Histol Histopathol (2008) 23: 1077-1091

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Ontogeny of the digestive tract in sharpsnout sea bream *Diplodus puntazzo* (Cetti, 1777)

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Summary. The ontogeny of the digestive tract was studied histologically and histochemically in sharpsnout sea bream *Diplodus puntazzo* from hatching (0 DAH, Days After Hatching) until day 57 (57 DAH). At hatching, the digestive tract appeared as a histologically undifferentiated straight tube lying dorsally to the yolk sac. When the mouth opened at 3 DAH, the digestive tract was differentiated into buccopharynx, oesophagus, incipient stomach and intestine. The pancreas, liver and gall bladder were also differentiated at this stage and both the bile and pancreatic duct had opened into the anterior intestine. Active feeding began in 50% of larvae at 4 DAH, although permanence of yolk reserves until 7 DAH suggests a period of both endogenous and exogenous feeding. Nutrient absorption was first visible from 5 DAH, as colourless supra- and infranuclear vacuoles in the anterior intestinal mucosa, suggesting a lipid content, as well as supranuclear, eosinophilic vacuoles, containing protein, in the posterior intestinal mucosa. Early caecal development could be detected from 10 DAH, whereas gastric glands appeared at 30 DAH, indicating the transition from larval to juvenile stage and the acquisition of an adult mode of digestion. Goblet cells appeared in the digestive tract of sharpsnout sea bream larvae shortly after first feeding. The mucus content of goblet cells varied with the digestive region and, in the buccal cavity and oesophagus, also with the developmental phase. This study provides knowledge for better husbandry practices in the aquaculture industry, as well as for the implementation of future nutritional studies.

Key words: Digestive system, *Diplodus puntazzo*, Larval development, Histology, Histochemistry

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Introduction

The sharpsnout sea bream *Diplodus puntazzo*, Cetti 1777, represents an appreciated fishery resource occurring throughout the Mediterranean, the Black Sea and the eastern coasts of the Atlantic ocean, from the Bay of Biscay to Sierra Leone, the Canary Islands and Cape Verde. It is considered as one of the most interesting species for diversification in aquaculture due to its high market price, ease of reproduction in captivity (Faranda et al., 1985; Caggiano et al., 1993; Greco et al., 1993), high growth rate and food conversion efficiency (Hernández et al., 2001; Favaloro et al., 2002). Although the first rearing trials date back to more than 20 years (Divanach and Kentouri, 1982), massive production of this species is still limited because of hatchery problems during larval rearing, particularly at transition from endogenous to exogenous feeding and weaning to artificial diets. Such problems, some of which arise from a lack of knowledge concerning nutritional requirements and feeding sequences, result in low and unpredictable survival rates (ranging from 3 to 40%) (Marangos, 1995). Better results have recently been obtained using the mesocosm larval rearing system, which attained 54% survival (Papandroulakis et al., 2004).

A detailed understanding of the development of the digestive tract, particularly as far as ingestion, digestion and assimilation mechanisms are involved, will contribute to the improvement of larval survival and growth in different rearing conditions, through the development of feeding protocols that match the nutritional requirements and digestive ability of the larvae. In this context, a histological and histochemical description of the larval digestive system represents the first step towards the determination of the functional relationships between feeding and assimilation.

The organogenesis of the digestive system has been investigated in a number of commercially valuable

finfish species, including the halibut (Blaxter et al., 1983), European turbot (Cousin and Baudin Laurencin, 1985), cod (Kjørsvik et al., 1991), the Dover and Senegal sole (Boulhic and Gabaudan, 1992; Ribeiro et al., 1999), summer and yellowtail flounder (Bisbal and Bengston, 1995; Baglole et al., 1997), gilthead seabream (Sarasquete et al., 1995; Elbal et al., 2004), haddock (Hamlin et al., 2000), sea bass (García-Hernández et al., 2001), clownfish (Gordon and Hecht, 2002), white seabream (Ortiz-Delgado et al., 2003), California halibut (Gisbert et al., 2004), common dentex (Santamaría et al., 2004), common pandora (Micale et al., 2006), shi drum (Zaiss et al., 2006), red porgy (Darias et al., 2007) and redbanded seabream (Sánchez-Amaya et al., 2007). These studies bear witness to a common ontogenetical process of the digestive system, even though considerable differences exist in the timing at which developmental events occur.

Different aspects of early life development of sharpsnout sea bream have been investigated in the last decade (Romano et al., 1999; Boglione et al., 2003; Sfakianakis et al., 2005; Korkut et al., 2006; Kouttouki et al., 2006; Saavedra et al., 2007; Suzer et al., 2007). However, the histological and histochemical changes that take place during gut ontogeny have never been reported. The purpose of this paper was to describe the morphogenesis of the digestive tract and its associated organs (liver, gall bladder and pancreas) in sharpsnout sea bream reared using the mesocosm technique (Papandroulakis et al., 2004), in order to provide the necessary knowledge for better husbandry practices in the aquaculture industry, as well as for the implementation of future nutritional studies.

Materials and methods

Larval rearing using the mesocosm technique

Larval rearing was carried out at the mesocosm hatchery of the Institute of Aquaculture, Hellenic Center for Marine Research (HCMR) (Crete, Greece). Two hundred thousand eggs obtained from a wild broodstock spawning naturally in tanks, were introduced into a 40 m^3 semi-indoor tank initially filled with surface sea-water (40 psu) and subsequently supplied with water from a shallow well (32 psu). The daily water renewal increased progressively from 20% to 400% at 42 days after hatching (DAH) and remained constant up to the end of the experimental trial (57 DAH). The tank was exposed to natural daylight, with additional light provided during the night (600 lux). During the experiment, oxygen saturation ranged between 90 and 95%. Water temperature decreased gradually from 22.3°C at the beginning of the experiment to 19.0±0.3°C at 7 DAH and remained constant thereafter. From 3 to 38 DAH, the culture was carried out in the presence of phytoplankton (*Chlorella minutissima*), which has been isolated from Heraklion bay since 1992 and mass produced in photo-bioreactors at HCMR (Hatziathanassiou et al., 2001). The phytoplankton concentration which was added daily was 300-400x10^3 cells ml^{-1} from 3 to 25 DAH, 200x10^3 cells ml^{-1} from 25 to 30 DAH and 100-150x10^3 cells ml^{-1} from 30 to 38 DAH, at which time phytoplankton use was discontinued. A surface skimmer was installed between 4 and 15 DAH to keep the surface free from oils, a prerequisite for successful swim-bladder inflation. From 4 to 30 DAH, larvae were fed on rotifers (*Brachionus plicatilis*) enriched with a commercial nutrient emulsion (DHA Protein Selco, INVE S.A., Belgium, containing 27% crude protein, 29% crude lipids and 5 mg/g Σω3 HUFA [Highly Unsaturated Fatty Acids]), supplied twice daily to maintain a concentration of 2-3 rotifers ml^{-1} in the tank. From 18 to 53 DAH, Artemia spp. nauplii enriched with a commercial nutrient emulsion (A1 DHA Selco, INVE S.A., Belgium, containing 67% crude lipids and min. 200 mg/g Σω3 HUFA) were provided continually in order to maintain a concentration of 0.05-0.35 nauplii ml^{-1}. Commercial feed crumbles of progressively larger size (R1 100; Proton 2/3, particle size 200-300 μm; NRD 3/5, particle size 300-500 μm; and NRD 5/8, particle size 500-800 μm, INVE S.A., Belgium) were provided continually from 29 DAH onwards, by means of electrical feeders. The biochemical composition of the commercial crumbles was 55-59% crude proteins, 14-16% crude lipids and 28-30 mg/g of Σω3 HUFA, with a DHA/EPA (Docosa-hexaenoic Acid/Eicosapentaenoic Acid) ratio of 2.

Morphology, histology and histochemistry

A random sample of 30 larvae was collected daily from hatching (0 DAH) to 20 DAH, every second day from 22 to 30 DAH, and every third day from 33 to 57 DAH, and anaesthetized with clove oil (Mylonas et al., 2005). A sub-sample of 10 larvae was observed under a stereoscope equipped with a graded eyepiece for morphological and morphometrical evaluations. Total length (TL) was measured to the nearest 0.01 mm. Wet weight (WW) was measured at 20, 30, 42, 51 and 57 DAH, while mortalities were recorded after 30 DAH, when siphoning of the bottom of the tank was initiated. For the histological study, 10 larvae were fixed in Bouin's solution, dehydrated in graded alcohols, embedded in a methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) and cut in 3-5 μm-thick sagittal sections. The Haematoxylin/Eosin (HE) stain was used for general histological observations under a light microscope to describe the development of the digestive tract and accessory glands. The following histochemical reactions were performed on the remaining 10 larvae, either 4% formaldehyde- or Bouin's fluid- fixed, and subsequently embedded in paraffin: Diastase/Periodic Acid Schiff (D/PAS) for glycogen (Bancroft et al., 1990); Alcian Blue 8GX pH 2.5/Periodic Acid Schiff (AB/PAS) for carboxylated (sulphated or not) and neutral glycoconjugates (Mowry, 1963); High Iron Diamine/Alcian Blue 8GX pH 2.5

(HID/AB) for sulphated and carboxylated, non-sulphated glycoconjugates (Spicer, 1965; Reid et al., 1989). Histochemical methods for general proteins (Bromophenol Blue, BB), as well as for proteins containing tryptophan (p-dimethylaminobenzaldehyde, DMAB), and proteins containing amino acids rich in disulfide and sulphydryl linkages, such as cysteine (performic acid-Alcian Blue method, followed by eosin, PFA/AB/EO), were performed according to Pearse (1985) and Bancroft et al. (1990).

