Anat Embryol (2006) 211: 507–518
DOI 10.1007/s00429-006-0103-3

ORIGINAL ARTICLE

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Histology of the digestive tract of the freshwater stingray *Himantura signifer* Compagno and Roberts, 1982 (Elasmobranchii, Dasyatidae)

Accepted: 20 April 2006 / Published online: 17 June 2006

Abstract We investigated the histology and histochemistry (of carbohydrates and proteins) of the digestive tract of the freshwater stingray *Himantura signifer*. The alimentary tract consists of a mouth, pharynx, esophagus, stomach (with a descending cardiac and ascending pyloric part), anterior intestine (with an initial portion and a spiral intestine) and posterior intestine, ending in a cloaca. Histologically, three layers—mucosa, *muscularis* and adventitia/serosa—were defined from the mouth to esophagus and in the posterior intestine, whereas in the stomach and anterior intestine four layers were present, including a submucosa. The epithelial lining of mouth, pharynx and cloaca was of the stratified cuboidal type, whereas that of the esophagus and posterior intestine was stratified columnar. The stomach and anterior intestine were lined by a simple columnar epithelium with microvilli. Goblet cells were observed along the alimentary tract, except in the stomach. In the descending cardiac portion of this organ, gastric glands composed of oxyntic, oxyntic–peptic and peptic cells were observed. The anterior intestine presented a spiral valve with 11 folds, formed by mucosa and submucosa. The posterior intestine was particular in displaying a three-layered *muscularis*. Mucosubstances secreted along the alimentary tract contained both neutral and acid mucins, but in the stomach only neutral mucins were detected. The stomach presented intense protein content in the epithelial lining of the gastric pits. Enteroendocrine cells were identified in the stomach and intestine. Overall, our data offer a baseline for comparative purposes and future detailed ultrastructural and immunohistochemical studies.

Keywords Histology · Mucins · Proteins · Elasmobranches · Stingray

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Introduction

The white-edge freshwater whip ray *Himantura signifer* (Compagno and Roberts 1982) is a stingray species, distributed in tropical fresh and brackish water habitats, namely in the sandy bottoms of estuaries and rivers of some Asian countries (Indonesia, Malaysia and Thailand) (Tam et al. 2003). It is a medium-sized benthopelagic whip ray, feeding mainly on small prawn and bottom-dwelling invertebrates (Wongrat 1998). *H. signifer* is considered a rare species, known from only a few specimens (Hilton-Taylor 2000). Despite recent research on the osmoregulation mechanism and spermiogenesis (Tam et al. 2003; Chatchavalvanich et al. 2005a, b), *H. signifer* continues to be a largely unstudied species. The digestive apparatus has never been characterized, at either anatomical or histological levels. Nevertheless, the study of the structure, types of epithelium and mucin secretion would be helpful for understanding the digestive physiology and feeding habits, and even for formulating a diet to support the artificial production of this whip ray. This gains relevancy if we consider that *H. signifer* is on the list of conserved species by IUCN-World Conservation Union. The number of animals has

been greatly reduced in recent years, due to their capture for human consumption and for commercial ornamental fish business (Compagno 1995), and so the degree of threat to this species is now considered to be substantial (Hilton-Taylor 2000).

In addition to histophysiology interests, the analysis of the digestive apparatus has a zoological and phylogenetic importance. It has been reported that chondrichthyes, and the elasmobranch subclass in particular, always present a small intestine with a spiral valve to increase the area for enzymatic treatment (Holmgren and Nilsson 1999). Nevertheless, the study of the digestive apparatus morphology and histochemistry has been neglected in this class, in clear opposition to teleosts, for which the digestive system, including its development, has been extensively characterized (Kapoor et al. 1975; Reifel and Travill 1979; Sarasquete et al. 1993).

The aims of the present study were to establish the normal histological structure of the digestive tract in the freshwater stingray, *H. signifier*, to identify the general types of mucins and proteins present in the mucosa epithelial cells and to investigate the presence of enteroendocrine cells. The information gathered could offer baseline knowledge for future studies on the digestive tract whereas being relevant for understanding the nutritional physiology of this threatened whip ray.

