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A histochemical and immunohistochemical study of digestive enzymes and hormones during the larval development of the sea bream, *Sparus aurata* L.

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Summary

The distribution of different hydrolytic enzymes and the localization of the hormones which regulate glucose metabolism during development of the digestive tract of the sea bream, *Sparus aurata* L., were studied. The yolk sac contains trypsin, glucose-6-phosphatase, ATPases and acid and alkaline phosphatase activities. Positive insulin, glucagon and somatostatin cells were observed in the pancreas and in the lumen of the intestinal tract during endogenous feeding. From hatching until 3 days later, the digestive tract of sea bream larvae shows no enzymatic activities. During exogenous feeding, the activities of the phosphatases and trypsin generally increase, as do the amounts of the hydrolytic enzymes and trypsin, as well as the pancreatic and intestinal hormones. The enzymatic activities gradually decrease from the anterior part towards the posterior part of the digestive tract.

Introduction

The sea bream, *Sparus aurata* L., is a species of great commercial interest; therefore, the year-round mass production of viable eggs and larvae has been the object of many studies (Devauchelle, 1984; Kadmon *et al.*, 1985; Pascual *et al.*, 1989). In this species, as in others with similar characteristics, the critical stage during larval development is the transition between the lecitotrophic phase and the start of exogenous feeding; it is therefore of great interest to understand the functional capacity of the digestive tract at that time.

The digestive tract of teleost larvae is morphologically, histologically and physiologically less developed than it is in adults. There is evidence that during the larval stage, the rates of ingestion and digestion, as well as the efficiency of assimilation, adapt in order to optimize larval growth. These adaptations show interspecific differences which depend on the ingested prey and their size (Polo, 1991). When larvae begin to feed the digestive tract is already functional and shows particular histophysiological characteristics (Govoni *et al.*, 1986).

During the endogenous feeding stage in most sea fish, the absorption of nutrients takes place through the syncytium surrounding the yolk sac, where reserves have been accumulated during vitellogenesis in the oocytes (Mommsen & Walsh, 1988; Sarasquete *et al.*, 1990a). Through an intracellular endocytosis and digestion mechanism within the yolk syncytium, the metabolites pass to the larval circulation in the walls of the yolk sac (Heming & Buddington, 1988). Two areas may be distinguished in the syncytium of the yolk sac of teleost larvae: one is made up of smooth endoplasmic reticulum, numerous mitochondria and glycogen granules, and is responsible for the metabolism of carbohydrates and lipids. The other area contains rough endoplasmic reticulum and Golgi complexes in a stratified structure. In this yolk-zone the proteins are synthesized and transported. Besides the syncytium, the yolk sac larvae possess enzymes which take part in degrading the yolk reserves (Hamor & Garside, 1973; Vernier & Sire, 1976, 1977a, b). In most teleosts, the stomach and pyloric caeca are not developed until metamorphosis. During a great part of the larval exogenous feeding stage the acid digestion of proteins and the ability to absorb nutrients differ from those in adults. During larval development in teleosts, proteolytic digestion seems to be compensated by the pinocytic process, in the lower intestine, resulting in acidophilic supranuclear vacuoles, which may disappear or persist in adult specimens (Stroband *et al.*, 1979; Watanabe, 1984a, b; Elbal & Agulleiro, 1986; Vernier & Sire, 1977b; Polo, 1991).

Whereas the ontogeny of the digestive tract in teleosts does not present great interspecific differences, the enzymatic system presented by larvae at hatching and the enzymatic changes which take place during larval development are controversial (Tanaka *et al.*, 1972; Barr *et al.*, 1985; Baragi & Lovell, 1986; Pedersen *et al.*, 1987).

This paper describes the histological changes in the digestive tract of *Sparus aurata* from hatching through the first month of larval life, as well as the distribution of pancreatic and intestinal hormones and of enzymes which take part in carbohydrate metabolism, in proteolytic digestion, and in the absorption and transport of macromolecules through membranes.

