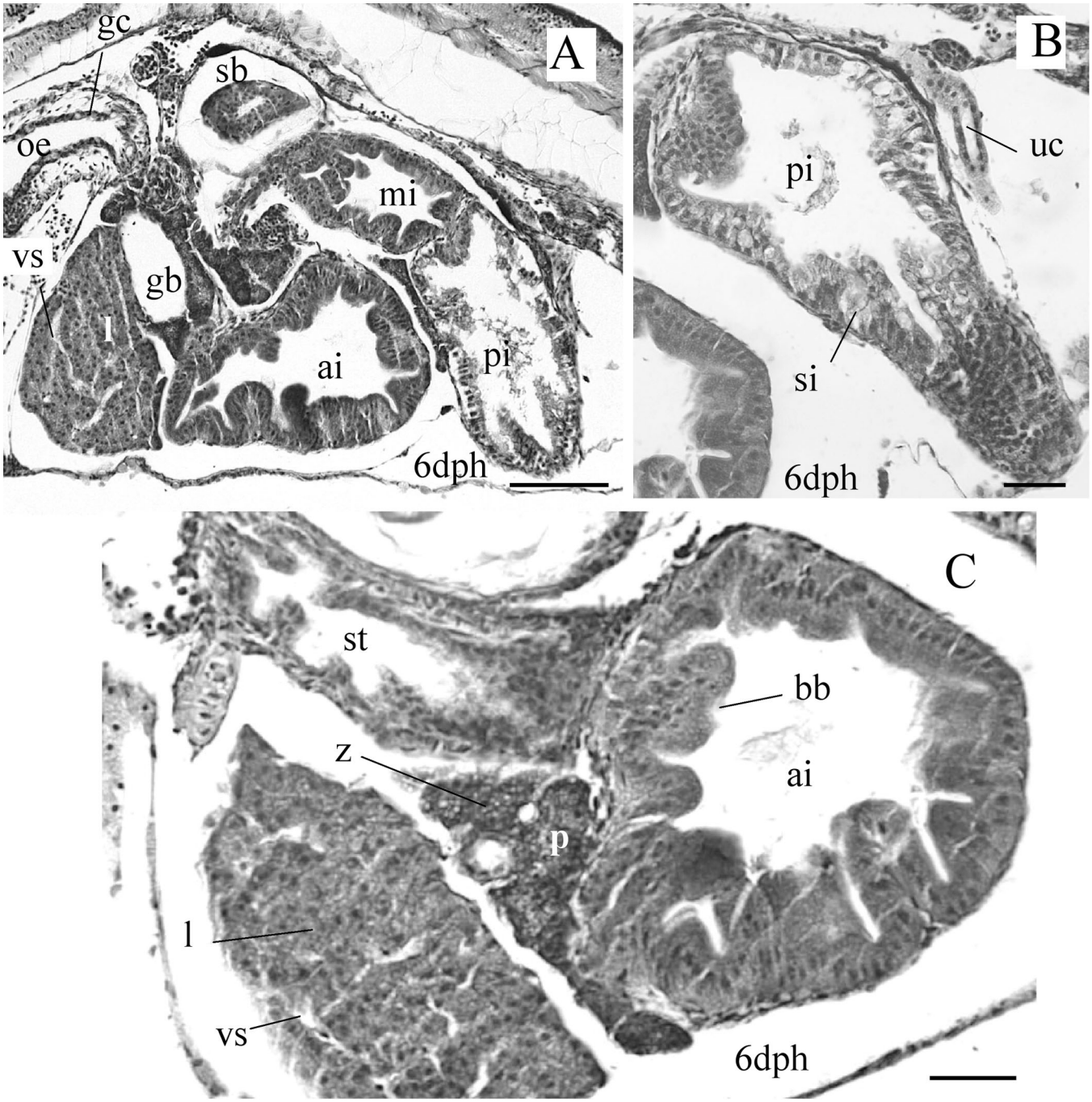

Fig. 3. Histological sections of *Diplodus sargus* larvae at 6 dph. **A.** Goblet cells start to be evident in the oesophageal epithelium. A differentiated liver with an evident vascular system, a functional exocrine pancreas with acidophilic zymogen and three different portions of the intestine (anterior, median and posterior) which shown an evident columnar epithelium are observed. Haematoxylin-eosin. Scale bar: 50 μm. **B.** Presence of acidophilic supranuclear inclusions in the enterocytes of the posterior intestine. Haematoxylin. Scale bar: 100 μm. **C.** Stomach developping appearing like a little pocket. A brush border is evident in the anterior portion of the columnar intestinal epithelium The hepatocytes shown a granular and vacuolized cytoplasm and the vascular system is observed clearly. The pancreocytes are organized in acini showing acidophilic zymogen granules. Haematoxylin-VOF. Scale bar 100 μm. ai: anterior intestine; bb: brush border; gb: gall bladder; gc: goblet cells; l: liver; oe: oesophagus; p: pancreas; st : stomach; mi: medial intestine; pi: posterior intestine; si : supranuclear inclusions; uc: urinary conduct; vs: vascular system; z: zymogen

loose connective tissue were evidenced in larvae from 3-4 dph. The lumen was lined by cells with short regular microvilli.

