Histol Histopathol (2001) 16: 511-521

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Histological and histochemical observations in the stomach of the Senegal sole, *Solea senegalensis*

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Summary. An histological and histochemical study was conducted on the stomach of adult Senegal sole, *Solea senegalensis* specimens. The stomach was made up of four distinct layers: mucosa, lamina propria-submucosa-, muscularis and serosa. Surface epithelial, glandular and rodlet cells were present in the mucosa. Cells of the columnar epithelium contained a basal nucleus. Numerous mitochondria, granular endoplasmic reticulum and Golgi apparatus consisting of several parallel cisternae and vesicles were observed in the cytoplasm of these cells. The lysosomes were small, round and dense. The gastric glands were numerous in the pyloric and fundic regions but absent in the cardiac stomach. These glands were formed by two cell-types: light and dark cells. The light cells were characterised by numerous mitochondria, while dark cells had slightly fewer mitochondria and a tubulo-vesicular system. Rodlet cells similar to those observed in other teleostean fish were present among the epithelial cells.

Although the epithelial cells of the mucosa contained a weak presence of neutral and acid mucopolysaccharides/mucosubstances, these substances were abundant in the lamina propria-submucosa. Proteins rich in arginine, lysine, cysteine and cystine were rarely present in the mucosa and lamina propria-submucosa of stomach, while proteins rich in tyrosine were abundant in these layers. Acid phosphatase, and ATP-ase (pH 7.2 and 9.4) activities were detected in the mucosa and lamina propria-submucosa. Alkaline phosphatase activity was not detected.

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

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Introduction

The structure of the gastrointestinal system of teleostean fish is well documented in the literature (Kapoor et al., 1975; Elbal and Agulleiro, 1986; Cataldi et al., 1987; Morrison, 1987; Grau et al., 1992; Domeneghini et al., 1998; Sarasquete et al., 1998), however, there are few studies on the morphology of the alimentary canal of pleuronectid species. Those available include investigations of gut of the adult fish (McLeese and Moon, 1989; Jenkins et al., 1992; Murray et al., 1994; Arellano et al., 1999) and larval and juvenile specimens (McDonald, 1987; Kjorsvik and Reiersen, 1992; Murray et al., 1993; Sarasquete et al., 1996; Ribeiro et al., 1999), or both (Veggetti et al., 1999). Histological aspects of the alimentary canal in pleuronectid species have not been studied in depth, however such research is becoming more valuable as interest in the culture of new species expands and, workers require more information related with histological and physiological aspects.

Histology of fish stomach mucosa is generally simpler than that of higher vertebrates. The gastric glands of fish contain only one cell-type that secretes both pepsinogen and hydrochloric acid (Reifel and Travill, 1978; Rebolledo and Vial, 1979).

The presence of mucosubstances in gastric epithelial cells has been observed in most teleosts (Kapoor et al., 1975). However, the literature contains few studies of the histochemistry of mucosubstances in the fish stomach (Reifel and Travill, 1978; Murray et al., 1994) and the investigations that have been done concern the variability of the epithelial mucosubstances in different groups of animals (Suganuma et al., 1981).

Senegal sole, *Solea senegalensis* is a flatfish (Fam. Soleidae) occurring along the west African coast and Atlantic coasts of Europe and Occidental Mediterranean (Arellano, 1999). This species is exploited extensively for aquaculture in southern European countries, such as Spain (Drake et al., 1984) and Portugal (Dinis, 1992; Dinis et al., 1999). Some studies have been performed at the histological level in larvae and juveniles (Sarasquete

Table 1. Histochemical reactions used to detect carbohydrates and proteins. Staining techniques for carbohydrates and proteins were taken from monographs by Martoja and Martoja-Pierson (1970) and Pearse (1985).

STAINING TECHNIQUES	FUNCTION AND/OR COMPONENTS DEMONSTRATED
Carbohydrates	
Periodic acid-Schiff (PAS)	Glycogen, neutral mucosubstances and/or glycoconjugates
Diastase-PAS	Glycogen
Alcian Blue (AB) pH 2.5	Carboxyl-rich glycoconjugates (sulphated or not)
HCl hydrolysis-AB pH 2.5	Sialic acid
AB pH 1.0	Sulphated glycoconjugates (weakly ionised)
AB pH 0.5	Sulphated glycoconjugates (strongly ionised)
Proteins	
Bromophenol blue	Proteins in general
Ninhydrin-Schiff	Proteins rich in lysine (-NH_2 groups)
Thioglicolate-potassium ferricyanide (Fe III)	Proteins rich in cystine (-S-S- groups)
1,2 Naphtoquinone-4-sulphonic acid, sodium salt	Proteins rich in arginine
Hg sulphate-sulphuric acid sodium nitrate	Proteins rich in tyrosine
Ferric ferricyanide (Fe III)	Proteins rich in cysteine (-SH- groups)
p-Dimethylaminobenzaldehyde	Proteins rich in tryptophan

et al., 1996, 1998; Ribeiro et al., 1999; Vieira, 2000) but references to Senegal sole adult specimens are scarce (Fehri-Bedoni, 1997).

The purpose of this study was to describe the histology (light and ultrastructural) and histochemistry of the stomach of adult Senegal sole, *Solea senegalensis* specimens, to be used as a basis for further works on nutrition, pathologies, etc, and to gather more information for the culture of this commercial flatfish species.