Materials and methods

Gilthead sea bream (*Sparus aurata* L.) larvae were studied from hatching until day 30. The larvae were fed with rotifers (*Brachionus plicatilis*) until day 14, and then *Artemia salina* nauplii until day 30, in tanks of 300–450 l at 19–20°C, according to the procedure described by Polo (1991). The length of the larvae varied from 2.5 mm at hatching to 5 mm 13 days after hatching and 9 mm after the first month. Some larvae were fixed in formaldehyde–phosphate buffer 0.1 M, pH 7.2, or in Bouin's solution, and embedded in paraffin for the histomorphological and immunocytochemical studies. Haematoxylin (Harris)–Eosin and Haematoxylin–Light Green–Orange G–Acid Fuchsin (Gutierrez, 1967) were used for morphological techniques. Other unfixed samples were frozen and sectioned in the cryostat (Cryocut E, Reichert–Jung) for demonstration of enzymatic activities (Table 1).

Controls of specificity were: incubation of active sections without substrate, and incubation of heat-inactivated sections in complete medium. Acid and alkaline phosphatases can hydrolyse glucose-6-phosphate at pH 6–7. Thus, controls for glucose-6-phosphatase included parallel incubations with β-glycerophosphate instead of glucose-6-phosphate as the substrate. Glycerophosphate is not hydrolysed by glucose-6-phosphatase. Besides L-cysteine, *N*-ethylmaleimide, L-tetramisole, L-phenylalanine, sodium fluoride, formaldehyde, 1,5-sorbitan-6-phosphate, *p*-chloromercuribenzoic acid and ouabain at concentrations between 1 and 20 mM were added to the incubation medium to inhibit different enzymatic activities (Lojda *et al.*, 1979; Culling *et al.*, 1985; Vacca, 1985).

The presence of trypsin, insulin, glucagon and somatostatin was investigated immunohistochemically using polyclonal antibodies anti-trypsin (1:1200), anti-insulin, anti-glucagon and anti-somatostatin (1:200 to 1:1000). Biotinylated goat anti-rabbit IgG or goat anti-rabbit IgG (second antibodies) and the biotin–streptavidin system (StrAvigen Supersensitive Universal Kit) supplied by Menarini Diagnostic, the avidin–biotin complex (Vectastain ABC kit) supplied by Vector Laboratories, or the PAP method (Sternberger *et al.*, 1970) were used. Peroxidase activity was detected using 3,3'diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO). Tests for specificity included: blocking endogenous peroxidase (0.3% H_2O_2 in methanol) and blocking biotin (avidin/biotin blocking) in tissues, replacement of antiserum with normal goat serum and omission of primary or secondary antibodies.

Results

The digestive tract of *Sparus aurata* L. is very undifferentiated at hatching. It is a straight tube leading to the yolk sac, with no folds or muscular layers, and it is histologically undifferentiated. Its epithelium is made up of a monostratified layer of columnar cells with basal nuclei and eosinophilic borders. There are no anterior or posterior openings. The accessory glands, liver and pancreas, are not yet developed (Fig. 1B). As the yolk sac is reabsorbed (Fig. 1A, B, C), the digestive tract changes its shape and structure. On day 1, the lower part curves and opens through the anus. On day 3, a loop begins to take shape in the digestive tract, and the mouth opens, presenting two epithelial folds made up of flat cells, surrounded by oral valves. Three areas may be distinguished in the digestive tract: an anterior area, made up of a monostratified epithelium of clear cubic cells with no brush border, a middle area with a prismatic epithelium and a striated acidophilic border, and a posterior area, similar to the middle portion, but with shorter epithelial cells.