Taste buds appeared in the epithelium which lied on a sub-epithelial connective layer. The oesophageal wall was surrounded by a circular striated muscle layer which became thicker as the larvae grew, and a thin tunica serosa was peripherally located. A second inner longitudinal thin muscle layer was observed by 13 dph.

No mucous/goblet cells were present in the epithelium of oesophagus until 6 dph (Fig. 3A). From this time, goblet cells were profusely distributed among the other epithelial cells and they are very abundant during the first month of the larval life (Figs. 4C, 5D). Around 15 dph, oesophageal goblet cells (Table 2) contained neutral mucosubstances and/or acidic

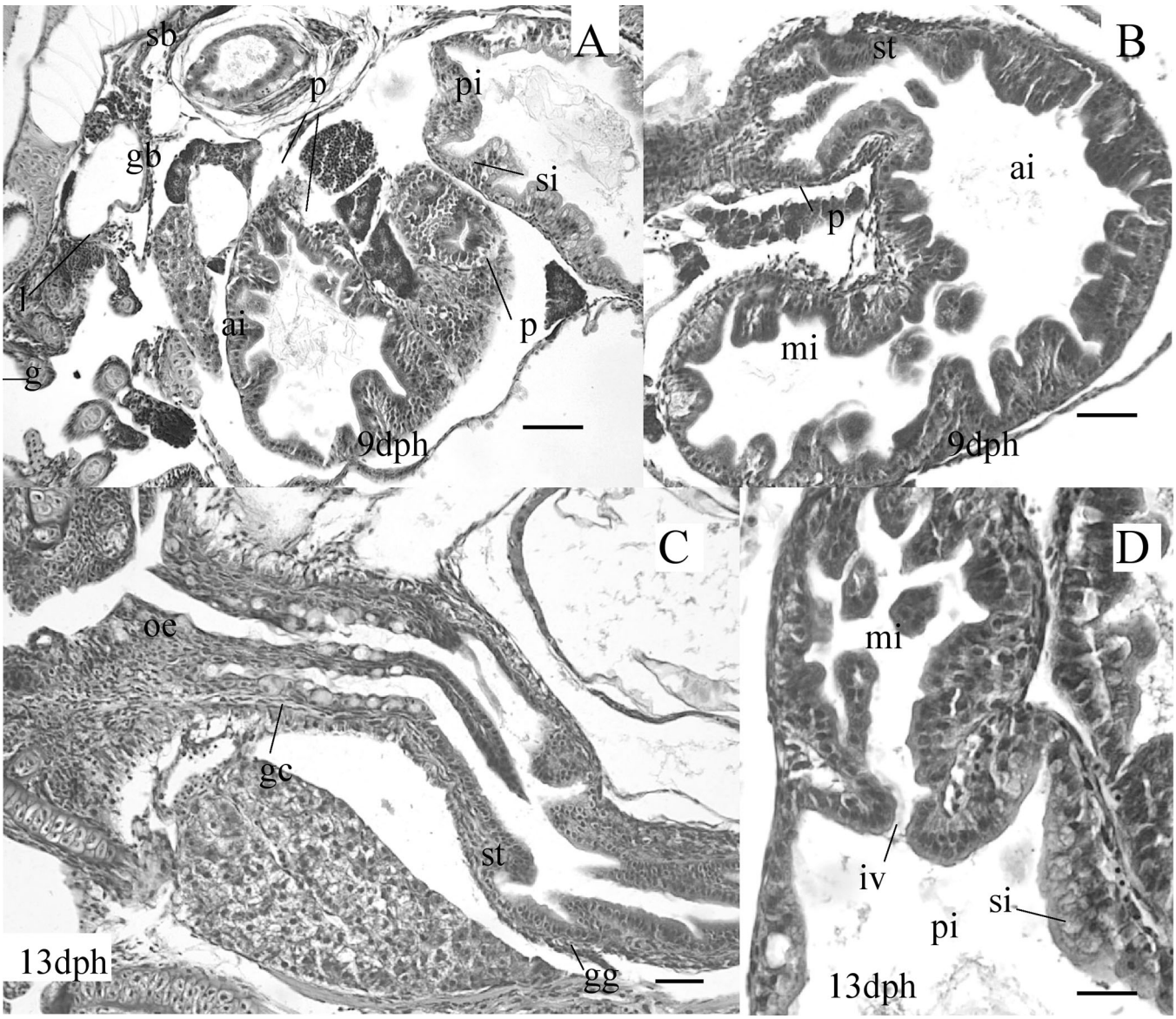

Fig. 4. Histological sections of *Diplodus sargus* larvae between 9 and 13 dph. **A, B.** Larvae at 9 dph showing several exocrine parcreatic portions in different localizations Acidophilic supranuclear inclusions are observed in the posterior intestine and the gastric glands are not evident in stomach. A. Haematoxylin-esosin; Scale bar 50 μm and B. Haematoxylin-VOF. Scale bar 75 μm. **C.** Oesophageal stratified epithelium of a larvae at 13dph with evident goblet or mucous cells. A simple columnar epithelium delineating the oesophagus-stomach transition is observed. First signs of gastric glands are apparent stomach. Haematoxylin-eosin. Scale bar 50 μm. **D.** Larvae at 13 dph. Ileo-rectal valve separating the medial and posterior portions of the digestive tract. Numerous supranuclear inclusions are evident in the epithelium of the posterior intestine. Haematoxylin-VOF. Scale bar 50 μm. ai: anterior intestine; gb: gall bladder; gc: goblet cells; l: liver; mi : medium intestine oe: oesophagus; pancreas; st : stomach; si : supranuclear inclusions; iv : intestinal valve.

