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# Histochemical aspects of the yolk-sac and digestive tract of larvae of the Senegal sole, *Solea senegalensis* (Kaup, 1858)

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**Summary.** Histochemical distribution of glycoproteins, carbohydrates and proteins rich in different aminoacids were studied using histological and histochemical procedures, in Senegal sole, *Solea senegalensis* (Kaup, 1858) larvae from hatching until day 15. Glycogen, proteins and glycoproteins were detected in the yolk-sac of the larvae at hatching and during the yolk-resorption. The epithelial digestive system (brush border, enterocytes and goblet cells) contained neutral and acid mucins (carboxylated and/or sulphated). Glycogen was observed in the cytoplasm of the digestive absortive cells (enterocytes) and in the liver (hepatocytes) on day 3-4 posthatching. Protein reactions, and specially those that showed proteins rich in arginine, tyrosine and tryptophan, were very intense in the zymogen granules of the pancreatic cells. Oesophageal and intestinal goblet cells contained glucose N-acetyl and sialic acid residues, but the mucin content of these mucous cells did not show affinity towards Con-A, suggesting the absence of glycoproteins with Mannose and/or glucose residues. WGA showed a very intense positivity in the microvilli of the digestive epithelium of the larvae and positive granules for both lectins, specially for Con-A, were detected in the cytoplasm of the anterior intestinal enterocytes.

**Key words:** Histochemistry, Glycoproteins, Carbohydrates, Proteins, Larvae, *Solea senegalensis*

## Introduction

Senegal sole, *Solea senegalensis* (Kaup, 1858) is a species well adapted to warm climates and commonly exploited in extensive aquacultural production of some southern European countries such as Spain (Drake et al., 1984) and Portugal (Dinis, 1992), but at the present time, there is little information on the biology of this species which has great economic-commercial interest (Rodríguez, 1984; Gutiérrez et al., 1985; Vázquez et al., 1994).

The vast literature regarding the digestive tract and digestion of teleosts includes observations on early ontogenic stages and on the morphological changes (Kapoor et al., 1975; Govoni et al., 1986; Bisbal and Bengston, 1995), as well as others related with the histochemical distribution and variation of proteic, lipidic and glucidic macromolecules (Cousin and Baudin-Laurencin, 1985; Ferraris et al., 1987; Segner et al., 1989, 1994; Deplano et al., 1991; Boulhic and Gabaudan, 1992; Sarasquete et al., 1993a,b, 1995). During endogenous feeding stage in most fish, the absortion of nutrients takes places through the syncytium surrounding the yolk-sac, where reserves have been accumulated during vitellogenesis of the oocytes (Mommsem and Walsh, 1988). The onset of feeding is a critical stage in the development of marine fish; when larval feeding begins, the digestive tract is already functional (Segner et al., 1994), although less developed than in adults (Govoni et al., 1986; Sarasquete et al., 1995). Lipids are one of the most important components of fish eggs, providing energy reserves and components of cell biomembranes (Sargent et al., 1989). In fish, lipids are used mainly after hatching, with protein and carbohydrate utilized before hatching (Kimata, 1983).

On the other hand, intestinal mucins are known to play an important role in the protection of the mucose against physical and chemical damage and bacterial attack (Allen et al., 1986; Chadee et al., 1987). The characteristics of mucins in the digestive system of fish have been widely studied by means of classical methods of histochemistry (Reifell and Travill, 1977, 1978, 1979; Gutierrez et al., 1986; Elbal and Agulleiro, 1986; Grau et al., 1992; Boulhic and Gabaudan, 1992; Sarasquete et al., 1995). Mucosubstances have been divided into two groups: neutral and acids, and two types of acid mucins

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have been identified: sulpho and sialomucins, depending on the presence of sulphate groups or sialic acid residues. Mucins must be considered neutral only if no acid residues are identified (Reid and Clamp, 1978).

Lectins are proteins or glycoproteins of non-immune origin with the ability to recognize specific saccharides, such as: N-acetyl-neuraminic acid or sialic acid (NANA), N-acetyl-galactosamine (GalNAc), N-acetyl-glucosamine (GlcNAc), Mannose (Man), glucose (Glc), galactose (Gal), Fucose (Fuc) residues, etc. (Pearse, 1985; Madrid et al., 1989a; Bancroft et al., 1990; Danguy et al., 1994; Menghi et al., 1994). In different teleost lectins have been found to be powerful and reliable tools to investigate the characteristics and distribution of glycoproteins of the digestive tract, reproductive system, etc (Gutierrez et al., 1985, 1986; Madrid et al., 1989a; Grau et al., 1992; Gonzalez de Canales et al., 1992; Sarasquete et al., 1993b, 1995). GlcNAc and/or sialic acid and GalNAc are the most common carbohydrate residues in the digestive tract of different vertebrates including fish, and generally the mucous cells do not show Man, Glc and Fuc residues of the glycoproteins (Madrid et al., 1989a).