Results

Growth and morphology

The growth of sharpsnout sea bream larvae followed an exponential curve (Fig. 1). Survival was 68% at the end of the mesocosm rearing phase, and the final number

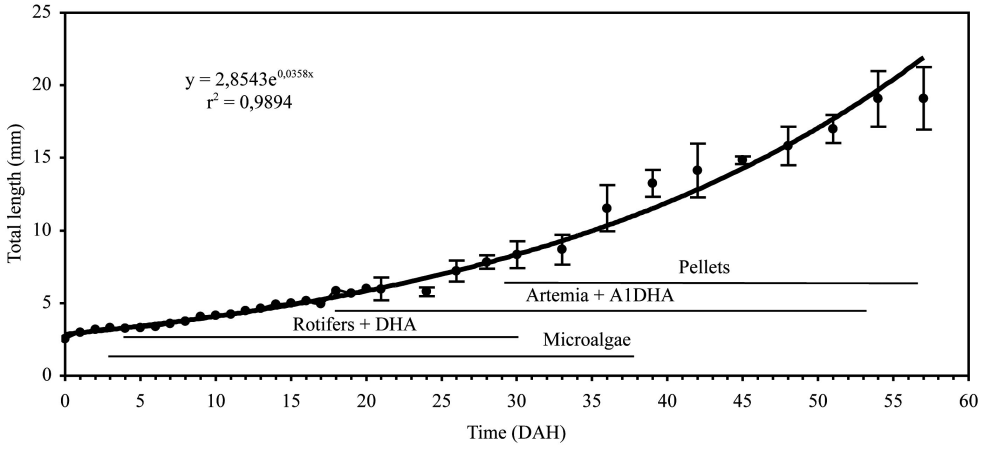

Fig. 1. Feeding protocol and growth in size (mean ± S.D., n=10) of sharpsnout sea bream larvae from hatching (0 DAH) to 57 days (57 DAH).

Table 1. Major events during larval development of sharpsnout seabream *Diplodus puntazzo*.

Stage	DAH	TL (mm)	Feeding mode	Histo-morphological observations
Endotrophic	0-3	2.55±0.05	Endogenous	Segmentation of gut into buccopharynx, oesophagus, stomach anlage, anterior and posterior intestine [2]. Differentiation of liver, gall bladder, exocrine and endocrine pancreas [2]. Mouth opening [3]. Intestinal looping and early mucosal folding [3]. Opening of biliary and pancreatic ducts into anterior intestine [3]. Eyes pigmented [3].
Endo-exotrophic	4-7	3.25±0.07	Endogenous and exogenous	Anus opening [4]. Protein and glycogen in the liver [4]. Yolk sac resorption [5]. Protein [5] and probable lipid [5] vacuolization in posterior and anterior intestine, respectively. Goblet cells in pharynx and oesophagus [5]. Mucosal folding of the oesophagus [6].
Exotrophic	8-57	3.74±0.20	Exogenous	Oil drop resorption [8]. Inflation of swim bladder [8]. Ileo-rectal valve fully developed [10]. Early caecal development [10]. Bucco-pharyngeal taste buds [11]. Notochord flexion [18]. Goblet cells in the buccal cavity [24]. Stomach segmentation [26]. Formation of gastric glands [30]. Anal and dorsal fins fully developed [33]. Goblet cells in the intestine [33]. Eruption of pharyngeal teeth [42-45].

DAH: Days After Hatching. Total length is expressed as mean±S.D. at the beginning of each stage. Numbers in brackets indicate the age in DAH.

of sharpsnout sea bream was 135,000 individuals. At 51 DAH, the mean (± SD) TL was 16.98±0.98, and the mean (± SD) WW was 540±135 mg.

At hatching (TL: 2.55±0.05 mm SD, standard deviation, *n*=10), the digestive tract appeared as a straight tube lying dorsally to the yolk sac. The anterior and posterior ends were closed. At 3 DAH (TL: 3.21±0.11 mm, *n*=10) the mouth was open, but no food could be identified in the digestive tract. At the same stage the eyes were pigmented and the pectoral fins were visible. At 4 DAH (TL: 3.25±0.07 mm, *n*=10) about 50% of the larvae were actively feeding and the yolk sac was almost completely resorbed. At the same stage the anus was open and the first chromatophores (4-6) appeared. At 8 DAH (TL: 3.74±0.20 mm, *n*=10) the oil drop was resorbed and the swim bladder was inflated in 100% of larvae. Notochord flexion began at 14 DAH (TL: 4.93±0.23 mm, *n*=10) in 30% of larvae and was completed in all fish by 18 DAH (TL: 5.85±0.25 mm, *n*=10). Anal and dorsal fins were completely formed by 33 DAH (TL: 8.69±1.03 mm, *n*=10).

Histology and histochemistry

The major ontogenetic events occurring during larval development of sharpsnout seabream are summarized in Table 1. The results of histochemical reactions showing the distribution of carbohydrates and proteins in the different gut regions throughout larval development are reported in Table 2.

At hatching the digestive tract appeared histologically undifferentiated (Fig. 2A). Within 2 days from hatching, sharpsnout seabream underwent rapid developmental changes, so that four segments could be distinguished in the digestive tract, namely the buccopharynx, oesophagus, presumptive stomach and intestine. At this stage, a conspicuous mass of strongly eosinophilic yolk globules was still visible in the resorbing yolk sac (Fig. 2B).

Buccopharynx

At mouth opening by 3 DAH, the oral cavity was lined by a simple squamous epithelium, while the pharynx was lined by a pseudostratified epithelium (Fig. 3A). The first goblet cells appeared at 5 DAH in the pharyngeal mucosa (Fig. 3B). These goblet cells were AB-positive with both the AB-PAS and HID-AB sequences, indicating the presence of carboxylated, non-sulphated glycoconjugates, but were negative to both PAS and protein reactions. The first buccopharyngeal taste buds appeared among epithelial cells at 11 DAH (Fig. 3C). Buccal goblet cells were visible from 24 DAH, especially in the ventral surface of the oral cavity (Fig. 3D), and were shown to contain carboxylated glycoconjugates (AB-positive) from 31 DAH. From 36 DAH they were HID-positive in the HID-AB sequence, showing the presence of sulphated glycoconjugates, in addition to carboxylated, non-sulphated ones (Fig. 3E). Taste buds and goblet cells became more numerous as larvae grew. Rudimentary pharyngeal teeth could be detected from 20 DAH as BB-positive (Fig. 3F) and

Table 2. Histochemical distribution of carbohydrates and protein in the digestive tract during larval development of sharpsnout seabream *Diplodus puntazzo*.

	Neutral glycoconjugates	Carboxylated glycoconjugates	Sulphated glycoconjugates	Glycogen	General proteins	Cysteine	Tryptophan
Buccal cavity							
Goblet cells	0	2-3 [31]	2 [36]	0	0	0	0
Pharynx							
Goblet cells	0	2-3 [5]	0	0	0	0	0
Oesophagus							
Goblet cells	0	2-3 [7]	2 [17]	0	3 [20]	3 [20]	0-1 [20]
Stomach							
Epithelium	2 [30]	0	0	0	0	0	0
Gastric glands	0-1 [30]	0-1 [30]	0	0	3 [30]	0	0
Intestine							
Epithelium/Enterocytes	3 [2]	0	0	0	2-3 [2]	0	2-3 [2]
Goblet cells	2-3 [33]	2-3 [33]	0	0	0	0	0
Supranuclear inclusions (posterior intestine)	0	0	0	0	3 [5]	0-1 [5]	0
Liver							
Hepatocytes	0	0	0	3 [4]	2-3 [4]	1 [4]	2-3 [4]
Pancreas							
Zymogen granules	0	0	0	0	3 [7]	0	1-2 [7]

Reaction intensity: 0, negative; 1, weak; 2, moderate; 3, strong. Timing of appearance of a positive reaction is reported in brackets as Days After Hatching [DAH].

erupted between 42 and 45 DAH, while mandibular and maxillary teeth, which were first visible at 30 DAH, had not yet erupted at 57 DAH.

Oesophagus

At 2 DAH the oesophagus could be distinguished as a short, narrow duct lined by a columnar, ciliated epithelium (Fig. 4A). Developing circular smooth muscle cells were visible in the oesophagus wall. At 5 DAH the inner mucosal surface of the oesophagus was expanded in several longitudinal folds. At the same stage, goblet cells appeared interspersed between epithelial cells. The oesophageal goblet cells were AB-positive from 7 DAH and AB- and HID-positive from 17 DAH (Fig. 4B), indicating the sequential appearance of carboxylated and sulphated glycoconjugates, respectively. On the other hand, these goblet cells contained no neutral glycoconjugates (PAS-negative) and were unreactive to protein reactions. The oesophageal goblet cells became more and more numerous throughout larval development, showing a strong positivity to protein reactions, particularly the PFA/AB/EO sequence for cysteine-rich proteins, from 20 DAH. A submucosa formed by loose connective tissue, a circular muscle layer (*tunica muscularis)* and a thin outer serosa completed the oesophagus wall.