glycoconjugates, the carboxylated ones being more abundant than the sulphated ones. Some mucous cells were only stained with PAS (neutral glycoconjugates), but other cells were stained in purple with Alcian Blue pH 2.5 + PAS double staining (neutral and carboxylated glycoconjugates). Goblet cells revealed their content of proteins to be rich in tyrosine, arginine and tryptophan from 15 dph (Table 3). No lipids were detected in these cells mucous cells.

Stomach

From 2-3 dph, the stratified epithelium of the oesophagus was replaced by a monostratified short-columnar epithelium at the entrance to the stomach anlage and a high columnar epithelium in the more caudal zone, which appears indistinguisable from the cells of the rest of digestive tract. By 6 dph, the future stomach appeared as a little pocket (Fig. 3 D) with a primordial pyloric sphincter. The mucosa was composed by a simple cubic epithelium with no signs of secretion and a subepithelial connective layer. The first longitudinal mucosa folds were observed on 3 dph. The epithelial cells with a thin granular cytoplasm gradually elongated and these columnar epithelial cells showed an oval nucleus in either basal or central position and short microvilli in their apical border. By 15 dph, a pyloric caecal ridge (pyloric sphyncter) divided the anterior intestinal region into the stomach and antero-median intestine.

The first signs of gastric glands were apparent by 13-15 dph and their number increased significantly by 23 dph (Figs. 4C, 5A-C), as did their content of proteins rich in tyrosine, arginine and tryptophan (Table 3). Like the columnar epithelial cells, the gastric glands were fully developed around 30-33 dph (Fig. 6A,B) forming