The liver and pancreas begin to appear between days 2 and 3, their cells being very similar to each other's and to those of the undifferentiated digestive tract. The liver appears at the back of the yolk sac as a compact, basophilic tissue. The pancreas is extrahepatic from hatching through the first month of larval life, and is divided into two parts: one attached to the liver and the other between the anterior and middle areas of the digestive tract. It is arranged in acini, made up of elongated cells with a basophilic cytoplasm and a prominent nucleus in which the nucleolus is visible. The zymogen granules are stored in the centre of the acinus from day 4. In the anterior part of the digestive tract, an elongated area of flat cells is differentiated into the oesophagus, and also a wider area with a cubic epithelium forms the beginnings of the stomach. The ileocaecal valve separating the anterior of the posterior intestine is also differentiated on day 4.

Table 1. Inhibition of enzymatic activities in the digestive tract of *Sparus aurata* larvae[a]

Alkaline phosphatase (EC 3.1.3.1)	*Acid phosphatase* (EC 3.1.3.2)	*Glucose-6-phosphatase* (EC 3.1.3.9)	*Adenosine triphosphatase* (EC 3.1.3.3)
L-Cysteine (10 mM)	Sodium fluoride (10 mM)	1,5-Sorbitan-6-phosphate (20 mM)	Ouabain (3 mM)
L-Tetramisole (5 mM)	Formaldehyde (20 mM)	Formaldehyde (20 mM)	*p*-Chloromercuribenzoic acid (10 mM)
L-Phenylalanine (5 mM)			

[a]Data given in the table are derived from the monographs of Lojda *et al.* (1979), Culling *et al.* (1985) and Vacca (1985).