In the present study, the distribution of carbohydrates (glycogen and mucins), proteins and glycoproteins was studied in the yolk-sac and digestive system of the Senegal sole, *Solea senegalensis* larvae from hatching until day 15 posthatching.

## Materials and methods

Larvae of *Solea senegalensis* (supplied by Cupimar SA fisheries from San Fernando, Cádiz, Spain) from hatching and until day 15 posthatching, were fixed in 10% v/v buffered formaldehyde (pH 7.2), dehydrated and embedded in paraffin wax. Sagittal sections of 6-8 $\mu$m thickness were stained with Haematoxylin-eosin or Haematoxylin-V.O.F. (Gutierrez, 1967). Histochemical methods for carbohydrates and proteins performed in this paper were taken from monographs by Pearse (1985) and Bancroft et al. (1990). Techniques for carbohydrates were: PAS and diastase or amylase-PAS (neutral glycoproteins and glycogen); Alcian Blue at pH 0.5, 1 and 2.5 (sulphated and carboxylated groups); sialidase and chlorhydric hydrolysis-Alcian Blue pH 2.5 (sialic acid); Alcian Blue at pH 0.5 or 2.5-PAS (sulphated or carboxylated glycoproteins in blue, and neutral glycoproteins in red). Bromophenol Blue (general proteins); Ninhydrin-Schiff (proteins rich in lysine), Ferric ferricyanide-Fe III and Tioglycollate reduction (proteins rich in cysteine and cystine residues); p-dimethylaminobenzaldehyde (proteins rich in tryptophan), Hg-sulphate-sulphuric acid-sodium nitrate (proteins rich in tyrosine); and 1,2-napthoquinone-4-sulphonic acid salt sodium (proteins rich in arginine) techniques were performed for proteins.

For study of lectins, endogenous peroxidase activity was destroyed by a 30 min treatment with 0.3% hydrogen peroxide in TBS (Tris buffer saline, pH 7.2-7.4). Sections were washed in three 5-min changes of TBS and then incubated, in a moist chamber, for 2 hr at room temperature in horseradish peroxidase-conjugated lectins (20 $\mu$g/ml, diluted in the same buffer): Con-A (Man and/or Glc) and WGA (GlcNAc and/or sialic acid). After three washes in TBS, the peroxidase activity was visualized with TBS containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide. When finally reacted, the sections were washed in running tap water (10 min), dehydrated, cleared and mounted in Eukkit. The following controls were performed for the lectin stainings: 1) substitution of lectin-HRP conjugates by TBS; 2) pre-incubation of the lectins with the corresponding sugar inhibitor (D-GlcNAc and Methyl-α-Man) (Sigma Chemical, St. Louis, MO).

## Results

At hatching, Senegal sole, *Solea senegalensis* (Kaup, 1858) larvae showed a homogeneous acidophilic yolk

**Table 1.** Histochemical distribution of carbohydrates, proteins and lipids during development of *Solea senegalensis* larvae.

| | YOLKSAC | LIVER | PANCREAS | DIGESTIVE EPITHELIUM | |
|---|---|---|---|---|---|
| | | | | Enterocytes | Goblet cells |
| Neutral glycoproteins | 1 | 1 | 1 | 1 | 2 |
| Sialoglycoproteins | 0 | 1 | 1 | 2-3 | 3 |
| Sialosulphated glycoproteins | 0 | 0 | 0 | 2-3 | 0-3 |
| Glycoproteins with α-D-Man, α-D-Glc | 1 | 1 | 1 | 2 | 0 |
| Glycoproteins with (ß-D-GlcNAc)n and/or NANA | 2 | 1 | 1 | 2 | 3 |
| Glycogen | 1 | 1-3 | 0 | 2 | 0 |
| Proteins in general | 3 | 2 | 3 | 1 | 0 |
| Proteins rich in lysine | 3 | 1 | 3 | 1 | 0 |
| Proteins rich in arginine | 3 | 1 | 3 | 1 | 0 |
| Proteins rich in tyrosine | 2 | 1 | 2 | 1 | 0 |
| Proteins rich in tryptophan | 2 | 1 | 3 | 1 | 0 |
| Cysteine residues | 2 | 1 | 2 | 1 | 0 |
| Cystine residues | 2 | 1 | 2 | 1 | 1 |