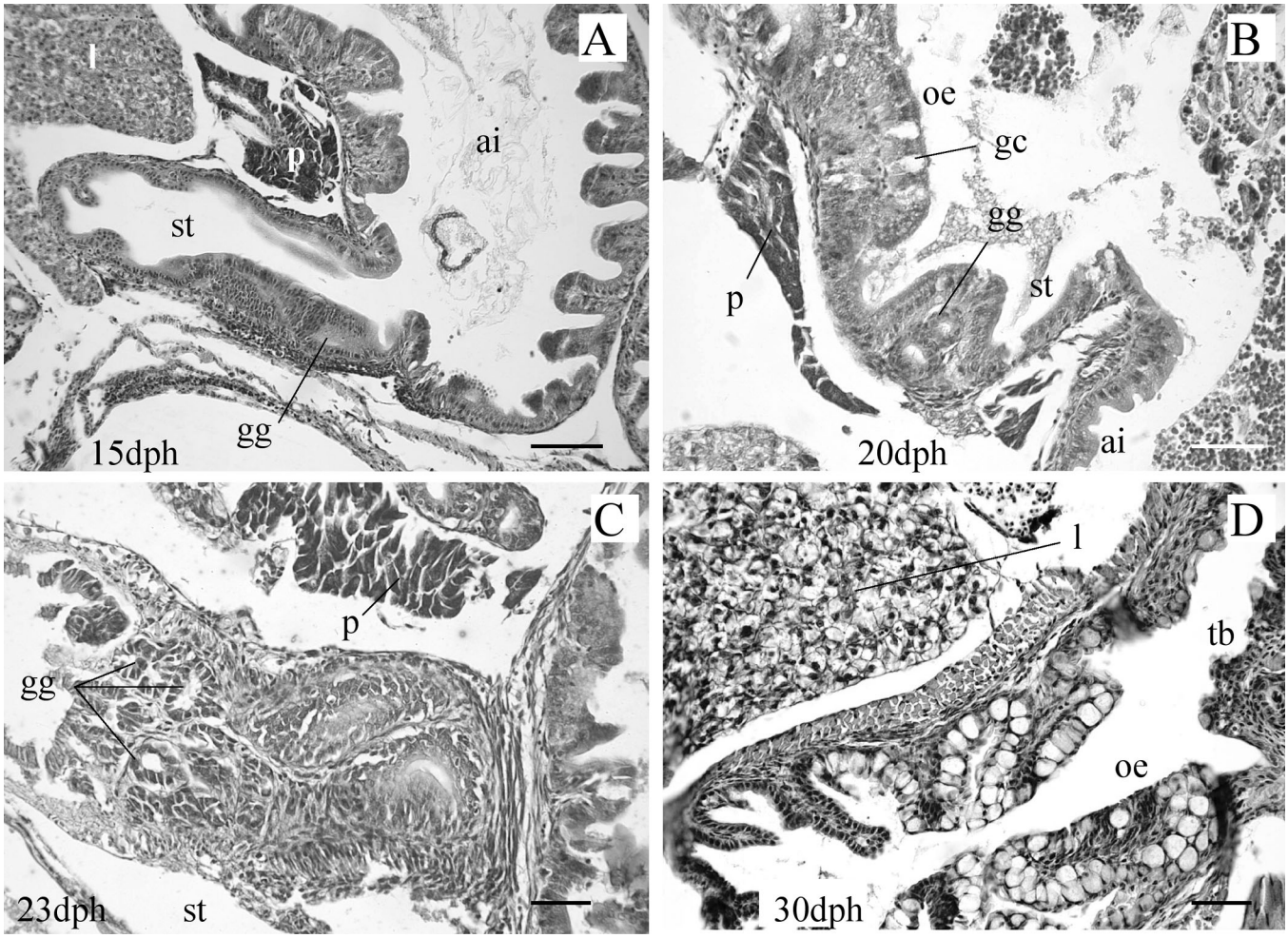

Fig. 5. Histological sections during the first month of the white seabream larval life. **A, B and C.** Progressive developping of gastric glands of the stomach at 15, 20 and 23 dph respectively, which are composed of a single type of secretory cell. Abundant goblet cells are observed in oesophageal stratified epithelium of larvae at 30 dph **D.** . Haematoxylin-eosin. ai: anterior intestine; gc: goblet cells; gg: gastric glands; l: liver; p: pancreas; st: stomach; tb: taste buds.

aggregates of cells connected to the lumen and surrounded by a delicate connective tissue layer. These tubular glands were composed of a single type of secretory cells devoid of microvilli on their apical border and lining their base with a simple cuboidal epithelium. The stomach wall was composed of mucosa, lamina propria-submucosa, muscularis and serosa layers. The deep mucosal layer was highly vascularised.

Three gastric regions could be differentiated: cardiac, fundic and pyloric. The muscular layer was thin in the cardiac portion but became thicker in the pyloric portion. Gastric glands were only located in the cardiac portion. In the stomach, the tunica muscularis consisted of two smooth muscle layers, an inner circular and an outer longitudinal. From the cardiac region towards the fundic region, a progressive change from striated into smooth muscle fibres was observed. The pyloric region (Fig. 6D) was relatively short, rich in connective tissue and showing an increase in the number of circular muscle fibres. The gastric serosa was formed by connective tissue containing capillaries and small blood vessels.

In the stomach, the epitelium and gastric glands contain glycogen, neutral and especially carboxylated glycoconjugates and proteins rich in different aminoacids, being those proteins rich in arginine, tyrosine and tryptophan specially abundant in gastric glands (Tables 2,3).

Table 3. Histochemical distribution of proteins in oesophagus, stomach and intestine in *Diplodus sargus* larvae between 6 and 15dph.

	GENERAL PROTEINS	LYSINE	TYROSINE	ARGININE	CYSTINE	CYSTEINE	TRYPTOPHAN
Oesophagus							
Epitelium/Enterocytes	0-1	1	2	1	2	3	0
Mucous Cells	0-1	2	0	0	2	2-3	0
Stomach							
Epithelium/Enterocytes	0-1	1	1	2-3	3	0-1	1
Gastric Glands/Larvae at 25 dph	2	1	2-3	2-3	1	1	2-3
Intestine							
Epitelium/Enterocytes	2	1	0	0	1	1	0
Mucous Cells	0	0	0	0	2	0	0
Supranuclear Inclusions/Posterior Intestine from 6dph	3	2-3	2-3	2-3	2-3	2-3	2-3

Intensity of reaction: 0, negative; 1, weak; 2, moderated; 3, strong.