Materials and methods

Adult specimens of the Senegal sole, *Solea senegalensis* body weight 370-430 g (total length ranging from 20-30 cm; 2-3 years old) were collected from "Cupimar, S.A" fisheries (San Fernando, Cádiz, Spain). After capture they were maintained in tanks of 2000 L in the Instituto de Ciencias Marinas de Andalucía (CSIC) until they were utilised. Fish were anaesthetised with benzocaine (50 mg/L); the abdominal cavity was opened and the stomach dissected away. Small samples of stomach were fixed for 24 h in Bouin's fluid. After dehydration in a graded series of ethanol, the samples were embedded in paraffin. Sections of 6-8 μm thickness were stained with haematoxylin-eosin and haematoxylin-VOF (Gutiérrez, 1967). Lipid and enzymatic techniques were performed on unfixed frozen samples sectioned in a cryostat (Cryocut-E). Histochemical techniques for carbohydrates, proteins, lipids and enzymatic activities are shown in Tables 1, 2.

Scanning electron microscopy (SEM)

Stomach samples for scanning electron microscopy were fixed in 4% glutaraldehyde in 0.1M Na-cacodylate buffer (pH 7.2), dehydrated through an ethanol series, critical point dried with liquid CO_2, coated with gold, and viewed in a Hitachi S 570 scanning electron microscope, operated at 20 kv.

Table 2. Histochemical reactions used to detect lipids and enzymes. Staining techniques for lipids and enzymes were taken from monographs by Martoja and Martoja-Pierson (1970), Pearse (1985) and Bancroft and Stevens (1990).

STAINING TECHNIQUES	FUNCTION AND/OR COMPONENTS DEMONSTRATED
Oil Red 0	Neutral lipids
Br- Oil Red 0	Unsaturated lipids
Fe (III) Haematoxylin	Phospholipids
Sudan Black B	Neutral and acidic lipids
Nile Blue	Neutral lipids
Alkaline phosphatase (Gomori-Takamatsu method)	Alkaline phosphatase activity
Acid phosphatase (Gomori method)	Acid phosphatase activity
ATP-ase (pH 7,2)	ATP-ase (pH 7,2) activity
ATP-ase (pH 9,4)	ATP-ase (pH 9,4) activity

Transmission electron microscopy (TEM)

Small pieces of the stomach were fixed for 2 h in cold cacodylate-buffered, 2.5% glutaraldehyde at pH 7.2 (with 6% sucrose added), rinsed several times in buffer (without sucrose) and postfixed with 1% OsO_4 in 0.1M cacodylate buffer. The samples were rinsed several times in buffer, then dehydrated in a graded series of acetone and embedded in Spurr's medium. Ultrathin sections of 60 to 80 nm thickness (Reichert Jung ultramicrotome) were stained with uranyl acetate and lead citrate prior to examination using the electron microscope (Zeiss EM 9S2), operated at 80 kv.

Results

Scanning electron microscopy observation of the *Solea senegalensis* stomach showed primary longitudinal folds, on which secondary folds were also observed (Fig.

1). The surface of the epithelial cells exhibited short apical microvilli. Histologically, the stomach was made up of four distinct layers: mucosa, lamina propria-submucosa, muscularis and serosa (Fig. 2).

The gastric mucosa was composed of a simple columnar epithelium, with oval nuclei and evident nucleoli showing a strong affinity for Orange G of VOF-trichromic (Haematoxylin/VOF according to Gutiérrez, 1967). Cylindrical rodlet cells full of highly acidophilic granules were observed in the gastric mucosa of cardiac stomach (Fig. 3).

The gastric glands were surrounded by a layer of connective tissue (Fig. 4), and they were numerous throughout the stomach (fundic and pyloric regions) and absent in the cardiac stomach. The lamina propria-submucosa was composed of dense irregular connective tissue near the glandular zone. The muscularis consisted of two layers of smooth muscle: a circular internal and a longitudinal external layer (Fig. 2). The gastric serosa was formed by connective tissue containing capillaries

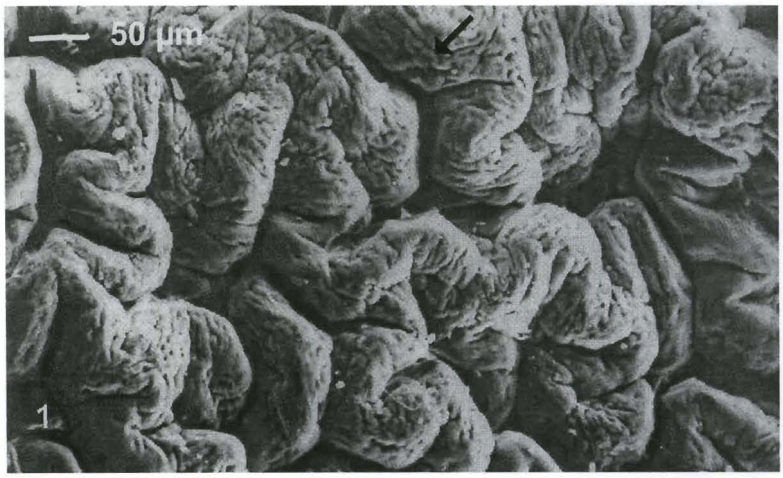

Fig. 1. A SEM picture of the mucosa gastric of *Solea senegalensis* stomach. Primary folds and secondary folds (arrow) are detected.