Results are expressed as semiquantitative assessment of colour intensities by independent scores of two investigators. Intensity of the reaction: 0, negative; 1, weak; 2, moderate; 3, intense.

surrounded by a monostratified layer of basophilic cuboid cells (Fig. 1). The yolk-sac showed affinity to eosin (Haematoxylin-eosin) and to light green and orange G (at the periphery) when Haematoxylin-V.O.F. morphological techniques were performed. Various vacuoles, probably corresponding to the neutral lipids dissolved during paraffin embedding process, were observed in the yolk-sac (Fig. 1A,B,C). The yolk matrix contained glycogen and proteins (Fig. 1B) rich in different aminoacids, but acid mucosubstances were not detected (Table 1).

At hatching, the alimentary canal of *Solea senegalensis* appeared undifferentiated as a straight tube attached dorsally to the yolksac. There were no anterior or posterior openings. The mouth opened on day 2-3 and simultaneously, one could observe the convolution of the gut. According to a saggital symmetrical plan, incipient stomach, anterior intestine, yolk and liver are positioned on the left and the pancreas, median and posterior intestine on the ride side. The yolk was gradually resorbed (Fig. 1D) and disappeared at the end of day 4. During this time, the structure and protein and carbohydrate distribution of the yolk-sac changed; the PAS (carbohydrates) and Bromophenol Blue (proteins) reactions being more intense in the acidophilic granular zone of the yolk than in the homogeneous portion.

The oesophagus differentiated on day 2. The folds of the mucosae consisted of a simple cubic epithelium that contained goblet cells with abundant secretion composed of sialosulphoglycoproteins (Table 1). These mucous cells increased in number and were more densely distributed in the anterior than in the posterior