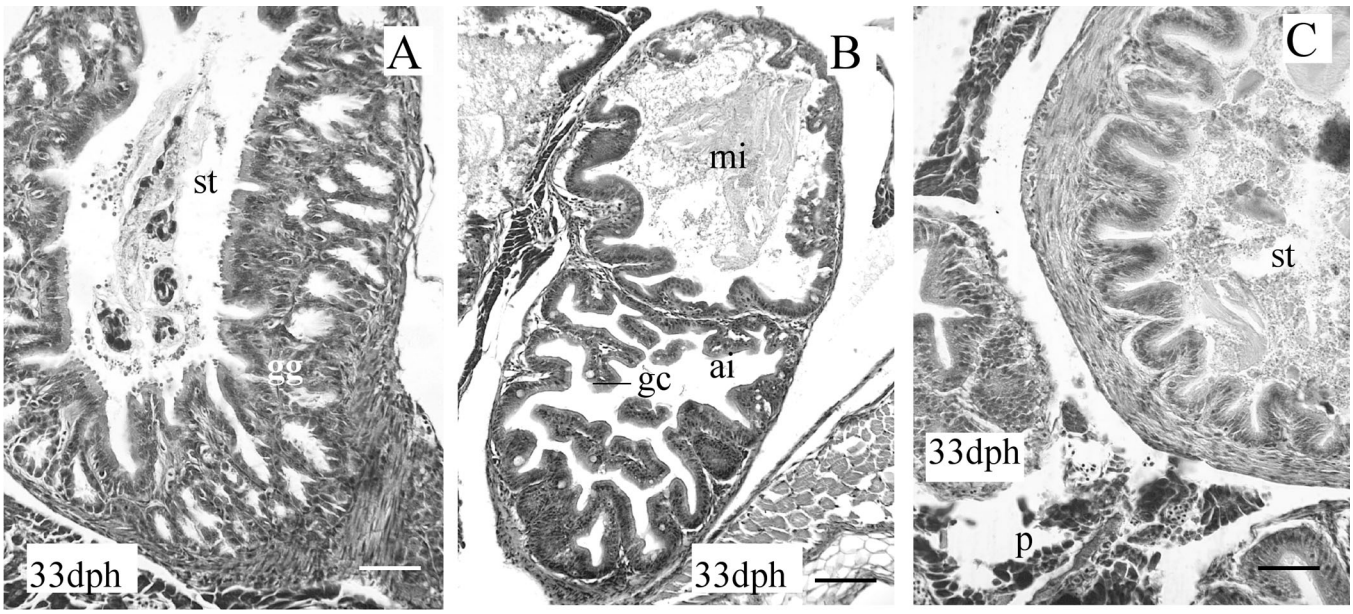

Fig. 6. Histological section of larvae at 33 dph **A.** Numerous developped gastric glands in the cardiac portion of stomach. Haematoxylin-VOF. Scale bar 75 μm. **B.** Antero and medial intestinal portions characterized by a high number of mucous cells and deeper mucosal folds. Haematoxylin-eosin. Scale bar 50 μm. **C.** Pyloric portion of stomach whitout gastric glands. Haematoxylin-eosin. Scale bar: 100 μm. ai: anterior intestine; gc: goblet cells; gg: gastric glands; mi: medium intestine; p: pancreas; st: stomach.

Intestine

The intestine was the longest portion of the digestive tract. By 2-3 dph, the luminal surface of the intestinal epithelium was composed of a monostratified columnar epithelium, which is bordered by a layer of microvilli or brush border at the apical surface. The intestinal abpsortive cells or enterocytes are arranged in a single layer and contain medium to basally located nuclei. The muscular layer was thin and composed of two muscular tissue layers: one circular internal and another longitudinal external separated by a very thin connective tissue layer.

By 2-3 dph a constriction correspondent to the future ileo-rectal valve appeared in the posterior third of the intestine clearly separating the antero-median from the posterior intestine. At 6 dph, the growing intestine formed a loop to accommodate in the visceral cavity, and three distinct regions could be differentiated. The antero-median portion received the pancreatic and biliary fluids and was characterized by a high number of goblet cells, very abundant close to the pyloric sphincter, which appeared by 15 dph and increased progressively during the larval development. These mucous cells exhibited the same histochemical characteristic described in the oesophagus (Tables 2, 3) containing neutral and/or carboxylated and/or sulphated glycoconjugates. The tall columnar enterocyte cells of this intestinal portion contained cytoplasmic vacuoles (neutral lipids dissolved during the histological procedure) which were stained with Red Oil O and Sudan Black in those fresh samples processed in a cryostat.

By 2 dph, mucosal transversal folds appeared in the anterior intestine and increased in size with increasing larval age. In the mid intestinal portion, the epithelial folds were deeper and more abundant. Finally, in the caudal intestinal portion, the enterocytes were not as tall as in the anterior intestine and the goblet cells were less numerous between 20 and 25 dph. Acidophilic supranuclear inclusions were already present in the enterocytes of the posterior intestine of 4-6 dph larvae (Fig. 3A,B). Active pinocytosis was evident at the base of microvilli of the enterocytes except for those near the anus. These acidophilic supranuclear inclusions were observed until 23-25 dph. They contained (Table 1) abundant proteins, especially rich in lysine and arginine, but also contained other aminoacids such as tryptophan, tyrosine, cysteine and cystine. PAS and Alcian Blue techniques were negative in these inclusions. The rectum was short and lined by a cubic epithelium. The enterocytes of this digestive portion were devoid of the supranuclear inclusions previously described when it was fully developed. The urinary bladder merged with the gut in this segment.