Stomach

The presumptive stomach started to differentiate at 2 DAH as a dilated tract posterior to the oesophagus, lined by a simple cuboidal epithelium with basal nuclei (Fig. 4A). At 5-6 DAH a primordial pyloric sphincter developed, separating the future stomach from the anterior portion of the intestine. The stomach epithelium close to the sphincter, from which the pyloric portion of the stomach would develop, became columnar (Fig. 5A). At this stage, the stomach appeared elongated and its mucosal surface was expanded in mainly longitudinal folds. At 10 DAH, the intestinal mucosa immediately posterior to the pyloric sphincter showed pronounced evaginations, indicating the beginning of caecal development (Fig. 5B). By the same time, the cardiac portion of the stomach, lined by a simple cuboidal epithelium, could clearly be distinguished from the fundic and pyloric ones (Fig. 5B), both lined by a thicker columnar epithelium, which appeared PAS-positive in its

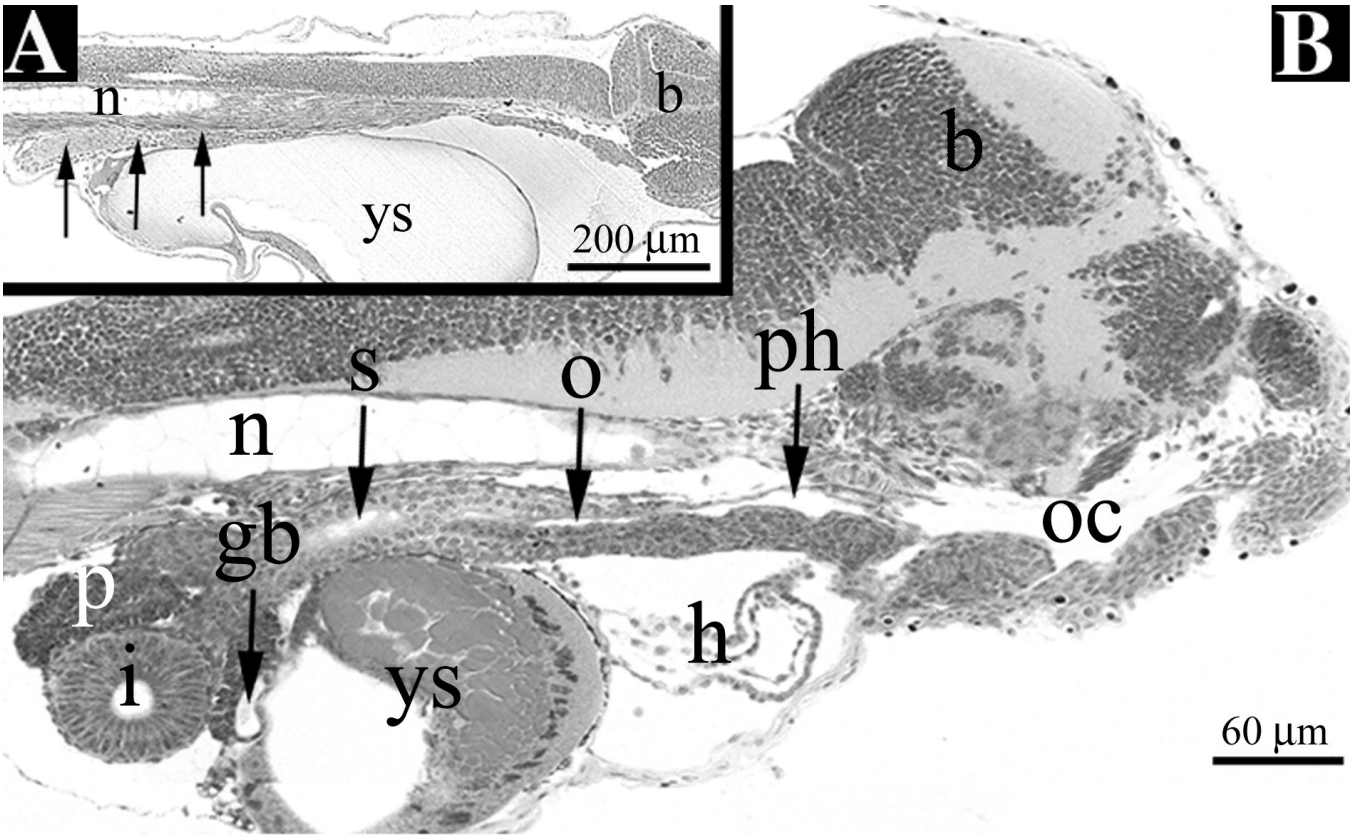

Fig. 2. A. General view of a sharpsnout sea bream larva at hatching (0 DAH), showing the undifferentiated digestive tract (arrows); HE. **B.** General view of the digestive tract in a 2-day-old yolk sac-larva of sharpsnout sea bream, showing the oral cavity (oc), pharynx (ph), oesophagus (o), future stomach (s), intestine (i), pancreas (p) and gall bladder (gb); HE. b: developing brain; h: primitive heart; n: notochord; ys: yolk sac.

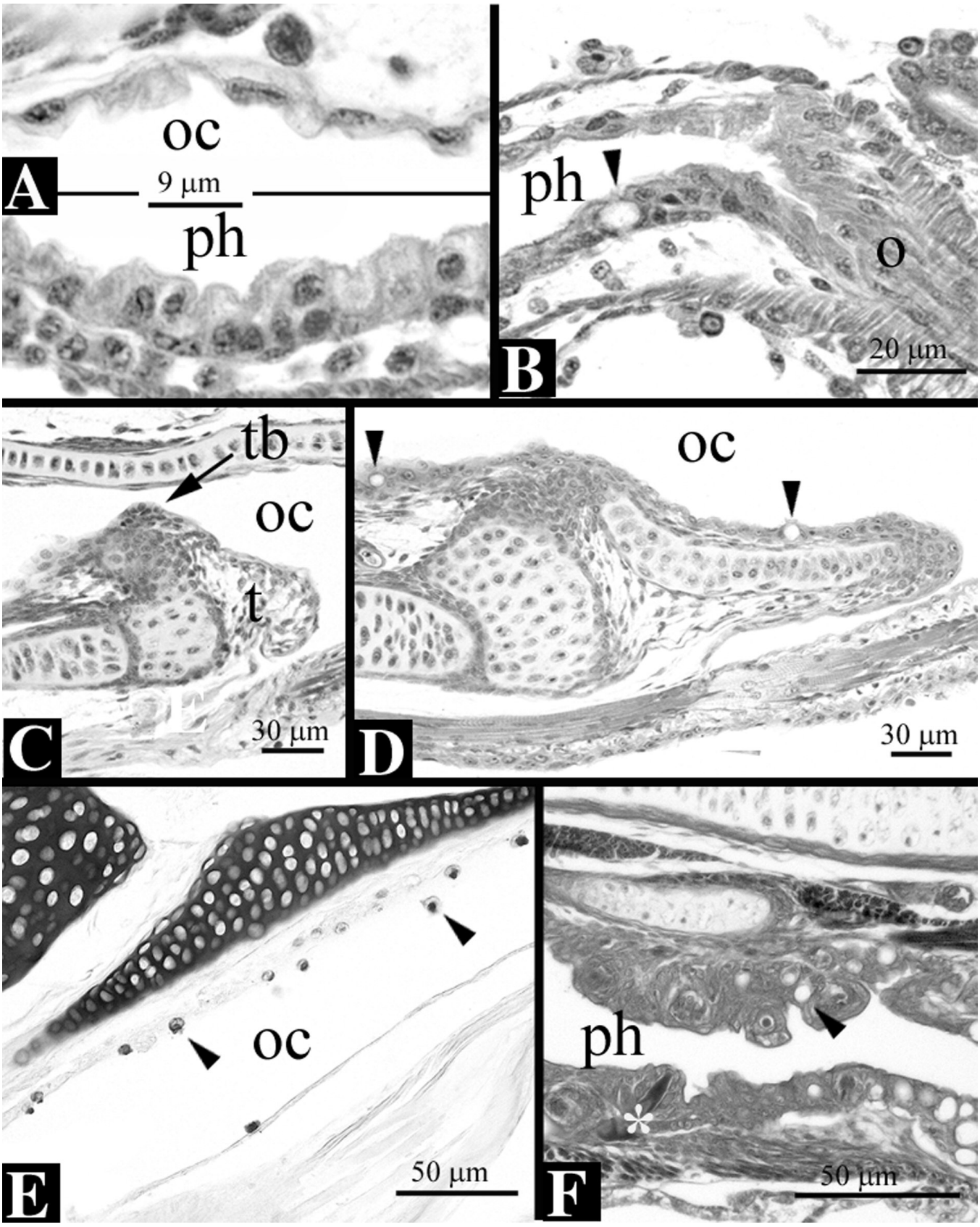

Fig. 3. A. Detail of the squamous buccal (upper picture) and pseudostratified pharyngeal (lower picture) epithelia in a 3-day-old sharpsnout sea bream larva; HE. **B.** Detail of pharyngeal cavity in a 5-day-old larva showing a goblet cell (arrowhead); HE. **C.** Oral cavity in a 11-day-old larva, showing a taste bud (tb) on the tongue (t) surface; HE. **D.** Oral cavity in a 24-day-old larva, showing the presence of two goblet cells (arrowheads); HE. **E.** Oral cavity in a 36-day-old larva, showing HID-positive goblet cells containing acidic sulphated glycoconjugates (arrowheads); HID-AB. **F.** Pharyngeal epithelium in a 21-day-old larva, showing developing teeth (asterisk); BB. o: oesophagus; oc: oral cavity; ph: pharynx; arrowheads: goblet cells.

apical zone from 30 DAH. The gastric epithelium appeared devoid of goblet cells.

Gastric glands appeared at 30 DAH as strongly BB-positive mucosal acinar cell aggregates in the fundic region (Fig. 5C), exhibiting also a weak AB- and PAS-positivity. By this time, a *muscularis mucosae*, a submucosa and a muscle tunica could be clearly distinguished in the stomach, surrounded by an outer serous membrane. The musculature surrounding the pyloric sphincter was well developed. Gastric glands proliferated as larval development proceeded, but only in the fundic portion, so that a fully developed glandular stomach could be observed by 39 DAH (Fig. 5D).