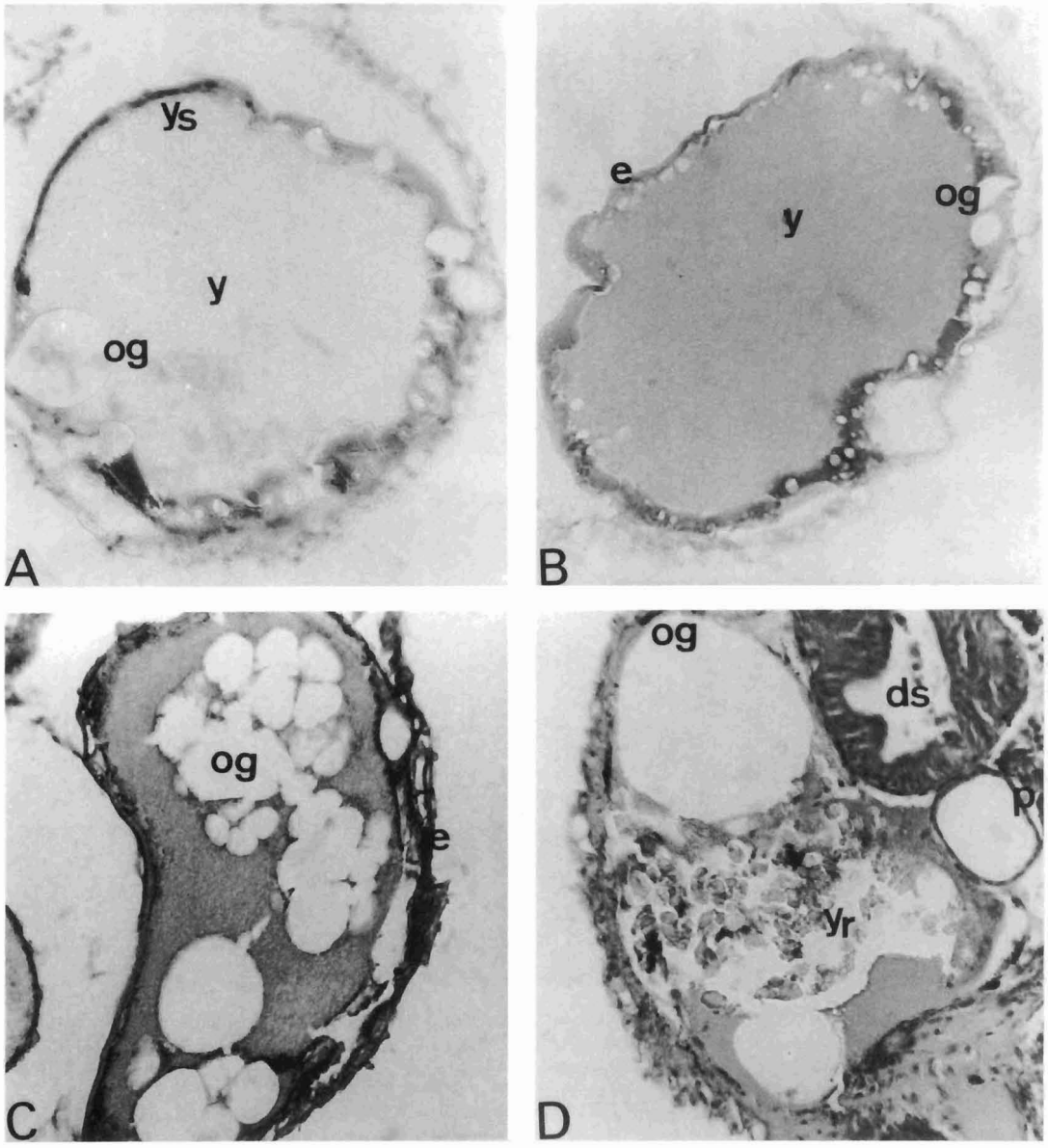


**Fig. 1.** Histological sections of the *Solea senegalensis* larvae at hatching and during the yolk resorption. **A.** Larvae at hatching showing the yolksac (matrix and envelope) with peripheral oil globules enclosed, corresponding to neutral lipids dissolved during paraffin embedding process. Haematoxylin-V.O.F. **B.** Presence of proteins in the yolk matrix and envelope of the yolksac and absence of proteic material in oil globules. Bromophenol blue reaction. **C.** Glycoproteins with ß-D-GlcNAc residues in the yolk-sac. WGA lectin. **D.** Resorption of yolk in a 3-day-old larva. Haematoxylin-eosin. ds: digestive system; e: envelope of the yolk-sac; og: oil globule; p: pancreas; y: yolk; yr: yolk-resorption; ys: yolk-sac. x 250

oesophagus. The stomach appeared, at hatching, as a little pocket with a simple cubic epithelium containing neither goblet cells nor gastric glands. Neutral glycoprotiens were present in the columnar epithelial cells. The intestine had a regular mucosae consisting of a pseudostratified layer of cells, and contained enterocytes with an acidophilic striated border, goblet cells and a very thin connective layer. These cells were more abundant in the anterior portion of the intestine and their number increased progressively from day 4. Enterocytes showed a basophilic granular cytoplasm and an acidophilic striated border (affinity to light green of the polychrome V.O.F.) containing neutral and acid mucosubstances. At this time very few goblet cells were stained with Alcian blue (pH 2.5)-PAS, suggesting the presence of carboxylated and neutral (PAS and diastase-PAS positive reaction) mucosubstances, as well as the presence of glycogen in the cytoplasm of the enterocytes (PAS positive and diastase-PAS negative reactions). Sulphated mucosubstances were not observed in the intestinal goblet cells (Fig. 2A). On day 3-4 post-hatching, the first signs of lipidic (vacuoles) and proteic absortion were observed in the anterior and posterior part of the intestine, respectively.

The oesophagus epithelium of 10-15-day-old larvae consisted of a stratified squamous epithelium containing