Extraparietal glands

At hatching, the liver was situated dorsally to the yolksac and ventral to the developing gut. At this time, the liver was differentiated and the hepatocytes were arranged in a chord-like pattern between the sinusoids. By 2 dph, the liver began to elongate and conform to the body cavity. The hepatocytes were loosely organized around a central vein and were not divided into distinct lobules. The bile duct connecting the liver and intestine was evident just before the total yolk resorption (3 dph) and it was lined by a single layer of epithelial cells. While in the early larval stages the hepatocytes had a basophilic homogeneous cytoplasm and a central nucleus, the granulation and vacuolization of the cytoplasm due to synthesis and storage of macromolecules (glycogen, proteins, lipids) increased progressively during larval development showing, around 4 dph, the hepatocytes a evident granular cytoplasm, a excentric nucleus, and a prominent nucleolus. Different histological aspects of the liver from hatching until the first month of larval life are shown (Figs. 1-5). Glycogen granules (PAS and distase-PAS positive) and proteins (Table 1), as well as oil globules stained with Black Sudan B and/or Red Oil O (unfixed samples processed in a cryomicrotome) or vacuoles (in paraffin sections) were easily detected in the cytoplasm of hepatocytes from 4-6 dph. The liver became bilobed at 15 dph.

In early larvae, the liver was located dorsally to the yolksac and the pancreas lies dorsal to intestine, and extends posteriorly to terminate occupying both sides of the abdominal cavity. By 2 dph, the basophilic cytoplasm of the exocrine pancreas was homogeneous and zymogen granules and pancreatic ducts were not apparent yet. In early larval stages, the pancreatic acinar cells resembled the hepatocytes in shape and they also display a spherical nucleus. Pancreatic ducts appeared just after yolk resorption by 3 dph. From this time, pancreatic acinar cells were grouped in rosette patterns around central canals that anastomosed with large ducts. Around 4-6 dph, in the exocrine pancreas, conspicuous acidophilic zymogen granules (Fig. 3A,C) containing proteins (Table 1) rich in tryptophan, cystine, tyrosine, lysine, cysteine and arginine were observed within the basophilic cytoplasm of the acinar pancreatic cells. These zymogen granules, which were abundant in the apical portion of cells, were strongly stained with orange G or eosin (H-VOF or H-E dyes), therefore distinguishing them from the hepatocytes and confirming the pancreas functionality. The exocrine pancreas lied primarily along the right side of the stomach but sending branches into the liver and the dorsal and ventral mesenteries of the gastrointestinal region. The pancreatic duct, like the bile duct, was lined with a cuboidal ephitelium and opened into the ventral part of the anterior intestine just after the pyloric sphincter. Neutral lipids were not detected in the exocrine pancreas.

Discussion

Metamorphosis from larvae to juvenile fish implies

important morphological and physiological changes. Such a transformation involves a progressive gut development and parallel changes in the feeding process. In species having a stomach, the complete development and functionality of the digestive tract is mainly related with the gastric gland formation in the stomach, the increment of pepsin activity and the apparition of an acidic digestion (Tanaka, 1971; Baglole et al., 1997; Douglas et al., 1999; Hamlin et al., 2000). It is evident that metamorphosis and specifically the gut development presents important inter-specific differences. Organogenesis of the digestive system and associated organs have been described for different marine fish species inhabiting cold waters, such as halibut, *Hippoglossus hippoglossus* (Blaxter et al., 1983; Luizi et al., 1999), cod, *Gadus morhua* (Bishop and Odense, 1966; Kjørvik et al., 1991) and haddock, *Melanogrammus aeglefinus* (Hamlin et al., 2000), and temperate waters such as gilthead seabream *S. aurata* (Cataldi et al., 1987; Sarasquete et al., 1993 a,b, 1995, 2001), seabass, *Dicentrarchus labrax* (Vu, 1976, 1980), *Senegal sole*, *Solea senegalensis* (Ribeiro et al., 1999; Sarasquete et al., 1996, 1998, 2001) and turbot, *Scophthalmus maximus* (Cousin and Baudin-Laurencin, 1985; Segner et al., 1994).

The gut of the marine teleostean fish develops from a straight and undifferentiated canal at hatching to a complex and segmented digestive tract in the juvenile stage. At the start of exogenous feeding, the digestive tract is generally anatomically differentiated and functional, except for the stomach which has just started to form at this time (Govoni et al., 1986; Segner et al., 1994; Sarasquete et al., 1995, 1996; Douglas et al., 1999; Ribeiro et al., 1999; Hamlin et al., 2000). In white seabream, as in other fish species (Boulhic and Gabaudan, 1992; Grau et al., 1992; Murray et al., 1994a,b; Bisbal and Bengtson, 1995; Arellano, 1995, 1999; Veggetti et al., 1999; Hamlin et al., 2000), the transition from oesophagus to stomach was evidenced by the total disappearance of the mucous/goblet cells and by the substitution of a stratified epithelium in oesophagus into a simple columnar epithelium in the stomach.