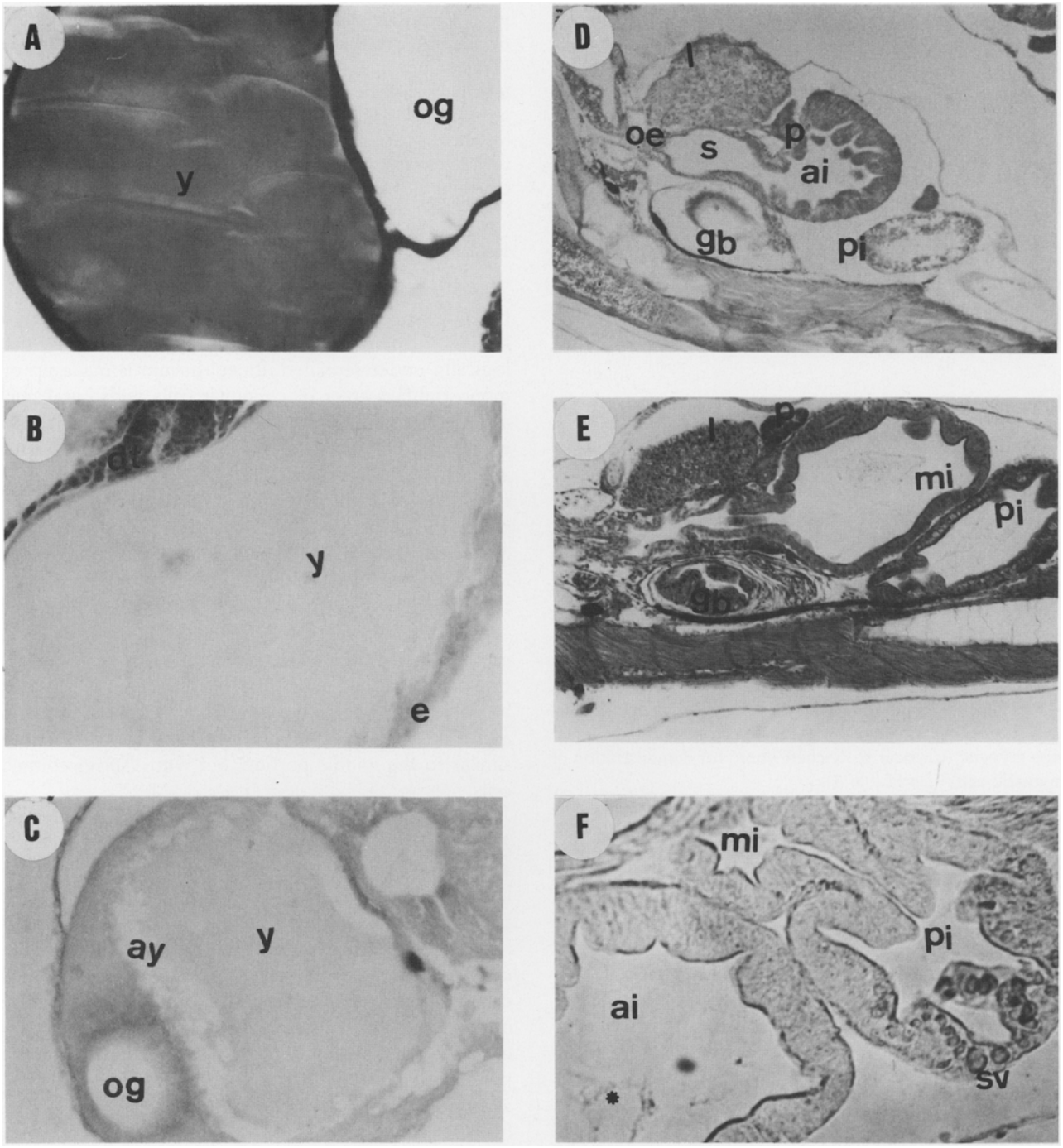

Fig. 1. (A) Yolk sac of *Sparus aurata* larva at hatching. Haematoxylin–Light Green–Orange G–Acid Fuchsin ×250. (B) Digestive tract and yolk sac of a larva at hatching. Haematoxylin–Eosin. ×250. (C) Yolk sac of a larva at day 2 of hatching. Absorption of yolk. Haematoxylin–Eosin. ×125. (D) Digestive tract and accessory glands of a 6-day-old larva feeding. Haematoxylin–Eosin. ×125. (E) Digestive tract of a larva feeding at day 10. Haematoxylin–Light Green–Orange G–Acid Fuchsin. ×125 (F) Mucosa of intestine of a 10-day-old larva feeding. Supranuclear vacuoles are seen in the posterior intestine and striated border of the enterocytes. Acid phosphatase and Haematoxylin–Eosin. ×250. ai = anterior intestine; ay = absorption yolk; e = envelope of yolk sac; gb = gas bladder; l = liver; mi = medial intestine; oe = oesophagus; og = oil globule; p = pancreas; pi = posterior intestine; s = stomach; sv = supranuclear vacuoles; y = yolk.

From days 5–6, transverse folds appear in the anterior intestine and increase with age. Their epithelium is monostratified, made up of cuboid cells and a striated border, with goblet cells is observed (days 14–17). From day 6 through the first month of larval life, acidophilic supranuclear vacuoles, whose appearance is related to exogenous feeding, are observed in the epithelial cells of the hindgut (posterior intestine, Fig. 1D, E, F).

During the period studied, *Sparus aurata* larvae have no gastric glands in the stomach, and their epithelium is made up of a layer of cuboid cells with no striated border. No pyloric caeca are observed at this stage, and the pancreas is extrahepatic.

Enzymes

Alkaline and acid phosphatase, adenosine triphosphatase (ATPase), glucose-6-phosphatase activities and trypsin are weakly positive in the yolk sac matrix of *Sparus aurata* larvae. The cellular envelope covering the yolk sac and