Intestine

Prior to mouth opening (2 DAH) an anterior (prevalvular) intestine could be distinguished from a bending posterior (postvalvular) intestine, by a constriction indicating the future ileo-rectal valve (Fig. 6A). No histological difference was observed between the two segments, both of them showing a single epithelial layer composed of columnar cells with basal nuclei, with a strongly eosinophilic brush border containing neutral glycoconjugates (PAS-positive), glycogen and proteins, particularly those rich in tryptophan. Loop formation started in the anterior intestine by 3 DAH. At the same stage primordial mucosal folding began, which increased with larval growth and appeared well developed in both portions of the intestine by 28 DAH. The ileo-rectal valve appeared completely formed by 10 DAH (Fig. 5B).

Soon after the beginning of exogenous feeding (5 DAH), unstained, supra- and infranuclear vacuoles could be seen in some tracts of the anterior intestinal epithelium (Fig. 6B). Such vacuoles, which were negative to both protein and carbohydrate reactions, increased in number as the larvae grew (Fig. 6D,E). Another type of inclusion was apparent in the epithelial cells of the posterior intestine from 5 DAH, consisting of large, eosinophilic, BB-positive supranuclear vacuoles, containing protein (Fig. 6C). These vacuoles remained a constant feature of the posterior intestinal mucosa throughout larval development (Fig. 6F). Goblet cells appeared interspersed between enterocytes of both anterior and posterior intestine from 33 DAH, some of them containing neutral mucins and some acidic ones (Figs. 6D-E).

Pancreas, liver and gall bladder

At hatching, the accessory digestive organs were absent. At 2 DAH the incipient exocrine pancreas was visible as polyhedral, strongly basophilic cells with a round nucleus and a prominent nucleolus, lying dorsally to the yolk sac wall and ventrally to the developing gut. The endocrine pancreas could already be distinguished at this stage as an islet of irregularly shaped cells with a pale cytoplasm and a large, central nucleus containing granular chromatin, lying within the exocrine cells (Fig. 7A). By 3 DAH conspicuous eosinophilic zymogen granules appeared within the exocrine cells, at the apical portion of their cytoplasm, and the main pancreatic duct was visible, opening into the anterior intestine (Fig. 7E). At this precocious stage the exocrine pancreas was proportionally very large in comparison to the last stages of larval growth. The zymogen granules were positive to protein reactions (BB and DMAB) from 7 DAH, while

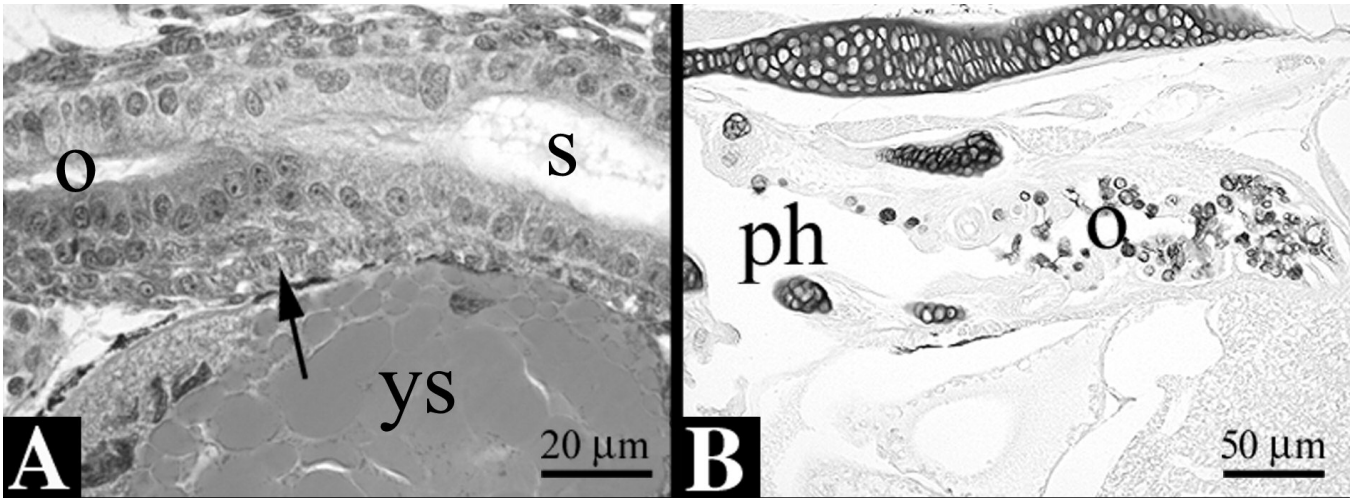

Fig. 4. A. View of the anterior portion of the digestive tract in a 2-day-old sharpsnout sea bream larva, showing the oesophagus (o), lined by a columnar, ciliated epithelium, and the dilated, presumptive stomach (s), lined by a simple, cuboidal epithelium. Developing smooth muscle cells (arrow) of the oesophageal wall are visible; HE. **B.** Pharynx (ph) and oesophagus (o) in a 23-day-old larva, showing HID-positive goblet cells, containing acidic sulphated glycoconjugates, particularly abundant in the oesophagus; HID-AB. ys: yolk sac.

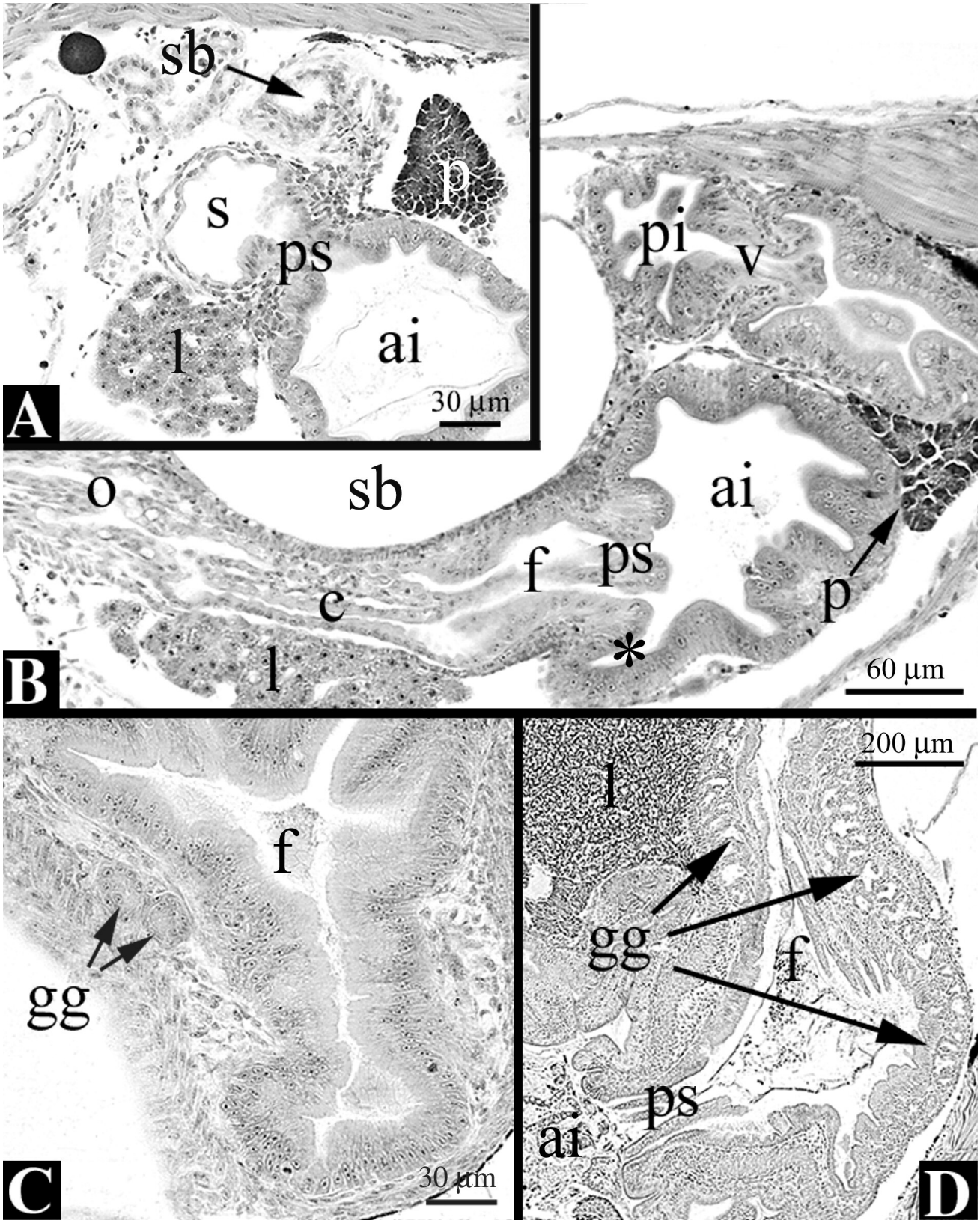

Fig. 5. A. View of the digestive tract in a 6-day-old sharpsnout sea bream larva, showing the pyloric sphincter (ps), separating the stomach (s) from the anterior intestine (ai). Note how the cuboidal gastric epithelium becomes columnar in proximity of the sphincter; HE. **B.** General view of the digestive system in a 10-day-old larva. A developing caecal ridge (asterisk) is visible just after the pyloric sphincter (ps). The fundic portion of the stomach (f), lined by a columnar epithelium, may be distinguished from the cardiac portion (c), lined by a cuboidal epithelium; HE. **C.** Fundic portion of the stomach of a 30-day-old larva, showing two gastric glands (gg); HE. **D.** Fully developed glandular stomach in a 39-day-old larva, showing numerous gastric glands (gg); HE. ai: anterior intestine; f: fundic stomach; l: liver; o: oesophagus; p: pancreas; pi: posterior intestine; ps: pyloric sphincter; sb: swim bladder; v: ileo-rectal valve.

they were negative to AB-PAS.