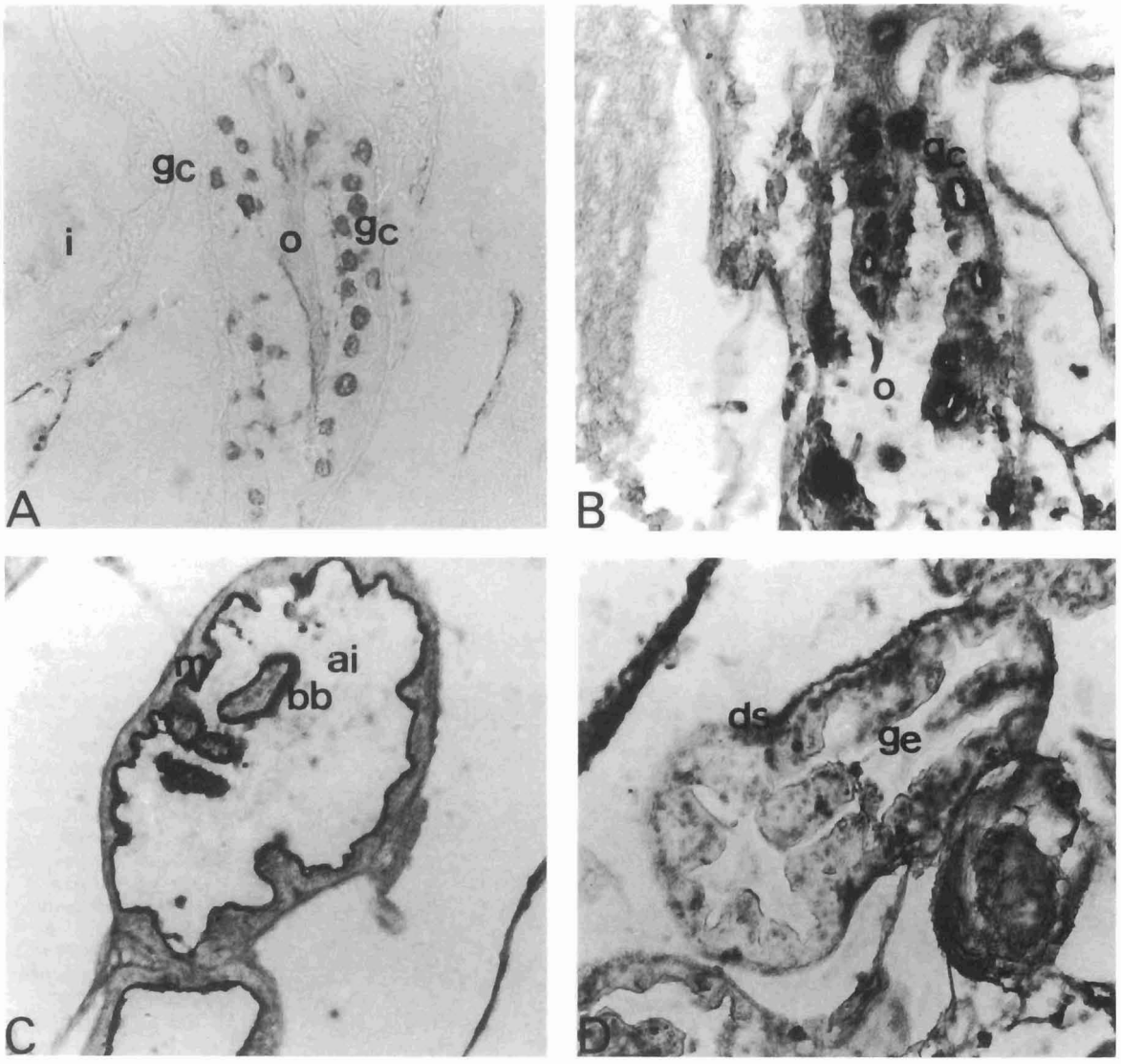


**Fig. 2.** Histological sections of the digestive system of *Solea senegalensis* larvae at different days posthatching. **A.** Sulphated mucosubstances in oesophageal goblet cells and negativity in the intestinal mucous cells of a larva at day 5. Alcian blue pH 0.5 reaction. x 250. **B.** Glycoproteins with GlcNAc and/or sialic acid residues in the mucous cells (goblet cells) of the oesophagus. WGA. x 400. **C.** Digestive epithelium of a larva at 9 day showing strong positivity to glycoprotein reaction in the brush border of the enterocytes. WGA x 250. **D.** Presence of glycoproteins (Man and/or Glc) in the granular enterocytes of the anterior intestine of a 9-day-old larva. Con-A x 250. ai: anterior intestine; bb: brush border of the enterocytes: ds: digestive system; gc: goblet cells; ge: granular enterocytes; i: intestine; m: mucose; o: oesophagus.

goblet cells that were more densely distributed in the anterior than in the posterior portion of the digestive system. The stomach epithelium consisted of cuboidal or columnar epithelial cells having nuclei in the middle or basal parts, and neutral mucins were detected in the epithelium. Goblet cells were absent, and during this time (15-days-old larvae) gastric glands were not observed in the stomach. A submucose, a muscularis of circular striated muscle fibers and a serosa completed the stomach wall. The epithelium of the intestine consisted of a single layer of columnar cells or enterocytes with an acidophilic striated border and goblet cells; these mucous cells decreased in number in the posterior part of the intestine. During this time we could observe evident signs of absortion in the shape of white infranuclear vacuoles (neutral lipids) in the anterior and middle intestine together with large eosinophilic and spherical inclusions (containing proteins) in the supranuclear areas of the enterocytes inside the hindgut (posterior part of the digestive tract).