Materials and methods

Animals

Ten mature white-edge freshwater whip rays, weighing an average of 750 g (SD = 144 g), were collected from the Chao Phraya River, Nakhon Sawan province, in the central part of Thailand. Animals were anaesthetized under deep cold and transcardiacally perfused (via conus arteriosus) with Bouin's fluid for 15 min. It is opportune to mention that several studies were and are still being conducted on these animals (Chatchavalvanich et al. 2005a, b), aiming to characterize different morphological, physiological and ecological aspects of this species.

Morphology

Different parts of the digestive tract (mouth to cloaca) were collected, sliced into small pieces and further immersed in Bouin for 18–24 h, at room temperature. All fragments were routinely processed for paraffin embedding (Merck, Histosech). Sections of 5–6 μm in thickness were prepared and stained with hematoxylin–eosin and Masson's trichrome, the latter for better differentiating collagen from muscle fibers. In addition, small pieces of the stomach and anterior intestine (about 1 mm^3) were fixed for 2 h at 4°C in 2.5% glutaraldehyde, diluted in 0.4 M cacodylate buffer (pH 7.4) with 5 mM $CaCl_2$. After washing in buffer, the fragments were postfixed with 2% OsO_4 buffered with cacodylate, also for 2 h at 4°C and then dehydrated in ethanol and embedded in Epon. Semithin sections (1 μm in thickness) were prepared and stained with methylene blue and azure II for light microscopy observation.

Histochemistry

Histochemical studies for neutral mucins identification were made using periodic acid Schiff (PAS)–hematoxylin and also diastase-PAS (D-PAS). For the identification of acid mucins, the alcian blue (AB) staining was used at pH 2.5 and 1.0, for preferential staining of mucins with carboxylated and sulfated groups, respectively (Cook 1990). The combined AB–PAS stain was also performed, to check the coexistence of the acid and neutral mucins (Mowry 1958). All these reactions were applied in paraffin and semithin sections. For PAS in paraffin, and after dewaxing, sections were treated with 1% periodic acid for 5 min, washed and stained with Schiff's reagent for 15 min. Nuclei were counterstained with hematoxylin. For semithin sections, the procedure was similar, but immersion times in reagents were doubled. In control sections, the oxidation with periodic acid was omitted. For AB, dewaxed sections were stained with 0.5% AB for 5 min in 3% acetic acid (pH 2.5), for primarily carboxylated mucins and in an HCl solution (pH 1.0), to demonstrate sulfated mucins. For semithin studies, sections were first treated with a saturated solution of NaOH in ethanol, to remove the embedding medium, and then stained with the above solutions for 30 min (Lobo-da-Cunha and Batista-Pinto 2003).

For protein histochemistry, the tetrazonium and the dimethylaminobenzaldehyde (DMAB) reactions were performed. The former identifies proteins rich in tyrosine, histidine, trytophan, arginine and cysteine, whilst the latter marks proteins rich only in trytophan. For the tetrazonium reaction, sections were treated for 10 min with a freshly prepared 0.2% solution of fast blue salt B in veronal acetate buffer (pH 9.2), washed and then treated for 15 min with a saturated solution of β-naphthol in veronal acetate buffer (pH 9.2). For the DMAB reaction, sections were dipped in 5% DMAB in an HCl solution for 1 min and then in 1% $NaNO_3$ in an HCl solution, washed and counterstained in 0.7% safranin in 50% ethanol for 1 min; after washing in water and ethanol, the slides were dipped in acetone, cleared and mounted in DPX.

For the histochemical detection of enteroendocrine cells (EC) a silver Grimelius staining was performed. This broad spectrum exploratory method detects a part of the whole population of gastroenteropancreatic endocrine cells, namely those rich in serotonin (Hould 1994).