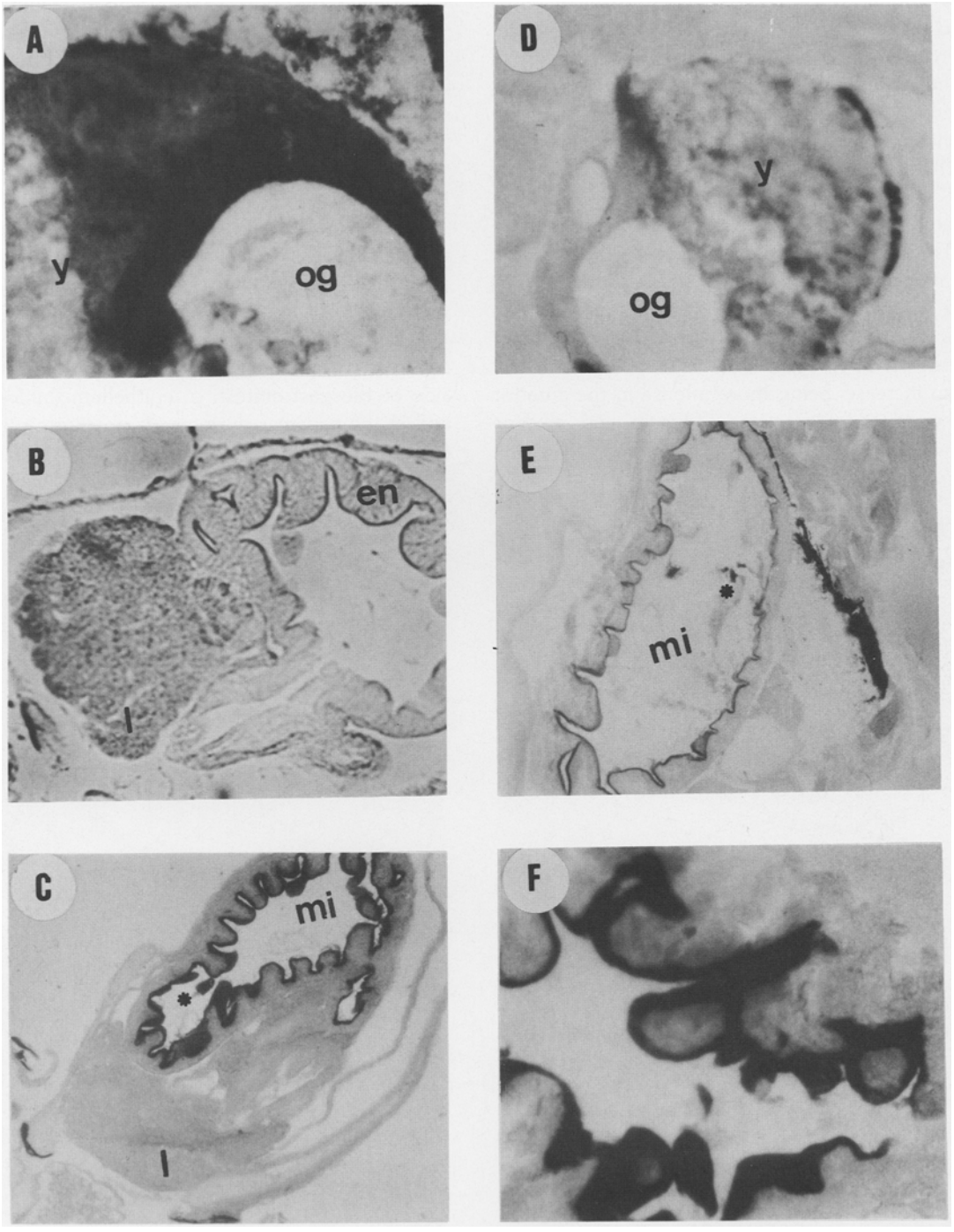

Fig. 2. (A) *Sparus aurata* larva at day 2 after hatching. Absorption of yolk. Acid phosphatase activity and Haematoxylin–Light Green–Orange G–Acid Fuchsin. ×400. (B) Larva at day 7. Enzymatic activity in liver and enterocytes of anterior intestine. Glucose-6-phosphatase and Haematoxylin–Eosin. ×250. (C) Alkaline phosphatase activity in the mucosa of the intestine of a 7-day-old larva feeding. ×125. (D) Glucose-6-phosphatase activity in the envelope of the yolk sac and granular yolk of a 3-day-old larva. ×125. (E) Acid phosphatase in the digestive tract of a 5-day-old larva. ×125. (F) ATPase activity in the brush border of enterocytes of the anterior intestine of a 7-day-old larva feeding. ×400. en = enterocytes; * = food ingested; l = liver; mi = medial intestine; og = oil globule; y = yolk.

show high levels of activity of these enzymes. The digestive tract shows no enzymatic activity until 3 days (Fig. 2A ,D).