There were some histochemical differences between the oesophageal and intestinal goblet cells of the *Solea senegalensis* larvae. These mucous cells were negative to the protein reactions (Table 1). The oesophageal goblet cells contained sialo and sulphomucin glycoproteins (Table 1; Fig. 2A), but those present in the intestine only contained sialomucins (sialidase-Alcian blue pH 2.5 negative reaction) (Table 1). Con-A and WGA stained the striated border of the oesophageal and intestinal epithelium and the cytoplasm of the absortive cells (enterocytes) (Fig. 2B,C). WGA and Con-A (Fig. 2D) -positive granules were detected in the absortive cells of the anterior intestine. Both lectins failed to stain or they stained the epithelium of the incipient stomach weakly. In the posterior intestine, Con-A only stained the cytoplasm of the columnar cells, but no reactivity, or very weak staining, was noted in the epithelium (microvilli) of these cells. Reactivity to WGA was

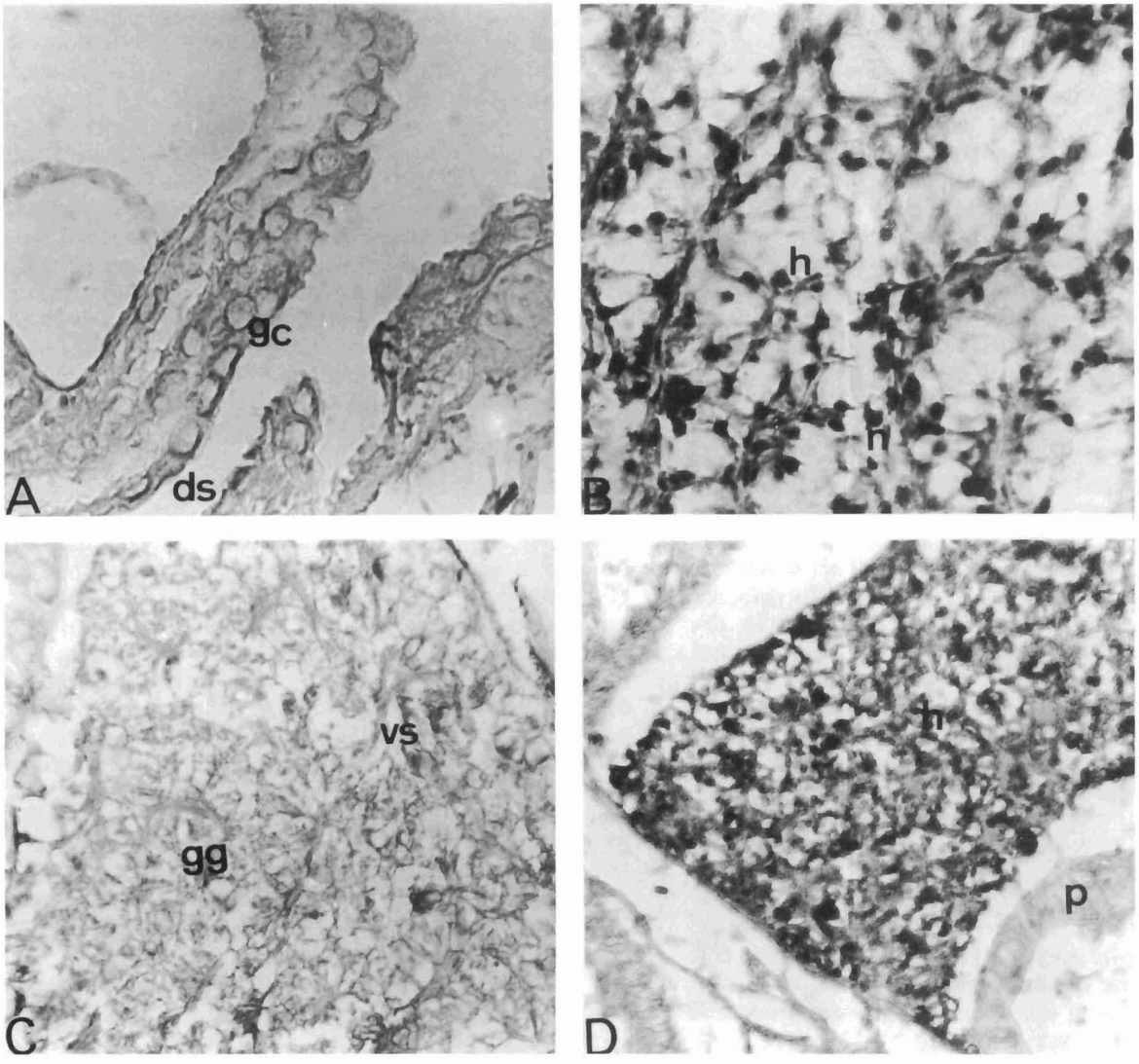


**Fig. 3.** Histological sections of the intestine and liver of larvae at day 15. **A.** Absence of glycoproteins with Man and Glc residues in the digestive goblet cells. Con A lectin. x 400. **B.** Hepatocytes and vascular system of the liver. Haematoxylin-eosin. x 400. **C.** Granules of glycogen (PAS positive and diastase-PAS negative) in the cytoplasm of the hepatocytes. PAS reaction. **D.** Glycoproteins with Man and/or Glc residues in the hepatocytes and vascular system of the liver. Con A lectin. x 250. ds: digestive system; gc: goblet cells; gg: granules of glycogen; h: hepatocytes; i: intestine; n: nucleus; p: pancreas; vs: vascular system.

identified in the digestive goblet cells (Fig. 2B) and in the brush border (microvilli) of the digestive epithelium (Fig. 2C) and Con-A-unreactive goblet cells (Fig. 3A) were detected in the digestive system of the *Solea senegalensis* larvae.

Histochemical results of the yolk-sac, digestive system, liver an pancreas of the larvae are summarized in the Table 1 and Figs, 1-3. Glycogen (Fig. 3C) and possibly neutral lipids were observed in the cytoplasm of the hepatocytes. Proteins specially rich in arginine, tyrosine and tryptophan were evident in the acidophilic granules (zymogen) of the pancreatic cells of the larvae on day 4 posthatching.