Results

Gross morphology

The digestive tract of *H. signifer* measured an average of 22 cm, being divided into the orobranchial cavity (6 cm), esophagus (1 cm), stomach (7.5 cm), anterior intestine (6 cm) and posterior intestine (1.5 cm), this ending in the cloaca (Fig. 1). The orobranchial zone was formed by a small oral cavity and a large pharynx (branchial cavity). The upper and lower jaws presented molariform teeth, forming 19–20 successive tooth bands. A short and straight tubular esophagus followed, ending with five transverse folds at the junction with the stomach. This organ was large and U-shaped, being anatomically divided into two regions, a descending "cardiac" part and an ascending "pyloric" part. These corresponded to approximately 2/3 and 1/3 of the stomach, respectively. The intestine was a short and straight tube, divided into anterior and posterior parts. The anterior intestine was originated just posterior to the pyloric sphincter, being subdivided into two portions: a short initial portion, which presented the openings of the bile and pancreatic duct, and a long spiral intestine; in related species, these regions have been called *duodenum* and *ileum*, respectively. The spiral intestine was large, occupying around 5/6 of the anterior intestine, and was characterized by having the spiral valve, composed of 11 folds arranged as funnels pointed forward (Fig. 2). In opposition, the posterior intestine—termed the large intestine or *rectum*, in related species—was narrow and presented no circular folds; it ended in a large cloaca. No rectal gland was observed.

Histological observations

Orobranchial cavity

This cavity was lined by stratified cuboidal epithelium, without cilia or microvilli (Fig. 3). Abundant mucous cells were observed, especially in the branchial region (Fig. 4). These were markedly globular, presenting abundant cytoplasm positive to PAS and AB (Table 1). Occasionally, small taste buds were found, interspersed in the epithelium of the oral and branchial cavities. Beneath the epithelium there was a *lamina propria* of loose connective tissue, presenting elements of a subepithelial nerve plexus. The branchial cavity presented a circumscribing band of hyaline cartilage with calcifying areas. Beneath the mucosa was the *muscularis*, composed of bundles of striated muscles organized in a thinner, nearly circular layer and a thick longitudinal outer layer (Fig. 3), which was continuous with the pharyngeal musculature. An abundant loose connective tissue area, with scattered ganglia and nerves of the myenteric plexus, was found amongst the muscular bundles.

Esophagus

The esophageal wall was composed of three layers: mucosa, *muscularis* and serosa (Figs. 5, 6). The mucosa formed longitudinal folds. It presented a stratified columnar epithelium without superficial specializations; occasionally taste buds were present (Fig. 7). Mucous cells were particularly abundant in this segment; these were large and columnar shaped or markedly globular, being positive for PAS and AB stains (Fig. 8; Table 1). The *lamina propria* was composed of loose connective tissue containing collagen fibers with a transverse and longitudinal orientation, numerous blood vessels and, occasionally, lymphatic tissue aggregates and elements of the nervous submucosal plexus. A *muscularis mucosae* was absent throughout the esophagus. The organ of Leydig was present in the deep part of the *lamina propria* (Fig. 6). It was formed by two strands (ventral and dorsal) of lymphomyeloid tissue, connected by numerous sinusoid-like capillaries. The lymphomyeloid tissue was composed of blast cells, granulocytes and lymphocytes, which can be difficult to distinguish with certainty in light microscopy, without the use of special stains. The *muscularis* consisted of thick circular striated muscles running along most of the organ, but, at the distal end, it became progressively arranged into a thin inner layer of circular smooth muscle and a thick outer layer of circular striated muscle. Externally there was a serosa, with a well-developed subserosal nerve plexus.

Stomach

The wall of the stomach (Figs. 5, 9) consisted of the four classical layers: mucosa, submucosa, *muscularis* and serosa. The mucosa was different in the descending "cardiac" versus ascending "pyloric" portions. In the descending part, the mucosa was indented by shallow gastric pits in which two or more gastric glands opened. A simple columnar epithelium with microvilli lined the surface and gastric pits. The cells presented oval nuclei located in the middle or upper half. In their apical portion cells were positive for PAS, tetrazonium and DMAB (Table 1). The gastric glands were long and tortuous non-ramifying tubules, occupying all the *lamina propria* extent in the descending region. The gland *isthmus* was clearly visible (next to the pit narrowing), but a differentiated neck versus base was difficult to recognize. In the glands, the epithelium varied from tall cubic cells, with oval nuclei and pale cytoplasm with long tubulovesicules (as confirmed at electron microscopy), close to the isthmus (Fig. 10), to large pyramidal cells, with round nuclei and dark cytoplasmic granules of variable sizes, close to the base (Fig. 11). These corresponded to oxyntic and peptic cells, respectively. Intermediate cells (oxyntic–peptic) were also observed in-between. All these cell types were negative for PAS and AB, but presented moderate staining with both tetrazonium and DMAB. In the epithelium of the sur-