The oesophagus has a pregastric digestion role (Reifel and Travill, 1977; Murray et al., 1994a). Increased epithelial stratification in correspondence with an important acidic glycoprotein secretion from the goblet cells has been related with a supportive function for the oesophageal mucosa (Hirji, 1983; Baglole et al., 1997). The goblet cells of *D. sargus* oesophagus, as those of *S. maximu*s (Cousin and Boudin-Laurencin, 1985) and *S. aurata* larvae (Sarasquete et al., 1995) appear a few days after feeding starts. In *M. aeglefinus* (Hamlin et al., 2000) around 10 days after feeding starts at 8 ºC. Nevertheless, the mucous secretion appears earlier in other species, coinciding with the mouth opening, i.e., *Solea senegalensis* (Sarasquete et al., 1996; Vieira, 2000) and Solea solea (Boulhic and Gabaudan, 1992).

Goblet cells are common components in the postgastric mucosa of larvae and adult fish (Sarasquete et al., 1995, 2001; Ribeiro et al., 1999; Arellano et al., 2002). In different vertebrates, enclosing man, these cells are involved in the process of transport, absorption and protection in the gut (Rhodes et al., 1985; Anderson, 1986; Pajak and Danguy, 1993; Park and Kim, 2001). Variability in staining within a given goblet cell could be attributed to a temporal sequence in the mucus biosynthesis (Harrison et al., 1987). The coexistence of neutral and acid glycoconjugates may indicate a cell differentiation with progressing development (Elbal and Agulleiro, 1986; Murray et al., 1996). In different fish species, according to Sarasquete et al. (2001), as previously was indicated in *Rama fuscigula* by Els and Hennerberg (1990), PAS-positive goblet cells might represent an early developmental stage, when the cells are producing mainly neutral glycoproteins. Goblet cells stain with Alcian Blue (pH 2.5) when the glycoproteins are carboxylated, and the presence of sulphated glycoproteins (Alcian Blue pH 0.5) coincides with the stage when sulphated groups are conjugated to the glycoproteins. In *D. sargus* larvae, as in other fish species (Arellano et al., 1999, 2002; Sarasquete et al., 2001), some digestive mucous cells were only stained with PAS and diastase-PAS (neutral glycoconjugates), but other cells were stained either a purple or blue colour when an Alcian Blue pH 2.5-PAS double-staining was performed, indicating either the presence of a neutral and acidic mucin mixture or a carboxylated secretion exclusively.

Although an incipient stomach was distinguished in white seabream larvae from 2 dph, the first sign of gastric gland formation appeared from 13-15 dph, increasing their number and development by days 22-23, and being fully developed around 30-33 dph. In other marine fish species, gastric glands appear by the same age or later, around 25 dph, as in *Dicentrarchus labrax* (Vu 1976, 1980) and *Solea senegalensis* (Sarasquete et al., 1996, 2001) at 19-20 ºC, by 29-36 dph in *Pleuronectes ferruginea* at 10 ºC (Baglole et al., 1997) and around 30- 33 dph in *Paralichthys dentatu*s (Bisbal and Bengtson, 1995) at 20 ºC, *Scophthalmus maximus* (Segner et al., 1994) and *Melanogrammus aeglefinus* at 8 ºC (Hamlin et al., 2000). Gastric gland secretions, pepsinogen and hydrochloric acid, provide preliminary extracellular protein digestion followed by membrane transport which replaces the less efficient processes of pinocytosis and intracellular digestion of proteins (Govoni et al., 1986). Walford and Lam (1993) reported that in the absence of a functional stomach, the anterior intestine is responsible for food digestion, with a pH that remains alkaline and where protease trypsin-like (serine type) activities (Moyano et al., 1996) take charge of this protein digestion. On the other hand, during white seabream larvae development, acidophilic zymogen-granules containing abundant proteins (enzymatic precursors) were detected in basophilic cytoplam of pancreocytes from 3-4 dph and especially by 6 dph. According to Hjelmeland (1995), yolksac larvae give

priority to synthesis and accumulation of digestive capacity in the form of pancreatic enzymes, suggesting a strategy of the larva to be ready to digest to the establishment of exogenous feeding.