## Discussion

In Senegal sole, *Solea senegalensis* (Kaup, 1858), the distribution of carbohydrates, proteins and lipids in the mature oocytes (Gutierrez et al., 1985) resembled that found in the yolk-sac of recently hatched larvae, such as has been observed in *Sparus aurata* (Sarasquete et al., 1993b, 1995). The yolk-sac of the Senegal sole contains glycogen, glycoproteins with Man and/or Glc residues, proteins rich in different aminoacids, specially proteins rich in arginine and lysine, and possibly neutral lipids (oil vacuoles). Phospholipids, glycolipids, lipoproteins and enzymes related to protein, carbohydrate and lipid metabolism have been reported in the yolk-sac larvae of different species (Manfredi-Romanini et al., 1969; Vernier and Sire, 1977; Sire and Vernier, 1981; Sarasquete et al., 1993a,b, 1995). In *Sparus aurata*, acid lipids present in the phospho-lipoglycoproteic yolk granules and neutral lipids present in the lipid globules of the oocytes are deposited, respectively, in the matrix and in the oil globules of the yolk-sac larvae (Sarasquete et al., 1993b).

During development of *Solea senegalensis*, neutral lipid (triacylglicerides and sterol esters) decreased and polar lipids increased, mainly due to a significant increase in phosphatidylserine (Vázquez et al., 1994). At histological level and during Senegal sole development, we also observed a decrease of oil vacuoles (dissolution of neutral lipids) in the yolk-sac, as well as its presence in the cytoplasm of the hepatocytes and in the enterocytes of the anterior intestine. In other species, glycogen, lipids and lipoproteins were reported (Vernier and Sire, 1977; Segner et al., 1994; Sarasquete et al., 1995). In the liver of the *Scophthalmus maximus* larvae and according to Segner et al. (1994), initially lipoid deposition occurs only along the periphery of glycogen fields, but later on the lipid droplets migrate into the glycogen fields, sometimes becoming the dominant energy store.