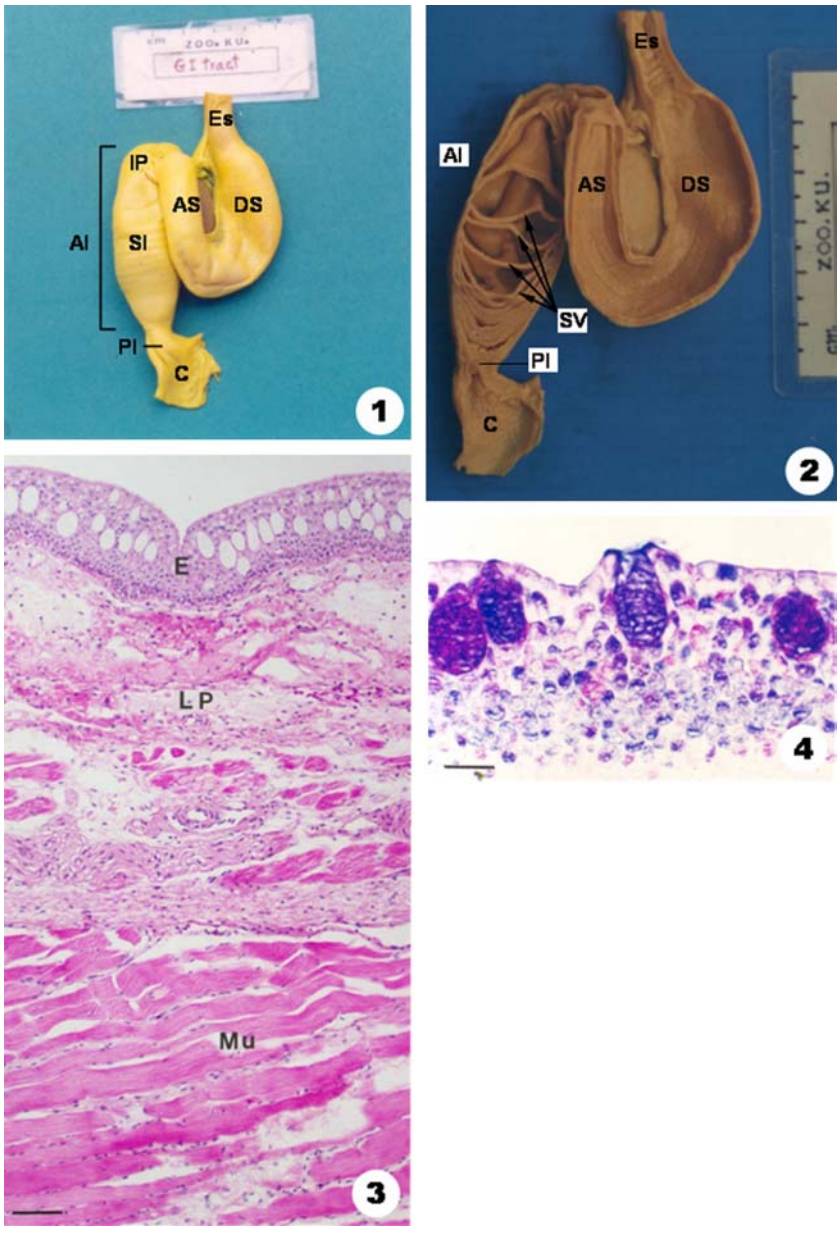

Fig. 1-4 **1** Gross structure of the digestive tract of *Himantura signifier*. *Es* esophagus, *DS* descending "cardiac" stomach, *AS* ascending "pyloric" stomach, *AI* anterior intestine (*IP* initial portion, *SI* spiral intestine), *PI* posterior intestine, *C* cloaca. **2** Longitudinal section of the digestive tract, revealing the internal morphology of the intestine. *Es* esophagus, *DS* descending "cardiac" stomach, *AS* ascending "pyloric" stomach, *AI* anterior intestine (*SV* spiral valve), *PI* posterior intestine, *C* cloaca. **3** Longitudinal section of the oral cavity, revealing the epithelium (*E*), *lamina propria* (*LP*) and the skeletal muscle of the *muscularis* (*Mu*). Hematoxylin and eosin. *Bar* = 100 μm. **4** Mucous cells in the oral cavity secreted both acid and neutral mucins. Alcian blue–PAS. *Bar* = 25 μm