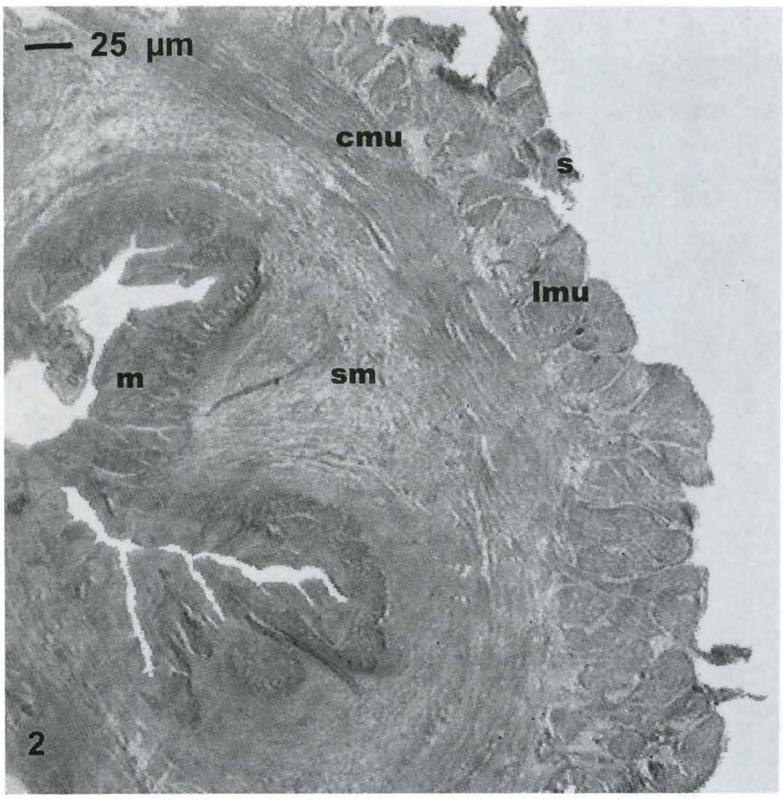

Fig. 2. Fundic stomach showing four distinct layers: mucosa (m), lamina propria-submucosa (sm), circular (cmu) and longitudinal muscular (lmu) and serous (s) layer. Haematoxylin/VOF.

and small blood vessels.

Surface epithelial cells

The columnar epithelium of the stomach contained a basally located nucleus. In the cytoplasm numerous mitochondria (n=40-50 in each observed section) were detected (Fig. 5). A granular endoplasmic reticulum and a Golgi apparatus consisting of several parallel cisternae and numerous vesicles were observed. The lysosomes were small, round and dense. Several dispersed granules and sometimes lipids droplets were observed in the

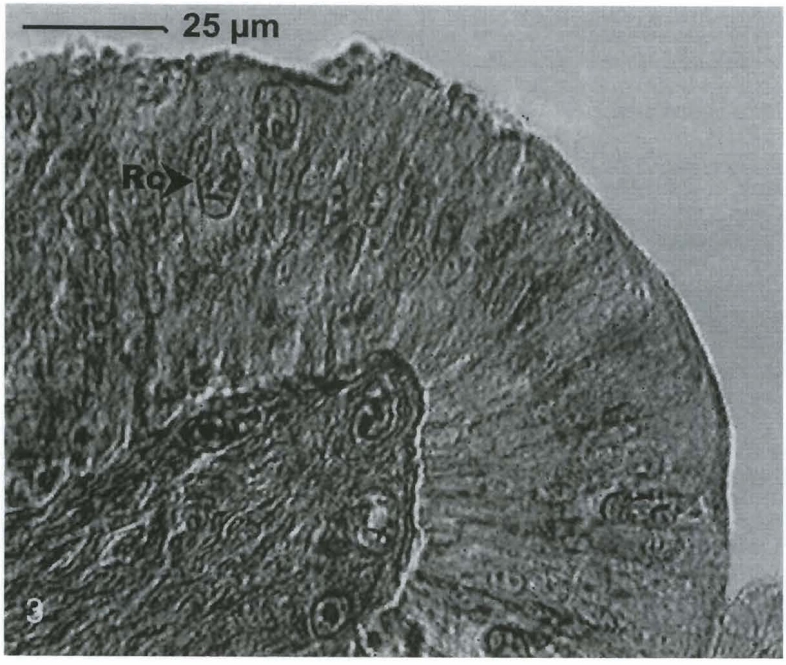

Fig. 3. Rodlet cells (Rc) in gastric mucosa of the anterior/cardiac stomach. Haematoxylin/VOF.

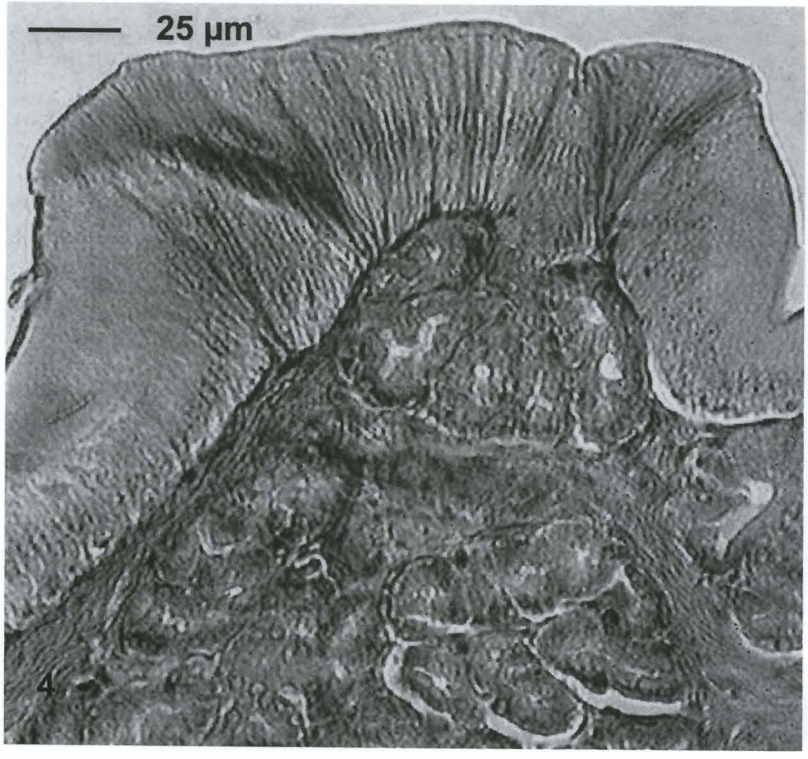

Fig. 4. High magnification of the gastric glands in the fundic stomach. Haematoxylin/VOF.