The immature liver could be distinguished at 2 DAH as a chord-like structure, close to the pancreas, consisting of swollen, conspicuously vacuolized cells, with the nuclei pushed towards the periphery (Fig. 7B). At the beginning of exogenous feeding by 4 DAH, the

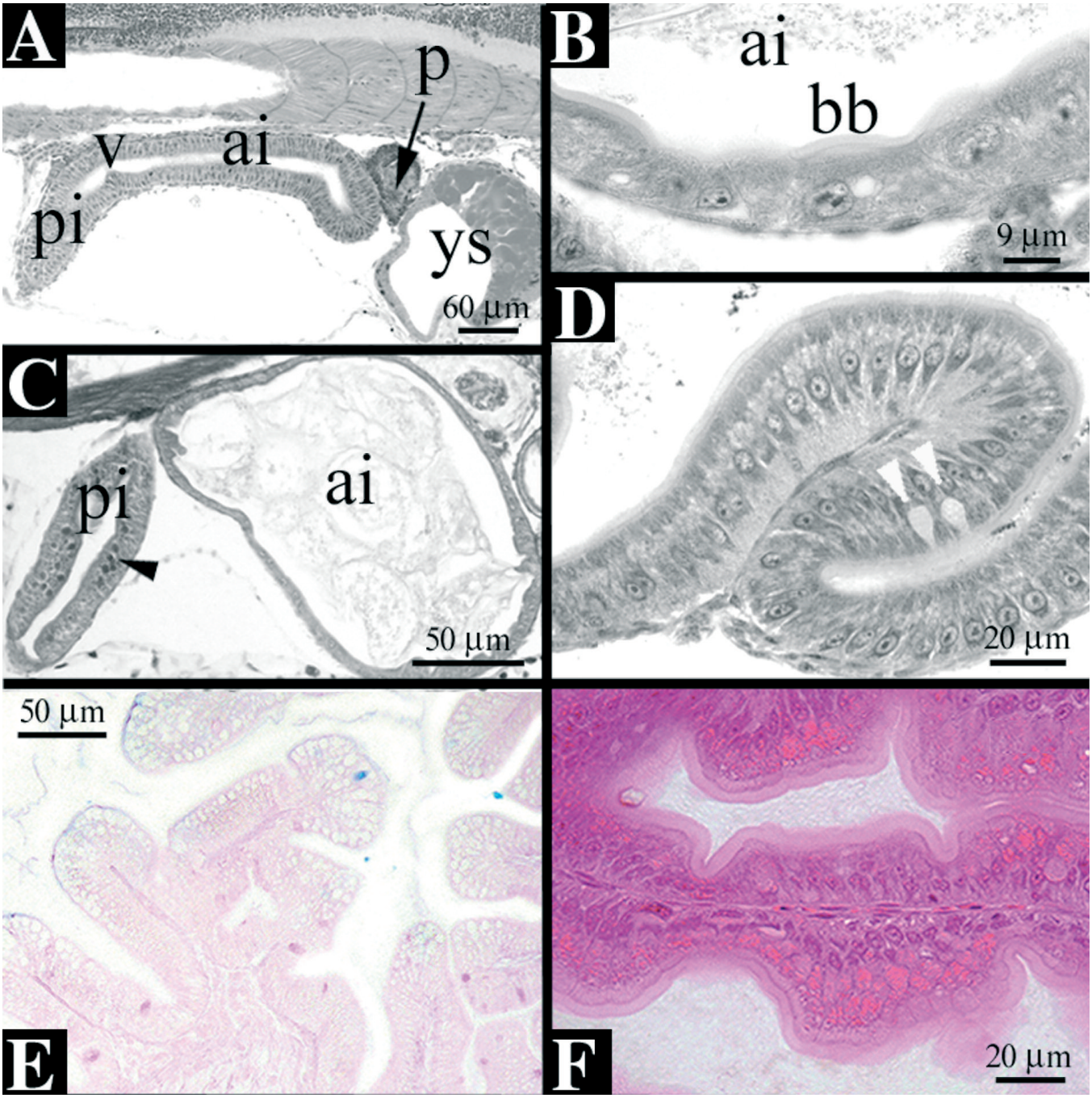

Fig.6. A. Detail of developing ileo-rectal valve (v) separating the anterior intestine (ai) from the posterior intestine (pi) in a 2-day-old sharpsnout sea bream larva; HE. **B.** Detail of anterior intestine in a 5-day-old larva, showing unstained vacuoles, indicative of probable lipid absorption; HE. **C.** Detail of digestive tract in a 6-day-old larva, showing supranuclear, BB-positive, protein absorption vacuoles (black arrowhead) in the posterior intestine (pi); BB. **D.** Detail of anterior intestinal epithelium filled with small vacuoles of probable lipidic nature in a 33-day-old larva. Two goblet cells are visible (white arrowheads); HE. **E.** Mucosal folds of anterior intestine in a 33-day-old larva; both PAS-positive (pink) and AB-positive (blue) goblet cells are visible, within the highly vacuolized epithelium; AB-PAS. **F.** Protein absorption vacuoles in the posterior intestine of a 57-day-old larva; HE. ai: anterior intestine; bb: brush border; p: pancreas; ys: yolk sac.

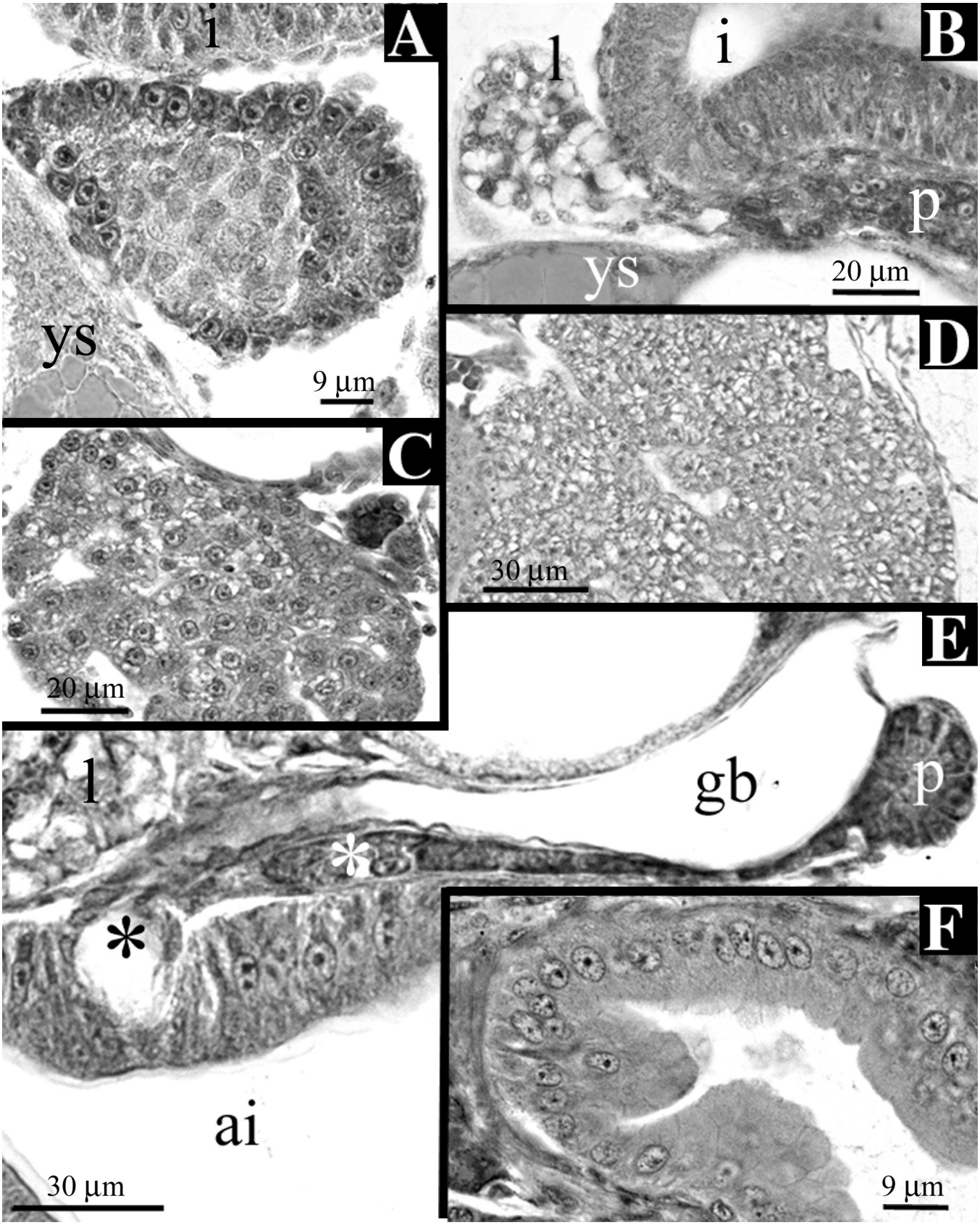

Fig. 7. A. Differentiation of the pancreas in a 2-day-old sharpsnout sea bream larva. The endocrine cells, with a pale cytoplasm and granular nuclear chromatin, are surrounded by the strongly basophilic exocrine cells, with a prominent nucleolus; HE. **B.** Immature liver (l) in a 2-day-old larva. The hepatocytes appear swollen, because of marked vacuolization; HE. **C.** Mature liver in a 4-day-old larva, showing moderately vacuolized hepatocytes; HE. **D.** Granulation and vacuolization of hepatic parenchyma in a 13-day-old larva; HE. **E.** Opening of the main pancreatic duct (white asterisk) and of the bile duct (black asterisk) into the anterior intestine (ai) in a 3-day-old larva; HE. **F.** Detail of the gall bladder in a 33-day-old larva, showing the thickened epithelium formed by cuboidal and cylindrical cells; HE. gb: gall bladder; i: intestine; l: liver; p: pancreas; ys: yolk sac.

mature hepatocytes appeared as polyhedral cells, tightly packed between sinusoids, often around a central vein, with a prominent, round nucleus and a granular, slightly vacuolar cytoplasm (Fig. 7C). The granules were shown to contain glycogen, as well as abundant protein, especially those rich in tryptophan, whereas the vacuoles appeared empty. Granulation and vacuolization of the cytoplasm increased as exogenous feeding proceeded (Fig. 7D).