The striated border of the intestinal enterocytes in 4-day-old sea bream larvae contains alkaline phosphatase and ATPase, the activities of acid phosphatase and glucose-6-phosphatase being very weak. Alkaline phosphatase and ATPase are more intense in the brush border of the enterocytes of larvae that have already re-absorbed their yolk reserves (day 5). All the enzymatic activities studied were more abundant in the anterior than in the posterior intestine (Fig. 2B, C, E, F).

The epithelium of the stomach mucosa, with no gastric glands, in 4-day-old sea bream larvae lacks alkaline phosphatase, but contains ATPase and acid phosphatase activities. The presence of this phosphatase activity in the supranuclear vacuoles of the lower intestine, which appear during the exogenous feeding stage, is worth noting. During this stage, trypsin is evident in the striated border of the intestinal enterocytes. Alkaline phosphatase and ATPase also increase, being more intense in the anterior than in the posterior intestine. Trypsin and alkaline phosphatase are also positive in the food ingested by the larvae (*Artemia* nauplii and rotifers) (Fig. 3A–D).

The liver of sea bream larvae contains glucose-6-phosphatase and acid phosphatase; the endothelium of the vascular system and the biliar cannalicula show alkaline phosphatase and ATPase activities (Fig. 2B).

Between the start of exogenous feeding and days 14–15, a weak (trypsin, glucose-6-phosphatase) or strong (phosphatases, ATPase) increase in the staining reaction of the enzymes was detected.

The effect of different inhibitors on these enzymatic activities is shown in Table 1.

Hormones

Three or four days after hatching, the sea bream larvae show positive insulin and glucagon reactivity in the portion of the pancreas attached to the liver, and glucagon-positive cells also in the pancreatic portion which is near the middle intestine. The first cells showing somatostatin immunoreactivity appear on the luminal side of the gastrointestinal epithelium (middle intestine) and in the pancreas during yolk absorption (day 3 or 4).