Table 1 Results from the histochemical techniques used to identify the mucosubstances and proteins secreted in the digestive tract epithelium of *Himantura signifer*

Organ	Cell	Methods						
		PAS	D-PAS	AB (pH 2.5)	AB (pH 1.0)	AB–PAS	Tetrazonium	DMAB
Mouth	Mucous cell	4	4	3	2	4	1	2
Pharynx	Mucous cell	4	4	3	2	4	1	2
Esophagus	Mucous cell	4	4	3	2	4	1	2
Stomach	Surface cell	4	4	0	0	0	3	2
Anterior intestine	Goblet cell	4	3	2	1	3	2	1
Posterior intestine	Goblet cell	4	3	3	2	3	1	2
Cloaca	Goblet cell	4	3	3	2	3	1	2

The intensity of the reaction is expressed in grades: 0, negative; 1, weak; 2, moderate; 3, intense; 4, very intense

face and crypts, numerous Grimelius-positive EC were observed; these cells were spindle or pear shaped, in close proximity to each other, and in contact with the lumen (Fig. 12). In the gland tubules, no positive cells for Grimelius were observed; nevertheless, rare presumptive EC were observed in semithin sections. These

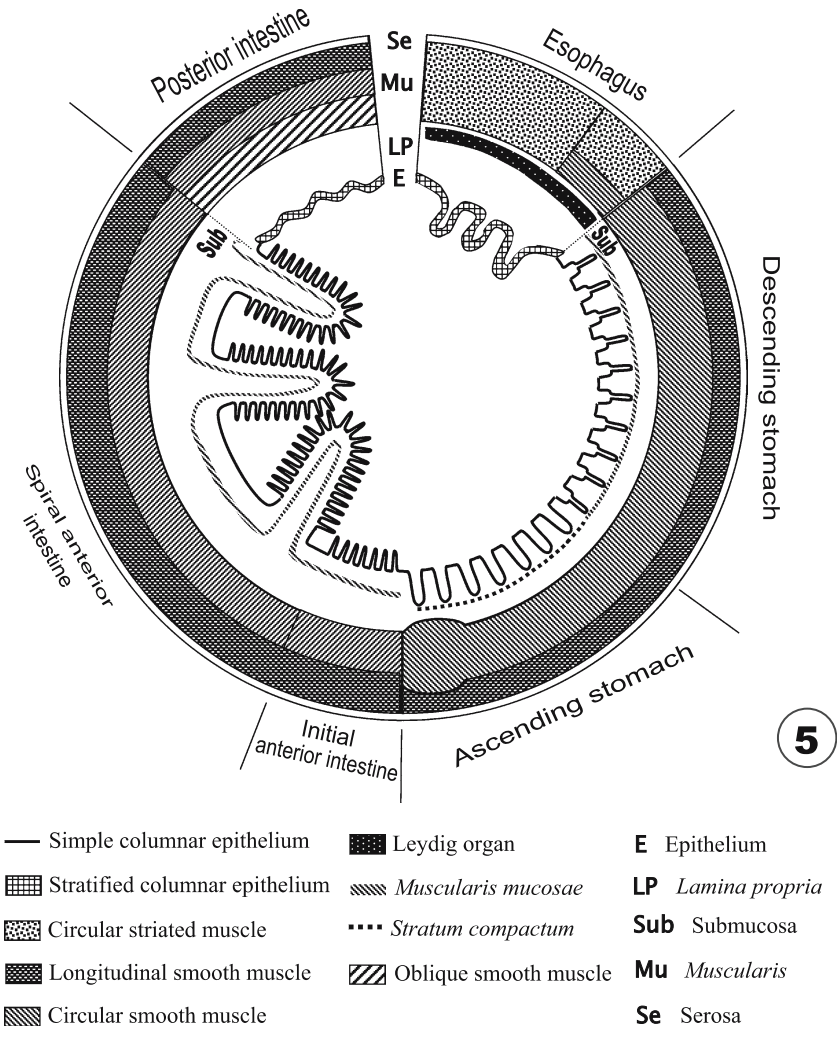

Fig. 5 Schematic organization of tubular digestive tract of *Himantura signifer*

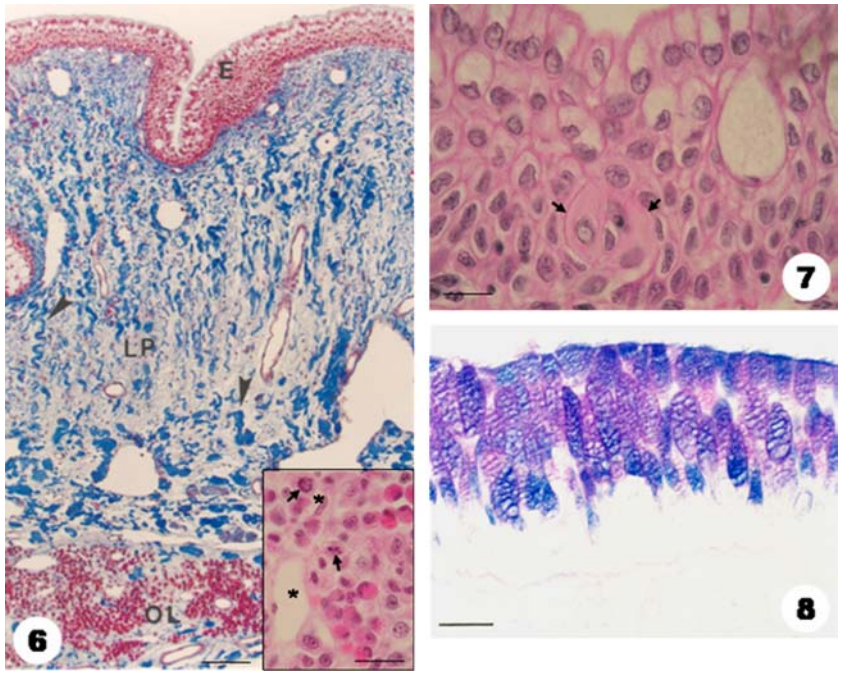

Fig. 6-8 **6** Cross-section of the esophagus. The epithelium (*E*) was stratified columnar. The organ of Leydig (*OL*) was present in the deepest part of the *lamina propria* (*LP*); it presented lymphomyeloid tissue (*inset*) composed of blast cells with mitotic activity (*arrows*) and leucocytes intermingled with sinusoid-like capillaries (*asterisk*). In the *LP*, abundant thick collagen fibers can also be observed (*arrowhead*). Masson trichrome. *Bar* = 100 μm. *Inset*: Hematoxylin and eosin. *Bar* = 25 μm. **7** Taste bud within *arrows* in the epithelium of the esophagus. Hematoxylin and eosin. *Bar* = 18 μm. **8** Mucous cells were particularly abundant in the esophagus, secreting both acid and neutral mucins. Alcian blue–PAS. *Bar* = 25 μm