The gall bladder, lined by a simple squamous epithelium, was visible between the pancreas and liver from 2 DAH (Fig. 2). At 3 DAH, the bile duct was already formed and opened into the anterior intestine (Fig. 7E). At 33 DAH the epithelium of the gall bladder appeared thickened, with cuboidal and cylindrical cells visible in the same section (Fig. 7F). A basal lamina separated the epithelium from the underlying layer of connective tissue, containing scattered smooth muscle cells.

Discussion

Larval development in sharpsnout seabream can be divided into three stages, based upon its feeding mode: an endotrophic stage from hatching to mouth opening (0-3 DAH), an endo-exotrophic stage (4-7 DAH) lasting until complete exhaustion of yolk reserves and a strictly exotrophic stage from 8 DAH onwards. Similarly to most marine fish species, sharpsnout seabream larvae possessed an undifferentiated digestive tract at hatching. Major developmental events took place between day 2 and 10, starting with regional gut differentiation into four histologically distinct segments (i.e. buccopharynx, oesophagus, incipient stomach and intestine), which occurred before mouth opening.

At mouth opening (3 DAH) the gall bladder, liver and pancreas were already differentiated and connected to the intestine by the bile and main pancreatic duct, respectively. A marked, colourless vacuolization of hepatocytes was visible before first feeding, suggesting accumulation of lipid from yolk and the oil globule by the primordial liver. Folding of intestinal mucosa, which would ensure more efficient digestion and absorption processes, was also apparent at mouth opening in *D. puntazzo*, as has also been reported in *D. sargus* (Ortiz-Delgado et al., 2003) and *P. auriga* (Sánchez-Amaya et al., 2007), and considerably earlier than in other Sparids studied so far (Santamaría et al., 2004; Micale et al., 2006; Darias et al., 2007). During this first phase, sharpsnout seabream larvae relied entirely on yolk reserves for their nutrition and showed a PAS-positive brush border of the intestinal epithelium, which could be related to the absorption of easily digested substances, such as disaccharides and short-chain fatty acids, similarly to what has been demonstrated in the gastric mucosa of the tilapia *Tilapia nilotica* (Osman and Caceci, 1991). The oesophageal epithelium of *D. puntazzo* exhibited ciliated cells during this stage, which have also been described in the oesophagus of *D. dentex* (Santamaría et al., 2004) before starting exogenous feeding, and could be related to water transport and osmoregulation. Histological and histochemical observations suggest that the development of the digestive tract, involving the presence of functional liver, pancreas and gall bladder, enabled early sharpsnout seabream larvae to ingest, digest and assimilate the first exogenous food even before endogenous reserves were completely resorbed.

Twenty-four hours after mouth opening, rotifers could be detected in the digestive tract of 50% of the larvae, although yolk reserves were completely exhausted at 8 DAH, indicating a brief period of combined endogenous and exogenous feeding. In a previous study with sharpsnout sea bream (Faranda et al., 1985), yolk consumption occurred faster than in the present study. The different rearing protocols between the two studies may account for this discrepancy: in fact, the mesocosm rearing technique, employed in the present study, is known to provide more natural conditions compared to intensive rearing techniques, as well as a more stable prey population, resulting in better feeding and, hence, slower yolk consumption (Zaiss et al., 2006). The absence of a transitional starvation period between endogenous and exogenous feeding, such as that reported in the yellowfin porgy *Acanthopagrus latus* (Leu and Chou, 1996) and gilthead sea bream *Sparus aurata* (Sarasquete et al., 1995), which may be caused by a too low frequency distribution of prey or their inadequate size (Parra and Yúfera, 2000), is indicative of an adequate feeding protocol employed in the present study during early larval stages. Glycogen and protein accumulation in the liver could be demonstrated as soon as exogenous feeding began, as PAS- and BB-positive granules, respectively. On the other hand, the large, unstained vacuoles of probable lipid origin, were reduced at the beginning of the endo-exotrophic stage. It could be suggested that lipid from yolk reserves accumulated by the liver before mouth opening was almost exhausted at transition from endogenous to exogenous feeding, as has been reported in other species (Diaz et al., 2002).

Soon after the beginning of exogenous feeding (5 DAH), intestinal absorption of nutrients was apparent as supra- and infranuclear unstained vacuoles in the enterocytes of anterior intestine, as well as supranuclear BB-positive vacuoles in the enterocytes of posterior intestine. The former suggested lipid absorption, as has been reported in other fish larvae (Deplano et al., 1991; Bisbal and Bengston, 1995; Diaz et al., 1997; Ortiz-Delgado et al., 2003; Zaiss et al., 2006; Sánchez-Amaya et al., 2007). On the other hand, protein absorption vacuoles in the posterior intestine, which have been described in both larval and adult gastric and agastric fish (Krementz and Chapman, 1975; Albertini-Berhaut, 1987; Micale et al., 2005, 2006), are involved in the pinocytotic uptake of proteins, which are subsequently digested intracellularly (Sire and Vernier, 1992). In some teleosts, they are present only until formation of the

gastric glands, suggesting a transient mechanism for protein digestion, related to the immaturity of the digestive tract (Tanaka, 1972; Luizi et al., 1999; Ortiz-Delgado et al., 2003; Elbal et al., 2004; Zaiss et al., 2006). Since no reduction in the number of protein vacuoles was apparent after a glandular stomach was formed in sharpsnout seabream, similarly to what has been reported in the European sea bass (García Hernández et al., 2001), common pandora (Micale et al., 2006) and red porgy (Darias et al., 2007), it can be suggested that a combined intra- and extracellular protein digestion mechanism is active in sharpsnout seabream.

The formation of the pyloric caeca has been considered in many species as the last major event during ontogeny of the digestive system in fish larvae, indicating the transition from a larval to a juvenile stage (Bisbal and Bengston, 1995; Hamlin et al., 2000; Chen et al., 2006; Micale et al., 2006). On the contrary, caecal development started precociously in sharpsnout seabream (10 DAH), well before the formation of the gastric glands, as in the European sea bass (García Hernández et al., 2001). In the absence of functional gastric glands, the precocious development of pyloric caeca, in which several enzymatic activities have been demonstrated (Cousin et al., 1987), may enhance the digestive capability of larvae. Moreover, as the pyloric caeca have the same histological structure as the intestine, their early development increases the total available absorption area (Houssain and Dutta, 1998), ensuring a more efficient utilization of feed.

The formation of pepsin-producing gastric glands during larval development in fish is crucial for extracellular luminal digestion of complex protein. In most examined gastric species, a glandular stomach is fully developed several weeks after the start of exogenous feeding (Stroband and Kroon, 1981; Segner et al., 1994; Ribeiro et al., 1999). In sharpsnout seabream, the first gastric glands were formed at the completion of the first month of life, similarly to what was observed in common pandora *Pagellus erythrinus* (Micale et al., 2006), and a fully developed glandular stomach could be observed by 39 DAH. This is in agreement with the sharp increase in pepsin-specific activity observed between 32 and 40 DAH in a recent study on digestive enzymes of larval sharpsnout seabream (Suzer et al., 2007). Gastric glands were detected more precociously in other Sparids, such as *Diplodus sargus* (Ortiz-Delgado et al., 2003), *Dentex dentex* (Santamaría et al., 2004), *Pagrus pagrus* (Darias et al., 2007) and *Pagrus auriga* (Sánchez-Amaya et al., 2007), but considerably later in the gilthead seabream *Sparus aurata* (Elbal et al., 2004). The location of gastric glands is species-specific and has been related to feeding habits (Ortiz-Delgado et al., 2003). In *D. puntazzo*, gastric glands occurred only in the fundic region of the stomach, as was also shown in the yellowtail flounder *Pleuronectes ferruginea* (Baglole et al., 1997), the summer flounder *Paralichthys dentatus* (Bisbal and Bengtson, 1995), the turbot *Scophthalmus maximus* (Segner et al., 1994) and the Dover sole *Solea solea* (Veggetti et al., 1999). On the other hand, gastric glands were only found in the cardiac region in *D. sargus* (Ortiz-Delgado et al., 2003), shi drum *Umbrina cirrosa* (Zaiss et al., 2006) and red porgy *P. pagrus* (Darias et al., 2007), whereas an entirely glandular stomach occurs in *P. erythrinus* (Micale et al., 2006). Neutral mucosubstances could be detected by the PAS reaction in the gastric epithelial cells at the same time as the gastric glands and could indicate the involvement of the former in mucosa protection from auto-digestion processes caused by hydrochloric acid and the enzymes secreted by the gastric glands, as in the milkfish *Chanos chanos* (Ferraris et al., 1987) and the gilthead sea bream (Elbal et al., 2004). On the other hand, neutral glycoconjugates have also been reported in the stomach epithelium of adult Mediterranean amberjack *Seriola dumerilii* (Grau et al., 1992) and larval white seabream *Diplodus sargus* (Ortiz-Delgado et al., 2003), and this fact has been related to the absorption of easily digestible substances, such as disaccharides and short-chain fatty acids. Neither proteins rich in tryptophan, which have been related to pepsinogen synthesis in the amberjack *S. dumerilii* (Grau et al., 1992), nor proteins rich in cysteine, were found in the gastric glands of *D. puntazzo*, whereas they do occur in the congener species *D. sargus* (Ortiz-Delgado et al., 2003). Similarly, cysteine- and tryptophan-rich proteins have been reported in the gastric glands of *P. pagrus* (Darias et al., 2007), but not in those of *P. auriga* (Sanchez-Amaya et al., 2007). This would suggest that the presence of these aminoacid residues in the gastric glands of fish is species-specific.