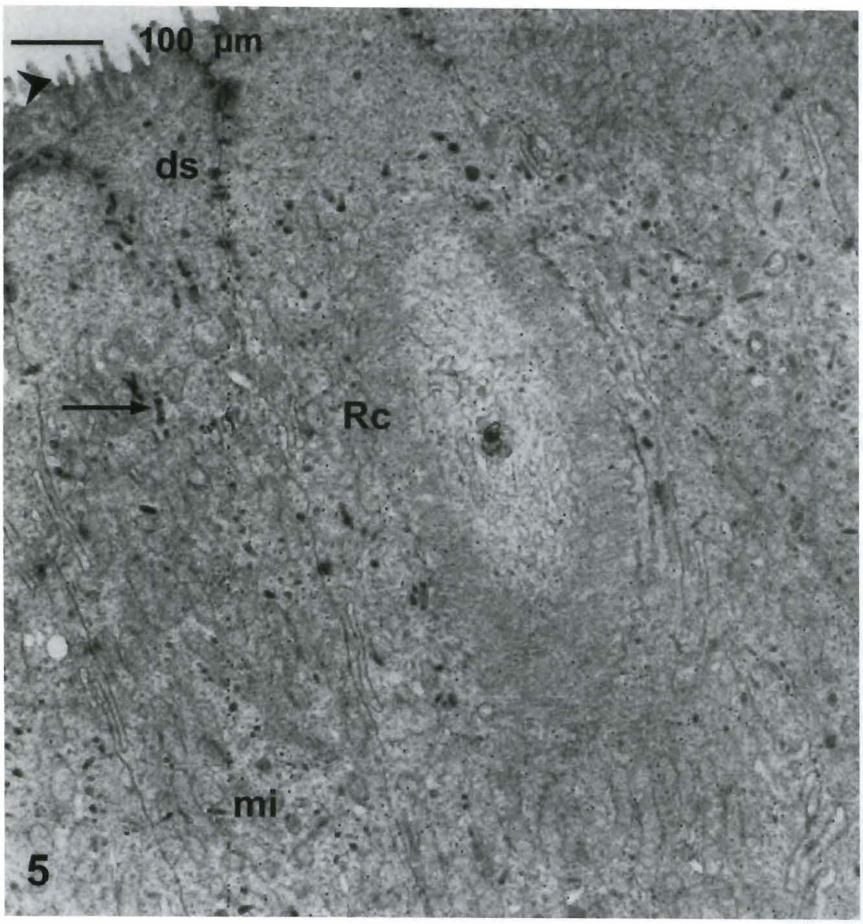

Fig. 5. Columnar cells of the stomach showing apical "microvilli" (arrowhead), desmosomes (ds), lysosomes of different sizes (arrow), numerous mitochondria (mi) and a rodlet cell (Rc).

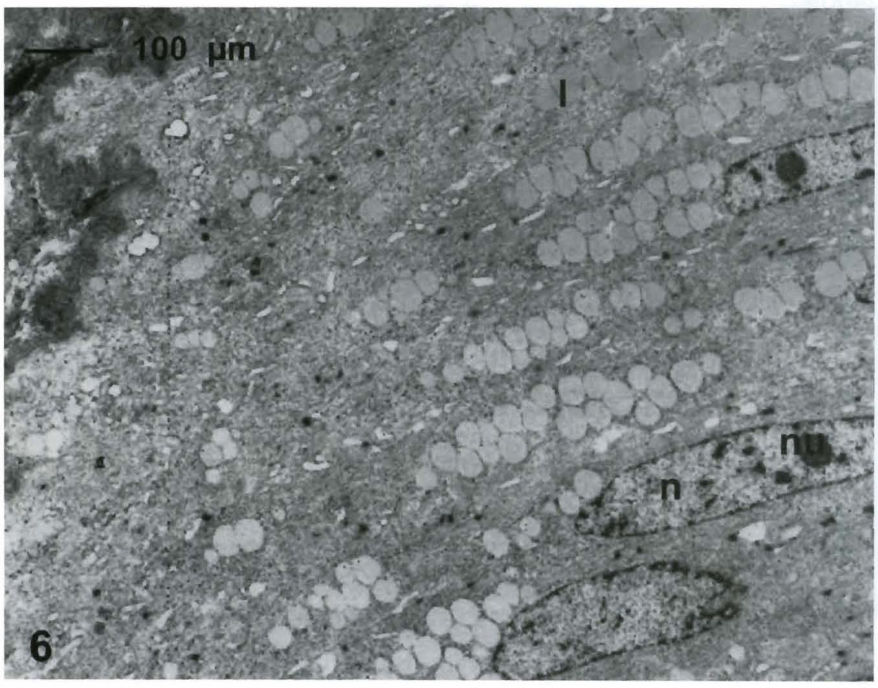

Fig. 6. Nucleus (n) and nucleolus (nu) of epithelial cells. Note the lipid droplets (l) in the cytoplasm of the cells.

Table 3. Histochemical evaluation of distribution of carbohydrates and proteins in stomach of *S. senegalensis*.