were triangular in shape and opened into the lumen, despite in a few occasions where they really appeared not to reach it (Fig. 13).

The stomach mucosa was separated from the submucosa by a thin *muscularis mucosae*, formed of circular strands of smooth muscle (Fig. 9). The submucosa was thick, composed of loose connective tissue, with abundant nerves (submucosal plexus) and blood and lymph-like vessels. The *muscularis* consisted of smooth muscle, arranged in a thick circular internal layer and a thinner longitudinal external layer, with loose connective tissue and the myenteric nerve plexus in-between. The serosa was similar to that described in the esophagus.

The mucosal transition from the descending to the ascending portion was marked by a sudden disappearance of gastric glands. Instead, the gastric pits were narrow and deep, extending throughout the mucosal thickness (Fig. 14). The surface epithelium was similar to that of the cardiac region. As in the descending portion, abundant EC were present. The *lamina propria* was more prominent, filling the space amongst the pits; although the *muscularis mucosae* disappeared in this region, a thin *stratum compactum* was observed and a marked structural difference between the *lamina propria* and the submucosa (with a looser connective tissue) was seen. Indeed, the submucosa was particularly extended in this portion, with many adipocytes and blood and lymph-like vessels. The *muscularis* was similar to the descending portion, except for the terminal region, in which there was a thickening of the internal circular layer that formed the pyloric sphincter.