The first goblet cells in the digestive tract of sharpsnout seabream were visible in the pharynx and oesophagus shortly after the first exogenous feeding, the buccal goblet cells appearing thereafter, while goblet cells in the intestine appeared finally at 33 DAH. Such a late appearance of intestinal goblet cells has been reported only in common pandora (Micale et al., 2006) and red porgy (Darias et al., 2007). The time at which mucus secreting cells are formed varies among species, although their sequence of appearance along the different segments of digestive tract (i.e. firstly, oesophagus, secondly, buccal cavity, and finally, intestine) is common to many examined species (García-Hernández et al., 2001; Ortiz-Delgado et al., 2003; Santamaría et al., 2004; Sánchez-Amaya et al., 2007). Glycoproteins produced by goblet cells may play an important lubricant role for the buccopharyngeal and oesophageal mucosa, which lack salivary glands. Mucosubstances may also play a role in protecting the digestive mucosa from bacterial and viral attacks (Gisbert et al., 2004), as well as in pre-gastric digestion (Baglole et al., 1997). The mucous content of goblet cells in *D. puntazzo* varied with the digestive region and, in some cases, with the developmental phase. Carboxylated glycoconjugates were detected in the

goblet cells from all segments of the digestive tract, whereas neutral glycoconjugates were found only in the intestine, where their presence could be related to absorption processes. On the other hand, the presence of sulphated glycoconjugates in the buccal and oesophageal goblet cells could be related to an enhancement of mucus viscosity, possibly supporting a particle trapping function, as has been reported in the carp, *Cyprinus carpio* (Sibbing and Uribe, 1984). Sulphated glycoconjugates could be detected only after the appearance of the carboxylated ones, suggesting a progressive development of the mucus content, as reported in human salivary glands (Harrison et al., 1987). Oesophageal goblet cells contained proteins as well, especially those containing cysteine, similarly to what has been reported in *D. sargus* and *P. pagrus* (Ortiz-Delgado et al., 2003; Darias et al., 2007).

Taste buds appeared in the bucco-pharynx by 11 DAH, suggesting that sharpsnout seabream larvae are able to select the prey on an organoleptic basis well before weaning. This information does not agree with what has been reported in a previous study (Boglione et al., 2003) using scanning electron microscopy. The different methodology might account for this discrepancy, since observation of histological structures by light microscopy may reveal some details that may be missed by surface examination, such as allowed by SEM.

The histological and histochemical description of digestive system ontogeny in sharpsnout seabream reported in the present study complements previous information on larval digestive enzymes in the same species (Suzer et al., 2007), and represents a first step towards the determination of the functional relationships between feeding and assimilation in this marine species of great interest to the Mediterranean aquaculture industry. The absence of a completely functional stomach during the first month of life, during which dramatic developmental changes take place, which are crucial for rearing success, would be considered problematic in routinely intensive rearing techniques. The appropriateness of the mesocosm rearing system for sharpsnout sea bream is witnessed by the high survival rate and body weight of the produced fry. In fact, in another study where larvae were reared with the green water technique, and the feeding regime also included rotifers, *A. salina* nauplii and enriched metanauplii, followed by artificial feed, the survival ranged between 18% and 22%, and the body weight at 60 DAH ranged between 0.15 and 0.18 g (Marangos, 1995), which is significantly less than in the present study. Also, in another study using the mesocosm rearing technique, albeit with an *Amylodinium* spp. infection at 34 DAH, the final survival rate of sharpsnout sea bream at 50 DAH was 54%, TL was 19.6±0.9 mm and body weight was 107±32 mg (Papandroulakis et al., 2004). Therefore, the use of mesocosm rearing technology may be the key for the success of sharpsnout sea bream larval rearing in the present study. This is due to the relatively long-term self-sufficiency of live-food items in the mesocosm culture system, both from endogenous and exogenous origin (Papandroulakis et al., 2004). The variety of digestible food items provided by this rearing system is likely to have allowed sharpsnout sea bream larvae, possessing an immature and incomplete digestive system, to fulfil their dietary requirements.

References

Albertini-Berhaut J. (1987). L'intestin chez les Mugilidae (Poissons ; Téléostéens) à différentes étapes de leur croissance. I – Aspects morphologiques et histologiques. J. appl. Ichthyol. 3, 1-12.

Baglole C.J., Murray H.M., Goff G.P. and Wright G.M. (1997). Ontogeny of the digestive tract during larval development of the yellowtail flounder: a light microscopy and mucus histochemical study. J. Fish Biol. 51, 120-134.

Bancroft J.D., Stevens A. and Turner D.R. (1990). Theory and practice of histological techniques. Churchill Livingstone. Edinburgh.

Bisbal G.A. and Bengston D.A. (1995). Development of the digestive tract in larval summer flounder. J. Fish Biol. 47, 277-291.

Blaxter J.H.S., Danielssen D., Moksness E. and Øiestad V. (1983). Description of the early development of the halibut *Hippoglossus hippoglossus* and attempts to rear the larvae past first feeding. Mar. Biol. 73, 99-107.

Boglione C., Giganti M., Selmo C. and Cataudella S. (2003). Morphoecology in larval fin-fish: a new candidate species for aquaculture, *Diplodus puntazzo* (Sparidae). Aquac. Int. 11, 17-41.

Boulhic M. and Gabaudan J. (1992). Histological study of the organogenesis of the digestive system and swim bladder of the Dover sole *Solea solea* (Linnaeus 1758). Aquaculture 102, 373-396.

Caggiano M., Canese S., Lupo A. and Cirillo A. (1993). Experiences of artificial reproduction and larval rearing of sheepshead bream (*Diplodus puntazzo*) in the South of Italy. In: From discovery to commercialization. Carrillo M., Dahle L., Morales J., Sorgeloos P., Svennevig N. and Wyban J. (eds). World Aquaculture "93, European Aquaculture Society Special Publication. Vol.19. p 326.

Chen B.N., Qin J.G., Kumar M.S., Hutchinson W. and Clarke S. (2006). Ontogenetic development of the digestive system in yellowtail kingfish *Seriola lalandi* larvae. Aquaculture 256, 489-501.

Cousin J.C.B. and Baudin-Laurencin F. (1985). Morphogénèse de l'appareil digestif et de la vessie gazeuse du turbot, *Scophthalmus maximus* L. Aquaculture 47, 305-319.

Cousin J.C.B., Baudin-Laurencin F. and Gabaudan J. (1987). Ontogeny of enzymatic activities in fed and fasting turbot, *Scophthalmus maximus* L. J. Fish Biol. 30, 15-33.

Darias M.J., Ortiz-Delgado J.B., Sarasquete C., Martínez-Rodríguez G. and Yúfera M. (2007). Larval organogenesis of *Pagrus pagrus* L., 1758 with special attention to the digestive system development. Histol. Histopathol. 22, 753-768.

Deplano M., Diaz J.P., Connes R., Kentouri-Divanach M. and Cavalier F. (1991). Appearance of lipid-absorption capacities in larvae of the sea bass *Dicentrarchus labrax* during transition to the exotrophic phase. Mar. Biol. 108, 361-371.

Diaz J.P., Guyot E., Vigier S. and Connes R. (1997). First event in lipid absorption during post-embryonic development of the anterior intestine in the gilt-head sea bream. J. Fish Biol. 51, 180-192.

Diaz J.P., Mani-Ponset L., Blasco C. and Connes R. (2002). Cytological detection of the main phases of lipid metabolism during early post-

embryonic development in three teleost species: *Dicentrarchus labrax*, *Sparus aurata* and *Stizostedion lucioperca*. Aquat. Living Resour. 15, 169-178.

Divanach P. and Kentouri M. (1982). Utilisation des techniques extensives pour la production à grande échelle d'alevins de sar *Puntazzo puntazzo*. C. R. Acad.. Sci. Paris 294 (3), 1017-1019.

Elbal M.T., García Hernández M.P., Lozano M.T. and Agulleiro B. (2004). Development of the digestive tract of gilthead sea bream (*Sparus aurata* L.). Light and electron microscopic studies. Aquaculture 234, 215-238.

Faranda F., Cavaliere A., Lo Paro G., Manganaro A. and Mazzola A. (1985). Preliminary studies on reproduction of *Puntazzo puntazzo* (Gmelin, 1789) (Pisces: Sparidae) under controlled conditions. Aquaculture 49, 111-123.

Favaloro E., Lopiano D. and Mazzola A. (2002). Rearing of sharpsnout seabream (*Diplodus puntazzo*, Cetti 1777) in a Mediterranean fish farm: monoculture versus polyculture. Aquac. Res. 33, 137-140.

Ferraris R.P., Tan J.D. and De La Cruz M.C. (1987). Development of the digestive tract of milkfish, *Chanos chanos* (Forsskal): Histology and histochemistry. Aquaculture 61, 241- 257.