Wormhoudt et al. (1980) indicated that arginine may regulate the synthesis of digestive proteases. The columnar epithelium of stomach may be the precursor of the pepsinogen-secreting gastric cells (Douglas et al., 1999). Neutral glycocjugates were detected in columnar epithelium of *D. sargus* larvae stomach, which also containe cysteine and especially cystine residues. It is interesting to remark that proteins rich in tyrosine, arginine and tryptophan were very abundant in gastric glands around 22-23 dph, while in *S. senegalensi*s stomach they became developed between 50 and 80 dph (Vieira, 2000, Sarasquete et al., 2001). The presence of neutral mucins, residues of cysteine and cystine in the columnar epithelium of stomach, as well as the presence of tryptophan, tyrosine and arginine particles in gastric glands could be related to the synthesis and secretion of enzymatic precursors, i.e. pepsinogen (Medeiros et al., 1970a,b; Gutiérrez et al., 1986; Grau et al., 1992; Gisbert et al., 1999; Vieira, 2000).

Reifel and Travill (1978) and Elbal and Agulleiro (1986) pointed out that the positive PAS reaction (neutral glycoconjugates) observed in the gastric epithelial cell surface resembles that observed in the striated border of intestinal enterocytes. It may indicate nutrient absorption occurring in the stomach. Neutral glycoconjugates have been detected in *D. sargus* stomach, and this fact has been related to the absorption of easily digestible substances such as disaccharides and short-chain fatty acids (Grau et al., 1992). On the other hand, the neutral mucous secretion in the stomach may serve to protect its epithelium from auto-digestion processes caused by hydrochloric acid and the enzymes secreted by gastric glands (Ferraris et al., 1987). Sulphated glycoconjugates are not present in gastric glands of some fish species (Sarasquete et al., 2001), including D. sargus larvae, but they have been observed in other species (Reifel and Travill, 1978; Grau et al., 1992). Spicer and Schulte (1992) speculated that sulphomucins may be able to form a complex with pepsin, thereby stabilizing or buffering the enzyme.

There are inter-specific differences in the gastric gland locations in the stomach of fishes. In some species, such as S. aurata (Elbal and Agulleiro, 1986), *Seriola dumerili* (Grau et al., 1992) and in *D. sargus* larvae, gastric glands were observed in the cardiac stomach region. In *Pleuronectes ferruginea* (Baglole et al., 1997), *Paralichthys dentatus* (Bisbal and Bengtson, 1995), Scophthalmus maximus (Segner et al., 1994) and *Solea solea* (Veggeti et al., 1999) larvae, gastric glands were located in the fundic stomach region. In both juvenile (Vieira, 2000) and adult *Solea senegalensis* specimens (Arellano et al., 2001), gastric glands were observed in fundic and pyloric regions. On the other hand, the stomach of adult *Hippoglossus hippoglossus* is entirely glandular, probably because it consumes large prey (Murray et al., 1994a).

Fat droplets in the midgut have been reported either in larvae (Kjørsvik et al., 1991; Sarasquete et al., 1995; Calzada et al., 1998) and adult teleost fish (Deplano et al., 1989, 1991; Arellano et al., 2002). Ingested lipids are hydrolysed, absorbed and after a resynthesis they are stored as lipid droplets in the enterocytes of epithelium (Iwai, 1969; Loewe and Eckmann, 1988). In *D. sargus* larvae, these lipid droplets are present in the antero-median intestine throughout development, although large and excessive lipid accumulations (steatosis/temporary storage) were never observed. In fish, lipid droplets seem to be a form of temporary storage of re-esterified fatty acids that accumulate when their uptake exceeds the enterocyte exporting capacities (Sheridan, 1988), or because an inability to metabolize lipids (Kjorsvik et al., 1991). Deplano et al. (1991) suggested that the excessive abundance of lipid droplets of varying size in the intestinal absortive cells/enterocytes could be the result of a default in the lipoprotein synthesis mechanism.

The final portion of the intestine is actively involved in the absorption of digestive products during the larval stage. Acidophilic supranuclear inclusions have been usually observed in the posterior intestine of larvae (Govoni et al., 1986; Kjorsvik et al., 1991; Sarasquete et al., 1993a, 1995; Ribeiro et al., 1999; Gisbert et al., 1999; Hamlin et al., 2000). In *D. sargus* larvae they are evident from a few days after feeding started up to 23 dph. These inclusions seem to reflect the absorption of protein macromolecules as an alternative pathway until the stomach develops a high proteolitic capacity. In fact, acidophilic inclusions were absent in starved larvae of other sparids (Yúfera et al., 1993; Crespo et al., 2001) and in well-fed *D. sargus* larvae they disappeared when gastric glands were formed and a general increase of enzymatic activities were measured (Cara et al., 2003).

The gut ontogeny in D. sargus follows the general pattern found in teleosts although there is some interspecific variability in the timing at which the different events occur. When comparing sparid species living in temperate waters in the same geographic area, it seem that *D. sargus*, *P pagrus* and *D. dentex* exhibit faster development than *S. aurata*. In fact, in *S. aurata* gastric glands were never detected during the first six weeks alter hatching (Domeneghini et al., 1998; Sarasquete et al., 2001) while the other gastric glands of the other three species gastric glands are well developed by the first month of life (Roo et al., 1999; Marín de Mateo et al., 2001).

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