	SURFACE EPITHELIUM	GASTRIC GLAND	LAMINA PROPRIA-SUBMUCOSA	MUSCULARIS
Glycogen	0	0	0	0
Neutral glycoproteins	1	1	2	1-2
Carboxylated groups	0-1	0	1	1
Sulphated glycoconjugates (weakly ionised)	0	0	0	0
Sulphated glycoconjugates (strongly ionised)	0	0	0	0
Sialic acid	1	0	1	1
Proteins in general	2	3	2-3	3
Proteins rich in lysine	1	1	1	1
Proteins rich in tyrosine	2	2	2	2
Proteins rich in arginine	0-1	1	1	1-2
Proteins rich in tryptophan	1	1-2	1-2	1
Proteins with cysteine residues	1	1	1	1
Proteins with cystine residues	0	0	0	0

Results are reported considering the intensity of histochemical reactions: 0, negative; 1, weak; 2, moderate; 3, intense.

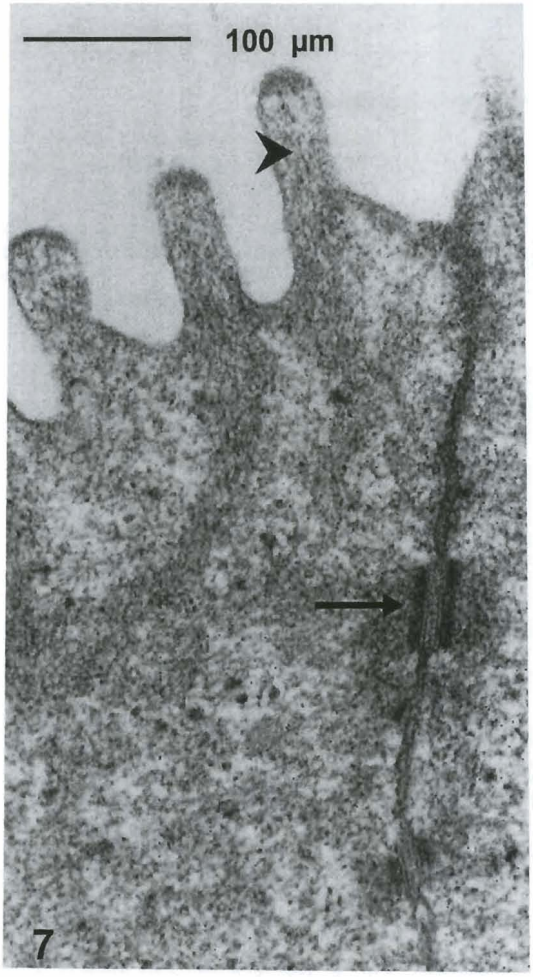

Fig. 7. A detail of the apical part of the columnar cell. Note the microvilli (arrowhead) and desmosomes (arrow).

cytoplasm of the epithelial cells (Fig. 6). Neighbouring cells were connected by desmosomes (Figs. 5, 7). The apical surface of epithelial cells exhibited short microvilli (Figs. 5, 7).

Gastric glands

The gastric glands consisted of pyramidal cells (Fig. 8) which could be classified as light or dark cells according to their electron density. The light cells (Figs. 8, 9) were more numerous, having a large, basal, euchromatin rich nucleus; the apical surface contained small microvilli. In the cytoplasm, the granular endoplasmic reticulum was distributed around the nucleus in several cisternae and in fragments by the cytoplasm; a Golgi apparatus consisting of 4 or 5 cisternae and numerous vesicles in the perinuclear region was observed. The irregular shaped lysosomes were scarce. The mitochondria were numerous (n=50-60 in each observed section) and were located in the basal region; the mitochondrial matrix had moderate density. Spherical secretory granules contained a homogeneous material and various granule-types of different electron-density were distinguished (Fig. 9).

Dark cells (Fig. 10) were inserted among the light ones, and characterised by a supranuclear tubulo-vesicular system and fewer mitochondria (30-45 in each observed section) and more numerous secretory granules than in light cells. Interdigitations or prolongation of the lateral membranes were observed (Fig. 10) between cells.

Rodlet cells

Rodlet cells were characterised by a distinctive cell capsule (Fig. 5) and conspicuous inclusions. The inner part of the plasma membrane was in contact with the cell capsule. The nucleus was round to oval with an irregular outline, and contained euchromatin scattered in its

nucleoplasm and heterochromatin ringing the periphery.

Histochemistry

The surface epithelium and gastric glands of stomach were weakly positive to PAS and Diastase-PAS reactions, while lamina propria-submucosa and muscular layers were moderately positive indicating the presence of neutral mucosubstances and/or glycoconjugates (Table 3).

Lamina propria-submucosa and muscular layers were stained with Alcian blue pH 2.5 indicating the presence of carboxylated mucopolysaccharides, and they were unreactive with Alcian blue pH 1.0 and 0.5 indicating the absence of acidic groups related with sulphated mucopolysaccharides. The gastric glands did

Table 4. Histochemical evaluation of distribution of lipids and enzymatic activities in stomach of *S. senegalensis*.

	SURFACE EPITHELIUM	LAMINA PROPRIA-SUBMUCOSA	MUSCULARIS
Insatured lipids	1	0-1	0
Neutral lipids	0-1	0	0
Acid lipids	0-1	0	0-1
Phospholipids	1	0-1	1
Alkaline phosphatase	0	0	0
Acid phosphatase	2-3	2-1	2
ATP-ase (pH 7.2)	2	1-2	0
ATP-ase (pH 9.4)	1	0-1	1

Results are reported considering the intensity of histochemical reactions: 0, negative; 1, weak; 2, moderate; 3, intense.