García-Hernández M.P., Lozano M.T., Elbal M.T. and Agulleiro B. (2001). Development of the digestive tract in sea bass (*Dicentrarchus labrax* L.). Light and electron microscopic studies. Anat. Histol. Embryol. 173, 1-19.

Gisbert E., Piedrahita R.H. and Conklin D.E. (2004). Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. Aquaculture 232, 455-470.

Gordon A.K. and Hecht T. (2002). Histological studies on the development of the digestive system of the clownfish *Amphiprion percula* and the time of weaning. J. Appl. Ichthyol. 18, 113-117.

Grau A., Crespo S., Sarasquete M.C. and Gonzáles de Canales M.L. (1992). The digestive tract of the amberjack *Seriola dumerilii*, Risso : a light and scanning electron microscope study. J. Fish Biol. 41, 287-303.

Greco S., Lo Paro G., Caridi D., Perdichizzi F., Cammaroto S., Micale V. and Genovese L. (1993). Controlled spawning and larval development in the sharpsnout seabream (*Diplodus puntazzo*, Sparidae). Oostende. European Aquaculture Society Special Publication. Vol.18. pp 185-188.

Hamlin H.J., Hunt von Herbing I. and Kling L.J. (2000). Histological and morphological evaluations of the digestive tract and associated organs of the haddock throughout post-hatching ontogeny. J. Fish Biol. 57, 716-732.

Harrison J.D., Auger D.W., Paterson K.L. and Rowley P.S.A. (1987). Mucin histochemistry of submandibular and parotid salivary glands of man: light and electron microscopy. Histochem. J. 19, 555-564.

Hatziathanassiou A., Divanach P., Kotzabassis K., Anastasiadis P. and Kentouri M. (2001). Performances of a solar tubular photobioreactor for hyperintensive mass culture of the microalgae *Chlorella minutissima* in Crete. Proceedings of 10th Panhellenic Conference of Ichthyologists, Chania (Greece). 18-20 October, 2001.

Hernández M.D., Egea M.A., Martinez F.J., Rueda F.M., Aguado F. and Garcia B. (2001). Effects of commercial diets with different P/E ratios on sharpsnout seabream (*Diplodus puntazzo*) growth and nutrient utilization. Aquaculture 195, 321-329.

Houssain A.M. and Dutta X.M. (1998). Assessment of structural and functional similarities and differences between caeca of the bluegill. J. Fish Biol. 53, 1317-1323

Kjørsvik E., van der Meeren T., Kryvi H., Arnfinnson J. and Kvenseth P.G. (1991). Early development of the digestive tract of cod larvae, *Gadus morhua* L., during start-feeding and starvation. J. Fish Biol. 38, 1-15.

Korkut A.Y., Saka S. and Firat K. (2006). The effects of different light intensities on early life development of sharpsnout seabream (*Diplodus puntazzo*, Cetti, 1777) larvae. Turk. J. Vet. Anat. 30, 381-387.

Kouttouki S., Georgakopoulou E., Kaspiris P., Divanach P. and Koumoundouros G. (2006). Shape ontogeny and variation in the sharpsnout seabream, *Diplodus puntazzo* (Cetti 1777). Aquac. Res. 37, 655-663.

Krementz A.B. and Chapman G.B. (1975). Ultrastructure of the posterior half of the intestine of the channel catfish, *Ictalurus punctatus*. J. Morphol. 145, 441-482.

Leu M.Y. and Chou Y.H. (1996). Induced spawning and larval rearing of captive yellowfin porgy, *Acanthopagrus latus* (Houttuyn). Aquaculture 143, 155-166.

Luizi F.S., Gara B., Shields R.J. and Bromage N.R. (1999). Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and Artemia prey. Aquaculture 176, 101-116.

Marangos C. (1995). Larviculture of the sheepshead bream, *Puntazzo puntazzo* (Gmelin 1789) (Pisces Sparidae). Cah. Options Méditerr. 16, 41-46.

Micale V., Genovese L., Costanzo M.T., Daprà F., Gai F., Muglia U. and Palmegiano G.B. (2005). Blackspot sea bream (*Pagellus bogaraveo*) fed on rice protein concentrate: intestinal morphology. In: Optimise the future. Howell B. and Flos R. (cpls.). European Aquaculture Society Special Publication. Vol. 35. pp 328-329.

Micale V., Garaffo M., Genovese L., Spedicato M.T. and Muglia U. (2006). The ontogeny of the alimentary tract during larval development in common pandora *Pagellus erythrinus* L.. Aquaculture 251, 354-365.

Mowry R.W. (1963). Special value of methods that colour both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of Alcian Blue 8GX and their combination with the periodic acid-Schiff reaction. Ann. NY Acad. Sci. 106, 402-443.

Mylonas C.C., Cardinaletti G., Sigelaki I. and Polzonetti-Magni A. (2005). Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (*Dicentrarchus labrax)* and gilthead sea bream (*Sparus aurata*) at different temperatures. Aquaculture 246, 467-481

Ortiz-Delgado J.B., Darias M.J., Cañavate J.P., Yúfera M. and Sarasquete C. (2003). Organogenesis of the digestive tract in the white seabream, *Diplodus sargus*. Histological and histochemical approaches. Histol. Histopathol. 18, 1141-1154.

Osman A.H.K. and Caceci T. (1991). Histology of the stomach of *Tilapia nilotica* (Linnaeus, 1758) from the river Nile. J. Fish Biol. 38, 221-223.

Papandroulakis N., Kentouri M., Maingot E. and Divanach P. (2004). Mesocosm: a reliable technology for larval rearing of *Diplodus puntazzo* and *Diplodus sargus sargus*. Aquac. Int. 12, 345-355.

Parra G. and Yúfera M. (2000). Feeding, physiology and growth responses in first-feeding gilthead seabream (*Pagrus aurata* L.) larvae in relation to prey density. J. Exp. Mar. Biol. Ecol. 243, 1-15.

Pearse A.G.E. (1985). Histochemistry. Theoretical and applied: Vol.2. Analytic Technology. Fourth Edition. Churchill Livingstone,

Edimburgh, 624 pp.

Reid P.E., Owen D.A., Fletcher K., Rowan R.E., Reimer C.L., Rouse G.J. and Park C.M. (1989). The histochemical specificity of high-iron diamine-Alcian blue. Histochem. J. 21, 501-504.

Ribeiro L., Sarasquete C. and Dinis M.T. (1999). Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larve. Aquaculture 171, 293-308.

Romano N., Fanelli M., Del Papa G.M., Scapigliati G. and Mastrolia L. (1999). Histological and cytological studies on the developing thymus of sharpsnout seabream, *Diplodus puntazzo*. J. Anat. 194, 39-50.

Saavedra M., Beltran M., Pousão-Ferreira P., Dinis M.T., Blasco J. and Conceição L.E.C. (2007). Evaluation of bioavailability of individual amino acids in *Diplodus puntazzo* larvae: Towards the ideal dietary amino acid profile. Aquaculture 263, 192-198.

Sánchez-Amaya M.I., Ortiz-Delgado J.B., García-López Á., Cárdenas S. and Sarasquete M.C. (2007). Larval ontogeny of redbanded seabream *Pagrus auriga* Valenciennes, 1843 with special reference to the digestive system. A histological and histochemical approach. Aquaculture 263, 259-279.

Santamaría C.A., Marín de Mateo M., Traveset R., Sala R., Grau A., Pastor E., Sarasquete C. and Crespo S. (2004). Larval organogenesis in common dentex *Dentex dentex* L. (Sparidae): histological and histochemical aspects. Aquaculture 237, 207-228.

Sarasquete M.C., Polo A. and Yúfera M. (1995). Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L.. Aquaculture 130, 79-92.

Segner H., Storch V., Reinecke M., Kloas W. and Hanke W. (1994). The development of functional digestive and metabolic organs in turbot *Scophthalmus maximus*. Mar. Biol. 119, 471-486.

Sfakianakis D.G., Doxa C.K., Kouttouki S., Koumoundouros G., Maingot E., Divanach P. and Kentouri M. (2005). Osteological development of the vertebral column and of the fins in *Diplodus puntazzo* (Cetti, 1777). Aquaculture 250, 36-46.

Sibbing F.A. and Uribe R. (1984). Regional specializations in the oro-pharyngeal wall and food processing in the carp (*Cyprinus carpio* L.). Neth. J. Zool. 35, 377-422.

Sire M.F. and Vernier J.-M. (1992). Intestinal absorption of protein in teleost fish. Comp.Biochem. Physiol. 103A, 771-781.

Spicer S.S. (1965). Diamine methods for differentiating mucosubstances histochemically. Histochem. J. 13, 211-234.

Stroband H.W.J. and Kroon A.G. (1981). The development of the stomach in *Clarias lazera* and the intestinal absorption of protein macromolecules. Cell Tissue Res. 215, 397-415.

Suzer C., Aktülün S., Çoban D., Kamacı H.O., Saka S., Fırat K. and Alpbaz A. (2007). Digestive enzyme activities in larvae of sharpsnout seabream (*Diplodus puntazzo*). Comp. Biochem. Physiol. A 148, 470-477.

Tanaka M. (1972). Studies on the structure and function of the digestive system in teleost larvae. V. Epithelial changes in the posterior gut and protein ingestion. Jpn. J. Ichthyol. 19, 172-180.

Veggetti A., Rowlerson A., Radaelli G., Arrighi S. and Domeneghini C. (1999). Post-hatching development of the gut and larval muscle in the sole. J. Fish Biol. 55, 44-65.

Zaiss M.M., Papadakis I.E., Maingot E., Divanach P. and Mylonas C.C. (2006). Ontogeny of the digestive tract in shi drum (*Umbrina cirrosa* L.) reared using the mesocosm larval rearing system. Aquaculture 260, 357-368.

Accepted March 19, 2008