In the rapid development of Senegal sole, triacyl-glycerol reserves were apparently used to obtain the necessary metabolic energy to enhance development. A significant increase in total carbohydrate and total polar lipid content between non-feeding yolk-sac larvae and first-feeding larvae was reported by Vázquez et al. (1994). In general, lipids and free aminoacids have been regarded as substrates for aerobic energy production in marine fish during early development (Fyhn, 1989; Sargent et al., 1989), wile the carbohydrates do not seem to have an important energetic role, since a significant increase was observed from yolk sac larvae to first-feeding larvae (Vázquez et al., 1994). During the histological development of *Solea senegalensis*, PAS (carbohydrates) and Bromophenol Blue (proteins) reactions were more intense during yolk-resorption than in yolksac of the larvae at hatching.

The distribution of carbohydrates and proteins in pancreas and liver of the *Solea senegalensis* larvae was similar to that detected during development of the *Sparus aurata* by Sarasquete et al. (1995). The presence of glycogen and oil vacuoles (neutral lipids) in liver and enterocytes of the larvae from 3-4th day posthatching, is noticeable as well as the important content of proteins and specially of basic proteins in the exocrine pancreatic cells. In the hepatocytes of the *Solea senegalensis* larvae, Con-A probably labels to Glc residues from glycogen. Ribelles et al. (1995) in *Sparus aurata* L. adults also observed the presence of glycogen and proteins in the liver parenchyma, as well as the great content of proteins in the pancreatic cells. However, proteins rich in lysine, tyrosine, arginine and tryptophan were not observed by these authors in the exocrine pancreatic cells of the seabream.

On the other hand, goblet cells which develop within oesophagus and intestine of *Solea senegalensis* are apparent when the mouth is open (on day 3 post-hatching) as in *Solea solea* (Boulhic and Gabaudan, 1992). This development occurs during later stages in other species such as turbot (on day 7; Cousin and Boudin-Laurencin, 1986) and seabream (on day 14; Sarasquete et al., 1995). In Senegal sole, oesophageal goblet cells contain neutral and sialosulphomucins and the intestinal mucous cells synthesize sialomucins. In vertebrates, the sulphate ions have a unique protective action against acid injury to oesophageal epithelium (Tobey et al., 1986) and acid mucins may protect the intestinal epithelium of the degradative action of glycosidases (Rhodes et al., 1985). The last authors suggested that sialitation and sulphation of the glycoproteins may be important for increasing the resistance of mucus to bacterial degradation. Neutral mucins were not observed in *Solea solea*, whose oesophageal mucous cells contain acid mucosubstances (Boulhic and Gabaudan, 1992). According to Eversole (1972), each individual goblet cell synthesizes a neutral glycoprotein which is subsequently carboxylated and finally sulphated. As in seabream (Sarasquete et al., 1995), the protein reactions were negative in the digestive mucous cells of the *Solea senegalensis* larvae. The presence of cystine bridges in the digestive goblet cells may be related with its glycoproteic nature.

The brush border of the intestinal enterocytes contains neutral and acid glycoproteins, and the epithelium of the incipient stomach of the larvae is

composed of neutral mucins. In various teleost fish (Reifel and Travill, 1978), the presence of neutral mucins at the stomach level has been related to the absorption of easily digested substances such as disaccharides and short-chain fatty acids. On the other hand in Senegal sole larvae, supranuclear inclusions containing proteins were observed in the epithelium of the posterior intestine once feeding began. According to different authors they are absent in starving larvae (Yufera et al., 1993) and they are the result of the pinocytosis of proteins, as demonstrated using peroxidase (Govoni et al., 1986; Georgoupoulou et al., 1986a,b).

The digestive epithelium of *Solea senegalensis* larvae contained glycoproteins with Glc, Man, GlcNAc and/or sialic acid residues. According to Madrid et al. (1989a), the presence of these carbohydrates, as well as the absence of Man and Fuc in the goblet cells is a common feature of the intestinal epithelium of different vertebrates including fish. The presence of WGA and Con-A-positive granules in the absorptive epithelium of the anterior intestine of the *solea senegalensis* larvae, may support the concept that in fishes the absorption of carbohydrates occurs in the anterior intestine, as suggested by Scherbina et al. (1978) and Madrid et al. (1989a).

The present study has been carried out in larvae growing under standard rearing conditions that are assumed to produce healthy larval development. Further studies, such as the appearance and development of gastric glands and other organs, enzymatic digestive activities and the study of the lipid metabolism during larval development of this species are under consideration at optical and ultrastructural level.

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