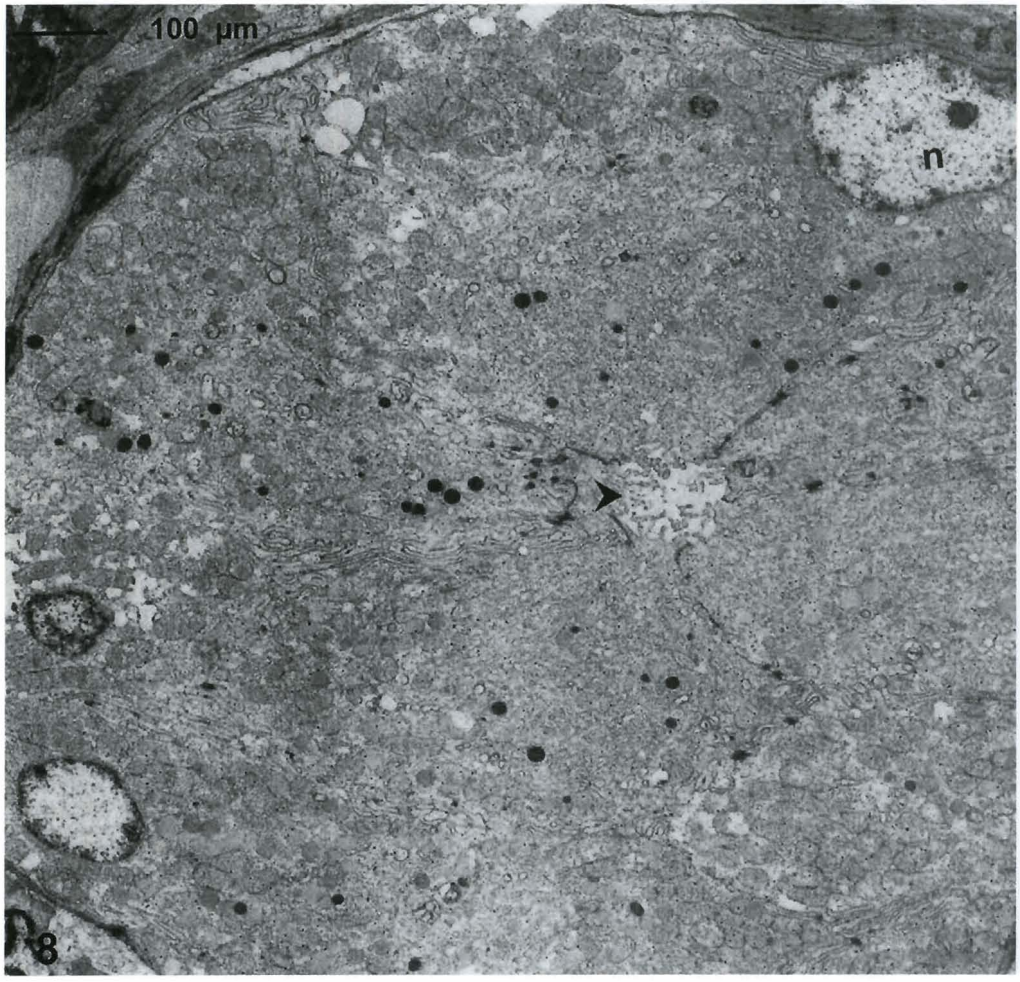

Fig. 8. Gastric glands showing basal nucleus (n) and the secretory granules (arrowhead) in the cytoplasm.

not contain carboxylated and sulphated mucosubstances (Table 3).

The reactions for proteins rich in arginine, lysine and cysteine were weakly positive in the surface epithelium, gastric glands, lamina propria-submucosa and muscular. Proteins rich in tyrosine were abundant in these areas (Table 3).

The histochemical reactions for acid, unsatured lipids and phospholipids were only faintly positive. Likewise, lamina propria-submucosa contained low amounts of unsatured lipids (Table 4).

Enzymatic histochemistry revealed that various enzymatic activities could be detected in the pyloric stomach of Senegal sole. Acid phosphatase and ATP-ase (pH 7.2 and 9.4) activities were detected in the mucosa and lamina propria-submucosa. In contrast, alkaline phosphatase activity was absent (Table 4).

Discussion

The mucosal epithelium of the stomach of *Solea senegalensis* is similar to that of other teleosts (Elbal and Agulleiro, 1986; Grau et al., 1992; Gargiulo et al., 1997) and it is entirely composed of a columnar epithelium. The existence of short microvilli and the presence of a small quantity of neutral mucosubstances in gastric epithelial cells could indicate that these cells play a certain role in the absorptive processes, in common with other teleostean fish species (Ezeasor and Stokoe, 1980; Grau et al., 1992). The granules dispersed in the cytoplasm of the surface epithelium of Senegal sole stomach were probably secretory granules and appeared weakly positive by histochemical techniques (PAS).

In the stomach of Senegal sole there were some cells similar in shape to mucous-secreting cells of the intestinal portion of Senegal sole (Arellano et al., 1999) but containing eosinophilic granules. These granular cells known as rodlet cells are common in oesophageal mucosa of other fish species, such as *Sparus aurata* (Cataldi et al., 1987). Rodlet cells have also been observed in stomach of *S. senegalensis* juveniles (Vieira, 2000).

Rodlet cells have been associated with secretory functions (Leino, 1982). The strong staining of rodlet cells with protein and enzymatic reactions observed in different teleostean fish (Leino, 1982; Iger and Abraham, 1997) suggests that the main component of the rodlet cell may be enzymatic.

The gastric glands of *S. senegalensis* are found throughout the stomach mucosa except in cardiac stomach. However, in *Seriola dumerili* no gastric glands can be seen in the pyloric region or in the oesophagus-stomach transition -oesogaster- (Grau et al., 1992).