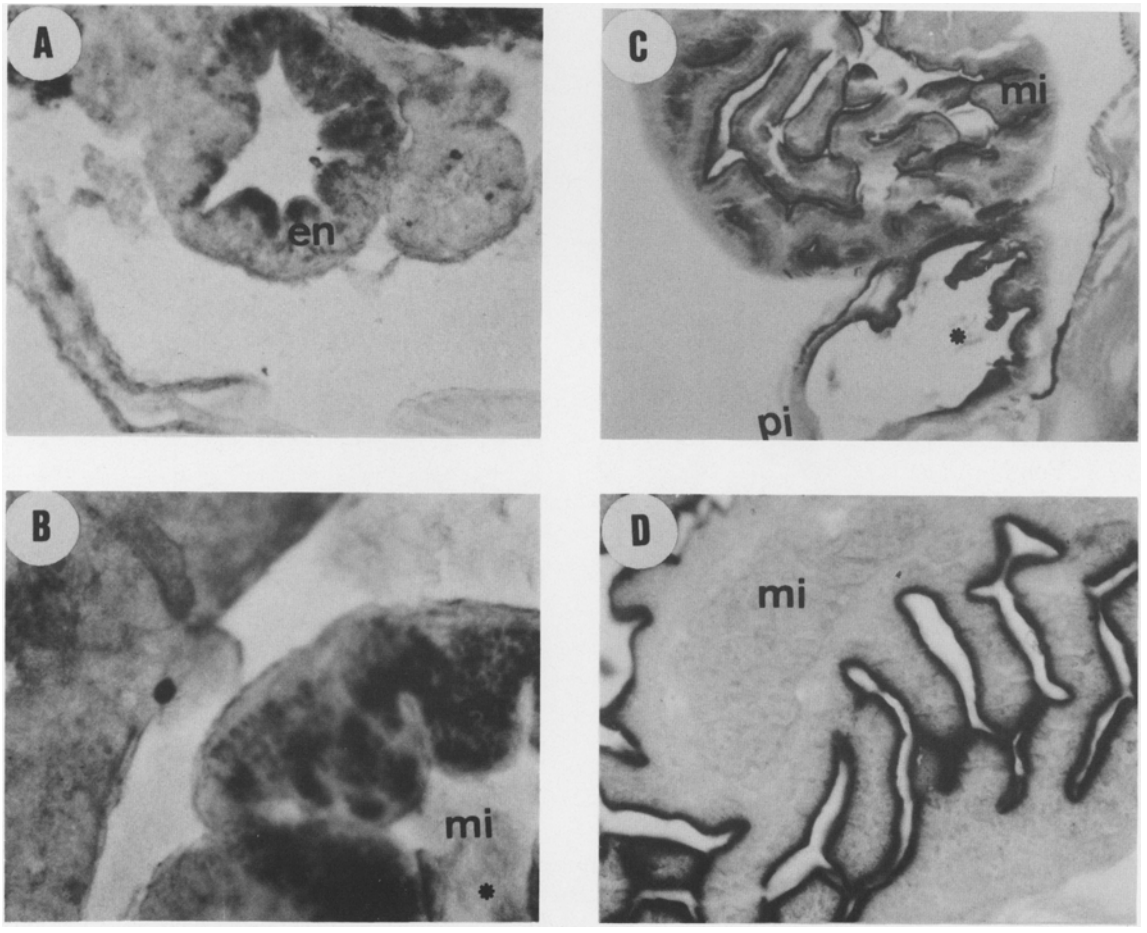

Fig. 3. (A) Trypsin in the cytoplasm of the intestinal enterocytes in a 6-day-old larva. ×250. (B) Enterocytes of the medial intestine in a 12-day-old larva feeding. Trypsin. ×400. (C) Digestive tract in a 15-day-old larva feeding. ATPase activity. ×125. (D) Alkaline phosphatase activity in the brush border of the intestinal mucosa of a 15-day-old larva. ×250. * = food ingested; en = enterocytes; mi = medial intestine; pi = posterior intestine.

Similar results were observed with all the immunocytochemical detection methods used.

Discussion

During the endogenous feeding of teleost larvae, yolk absorption, intracellular digestion and degradation of yolk platelets have been described by different authors (Hamor & Garside, 1973; Bashop & Schwartz, 1974; Vernier & Sire, 1977b; Sire & Vernier, 1991).

At hatching, the yolk sac of *Sparus aurata* larvae contains glycogen, glycoproteins and proteins rich in different amino acids (Polo, 1991), as well as glucose-6-phosphatase, trypsin, adenosine triphosphatase and acid and alkaline phosphatase activities. During the larval development of *Salmo irideus*, Manfredi-Romanini *et al.* (1969) detected ATPases, phosphatases, lipases, aminopeptidases and other enzymes, as much in the wall of the yolk sac as in the yolk syncytium. These authors pointed out the intense metabolic activity of the syncytium, and its role in carbohydrate, protein and lipid digestion, in the absorption processes and in the transport of macromolecules through the membranes.

The morphology, histology and histochemistry of the digestive system of *Sparus aurata* young and adults have been described in depth by Elbal & Agulleiro (1986) and Cataldi *et al.* (1987). There are some notable differences between the digestive tract of the young and adult fish and that of larvae. In young and adult specimens, the stomach is a well-differentiated organ, with gastric glands and four pyloric caecae in the anterior intestine, which respectively ensure the acid digestion of proteins (pepsin) and increase the intestinal surface (Govoni *et al.*, 1986). Sea bream larvae have no gastric glands or pyloric caecae during the first month, a fact which is true of most teleost species, in which the functional stomach, with gastric glands and musculature, and pyloric caecae appear late in larval life, near metamorphosis (Vu Tan Tue, 1976; Govoni, 1980; Cousin & Baudin-Laurencin, 1985; Avila & Juario, 1987; Ferraris *et al.*, 1987).

Although alkaline proteolytic enzymes (trypsin and chymotrypsin) have been described in nearly all species from the beginning of exogenous feeding of teleost larvae (Barr *et al.*, 1985; Baragi & Lovell, 1986; Pedersen *et al.*, 1987; Segner *et al.*, 1989) their activity is low, increasing during the development (Vu Tan Tue, 1983; Lauff & Hofer, 1984). The decrease in trypsin activity from the anterior to the posterior intestine in teleost larvae and adults suggests that most alkaline proteolytic digestion takes place in the anterior intestine (Stroband & Van der Veen, 1981; Segner *et al.*, 1989).

In the posterior intestine of *Sparus aurata* larvae which are ingesting food, a large number of acidophilic supranuclear vacuoles are detected; they are proteic in nature (Polo, 1991), and they contain acid phosphatase. These inclusions are not observed in starved sea bream larvae (Polo, 1991), and they have been described in larvae and adults of this and other species, representing the pinocytosis of proteins (Govoni, 1980; Watanabe, 1984a, b; Cousin & Baudin-Laurencin, 1985; Georgopoulou *et al.*, 1985, 1986; Elbal & Agulleiro, 1986; Avila & Juario, 1987).

The distribution of phosphatases, adenosine triphosphatase and glucose-6-phosphatase, and the effect of the inhibitors in these enzymes, in the digestive tract of sea bream larvae does not differ from that observed in *Sparus aurata* adults (Sarasquete *et al.*, 1990b), bearing in mind the incomplete development of the larval digestive system with an absence of gastric glands and pyloric caecae. There are inter- and intraspecific differences in relation to the effect of the inhibitors (Gonzalez de Canales *et al.*, 1987, 1990).

The presence of trypsin, phosphatases and ATPase in the yolk sac of *Sparus aurata* larvae, and later, as described by different authors (Stroband *et al.*, 1979; Lauff & Hofer, 1984; Cousin *et al.*, 1987; Ferraris *et al.*, 1987; Segner *et al.*, 1989; Verreth *et al.*, 1992), in the epithelium and prey present in the lumen of the digestive tract of other species, enables the larvae to digest proteins and to absorb and transport the macromolecules through membranes. Some of these authors also observed peptidases, aminopeptidases, amylases and esterases at the beginning of exogenous feeding of teleost larvae, which complement the proteolytic digestion and ensure digestion of fat and carbohydrate in larvae.

In *Sparus aurata* larvae, the presence of pancreatic hormones, insulin and glucagon, is evident 3 or 4 days after hatching, in *Clarias gariepinus* 14 hours after hatching (Verreth *et al.*, 1992), and later in other species (Youson & Cheung, 1990). The enzymes related to glycogen synthesis and degradation, and to glycaemia regulation (glucose-6-phosphatase) in yolk sac and liver, have been observed in sea bream larvae and in other species (Vernier & Sire, 1976, 1977a; Verreth *et al.*, 1992). Polo (1991) described the presence of glycogen in the oocyte yolk and in yolk sac and liver (day 4) during development of sea bream larvae.

The presence of somatostatin and glucagon in the pancreas and in the gastrointestinal luminal epithelium of the *Sparus aurata* larvae may be related to APUD-system cells, which regulate not only growth hormone but also the synthesis of insulin and glucagon in vertebrates (Junqueira & Carneiro, 1981).

In conclusion, during larval development of *Sparus aurata*, larvae already possess hormones (insulin, glucagon and somatostatin) and enzymes (glucose-6-phosphatase) which are able to regulate glucose metabolism. They also possess proteolytic enzymes (trypsin) and hydrolases (phosphatases and ATPase) which ensure protein digestion, as well as the absorption and transport of macromolecules through membranes. The absence of gastric glands, possibly related to the synthesis of pepsin, may be compensated for by the ability of the hindgut's vacuole system to carry out acid proteolytic digestion,

although this ability also persists in adults of *Sparus aurata* as in other teleost species.

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