Light and dark cells present in gastric glands of *S. senegalensis* stomach could be related to an alternative morphological expression of a single cell type (Elbal and Agulleiro, 1986), producing mainly pepsinogen (light cells) and acid (dark cells) such as was pointed by

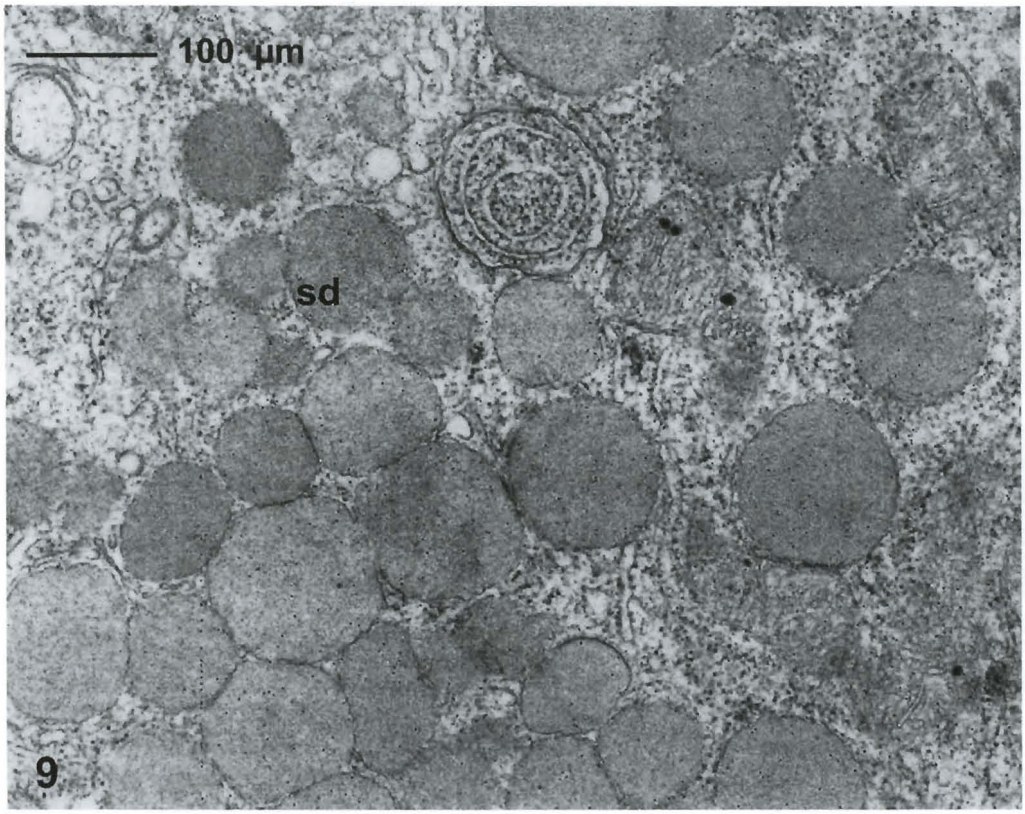

Fig. 9. Dense granules (sd) of light cells of the gastric glands.

Giraud et al. (1979) in a reptilian species, *Tiliqua scincoides*. In this species, the light cells were characterised by a granular reticulum, a well-developed Golgi apparatus and zymogen-like granules, while dark cells were characterised by more numerous mitochondria and by a tubulo-vesicular system. Similar formations in dark cells of *S. senegalensis* stomach have been reported in parietal cells of mammalian stomach and in cells involved in process of ion exchange: e.g. chloride cells in gills of fish (Pisam, 1981). As Western and Jennings (1970) pointed out, the tubulo-vesicular network participates in the production of hydrochloric acid. Thus the well developed mitochondria occurs close to the apical tubular system, indicating the energy demand of the tubules as ion-secreting structures (Mattison and Holstein, 1980).

The muscular layer of the stomach of *S. senegalensis* is similar to other fish species (Morrison, 1987; Grau et al., 1992; Murray et al., 1994). It is composed of two layers of smooth muscle: the inner circular muscle layer is well developed, and the outer layer is formed by longitudinal muscle fibres.

The surface epithelial cells of the stomach of *S. senegalensis* were only weakly positive for neutral and acid mucopolysaccharides/glycoproteins, which were abundant in the lamina propria-submucosa. A similar observation was described in *Solea solea* (Veggetti et al., 1999) and in seabream, *Sparus aurata* (Arellano, 1995). The presence of neutral glycoproteins in the stomach has been observed in the gastric epithelium of *Anguilla anguilla* (Gutiérrez et al., 1986), *Mugil saliens* (Elbal and Agulleiro, 1986), *Seriola dumerili* (Grau et al., 1992), *Sparus aurata* (Arellano, 1995; Domeneghini et al., 1998), *Hippoglossus hippoglossus*, *Pleuronectes americanus* and *Pleuronectes ferruginea* (Murray et al., 1994). Secretion of neutral glycoconjugates containing sugar residues in gastric glands may serve to protect the stomach epithelium from autodigestion caused by HCl and enzymes produced in gastric glands (Ferraris et al., 1987). These authors pointed out that the positive-PAS reaction seen on the surface of gastric epithelial cells resembles that seen in the striated border of intestinal enterocytes. In fact, the presence of neutral mucins in the stomach epithelium is a common characteristic in fish (Reifel and Travill, 1978; Grau et al., 1992; Arellano, 1995; Gutiérrez et al., 1986; Domeneghini et al., 1998; Gisbert et al., 1999; Vieira, 2000). The presence of these mucins has been related to the absorption of easily digestable substances, such as disaccharides and short-chain fatty acids (Grau et al., 1992). Neutral glycoproteins and ATP-ase activity present in the stomach mucosa of *Solea senegalensis* could also be involved in this process. In addition, the presence of neutral glycoconjugates may serve to protect the underlying layers from chemical and physical damage during trituration processes in order to compensate for the lack of a keratinized lining or another analogous structure (Gisbert et al., 1999).

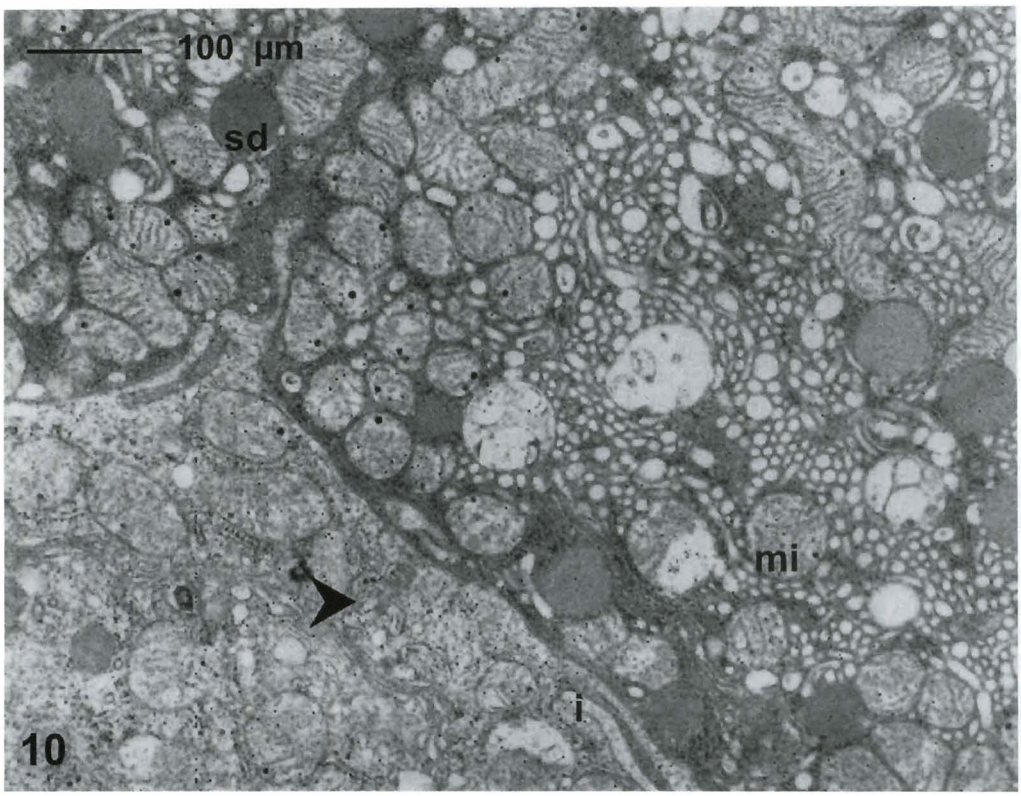

Fig. 10. Dark cell in gastric gland. Note dense secretory granules (sd) and mitochondria (mi), as well as interdigitations of the lateral membranes (i) with the light cells. Label the tubulo-vesicular system in the dark cell.

Sulphated glycoproteins were absent in the gastric glands of Senegal sole, likewise in *S. senegalensis* juveniles (Vieira, 2000) but they were detected in the stomach of different fish species (Reifel and Travill, 1978; Grau et al., 1992). Probably, this characteristic was related with the diet. Spicer and Schulte (1992) speculated that sulphomucins may be able to form a complex with pepsin, stabilising or buffering this enzyme.

Proteins rich in arginine, lysine, cysteine and cystine were scarce in the surface epithelium, gastric gland, lamina propria-submucosa and muscular layers of the Senegal sole stomach. However, proteins rich in tyrosine were abundant in these areas. In *Solea senegalensis* juveniles (Vieira, 2000) when gastric glands are developed, they are strongly positive to general protein reaction (Bromophenol blue) and contain proteins rich in tyrosine, arginine, lysine, tryptophan, cysteine and cystine. This protein content in gastric glands could suggest the presence of enzymatic precursors such as pepsinogen or other digestive enzymes (Medeiros et al., 1970a,b; Gutiérrez et al., 1986; Grau et al., 1992; Gisbert et al., 1999).

Epithelium of the Senegal sole stomach contained a weak presence of acid, and unsaturated lipids and phospholipids; lamina propria-submucosa showed a weak positivity to unsaturated and acid lipids. Transmission electron microscopic results demonstrated a low proportion of lipid droplets coinciding with the histochemical results.

Acid phosphatase and ATP-ase (pH 7.2 and 9.4) activities were detected in the mucosa and lamina propria-submucosa of the Senegal sole stomach, while alkaline phosphatase activity was not observed. However, in stealhead trout, alkaline phosphatase activity was located in the lamina propria and gastric glands, and the epithelium of gastric mucosa was devoid of this enzymatic activity (Prakash, 1960). In four fish species (*Clarias batrachus*, *Ophiocephalus punctatus*, *Ophiocephalus gachua* and *Barbus sophore*), Goel and Sastry (1973) pointed out that alkaline phosphatase activity was present in epithelium of the mucosa and gastric glands, and absent in lamina propria. The alkaline phosphatase activity supports the absorptive role of the epithelial cells, since absorptive processes are usually related to this enzymatic activity (Grau et al., 1992). Because the stomach of the Senegal sole does not present alkaline phosphatase activity, ATP-ase activity and neutral mucins, present in mucosa, could likely be related to this absorptive process.

Acknowledgements. The authors are grateful to Mrs. Mummert, Mrs. Adams, Mrs. Kempendorf and Mrs. Viaña for technical assistance. Special thanks to Dra M. Teresa Dinis. This work was supported by Interreg II Project EC (19/REGII/6/96; Portugal-Spain), as well as by a grant supplied to Dra Juana Arellano (AquaTT & Sata. Leonardo Programme EC).

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Accepted January 22, 2001