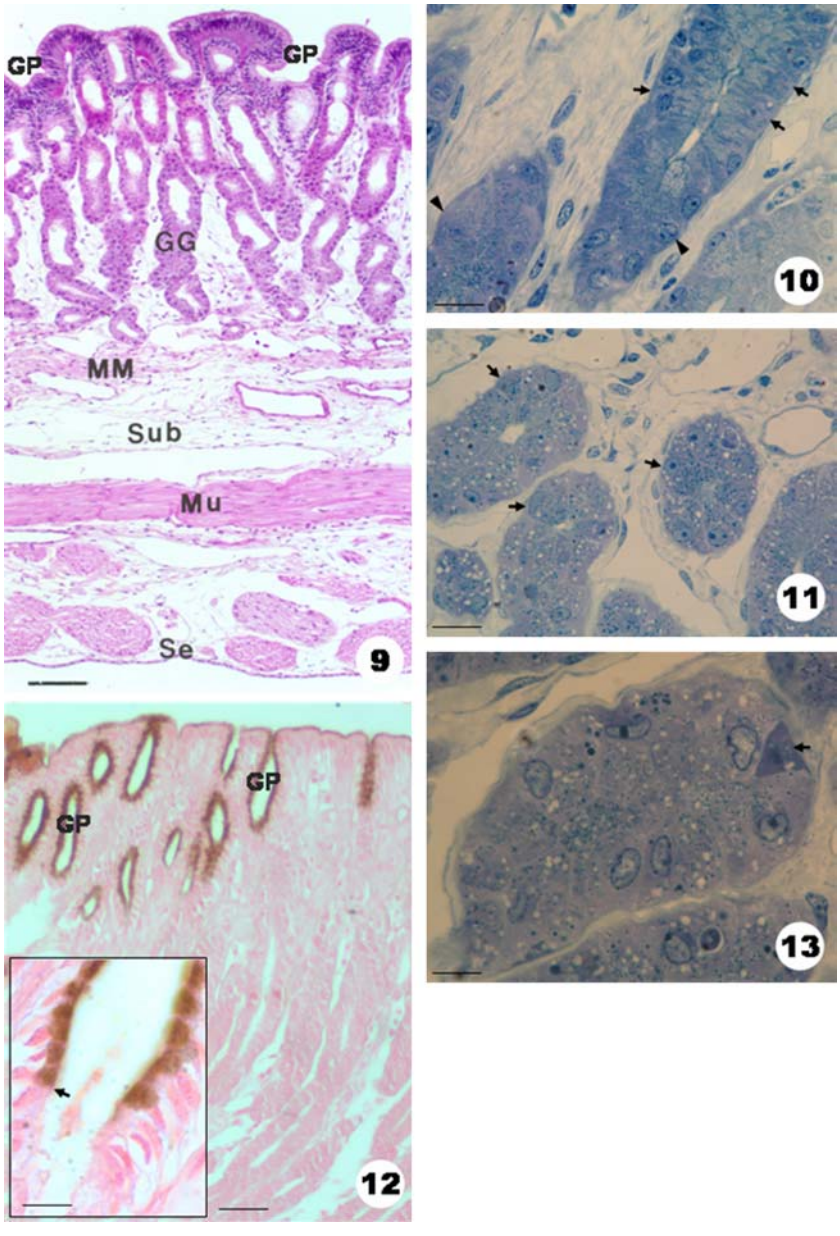

Fig. 9-13 **9** Cross-section of the descending cardiac stomach, revealing gastric pits (*GP*) and gastric glands (*GG*) in the mucosa. *MM muscularis mucosae*, *Sub* submucosa, *Mu muscularis*, *Se* serosa. Hematoxylin and eosin. *Bar* = 100 μm. **10** Semithin section of the descending "cardiac" stomach. In the gastric glands, oxyntic cells, with cytoplasmic tubulovesicules, were present close to the isthmus (*arrows*), whilst intermediate (oxyntic–peptic) cells were observed in the middle part of the glands (*arrowheads*). Methylene blue-Azur II. *Bar* = 15 μm. **11** Semithin section of the descending "cardiac" stomach. In the deep part of the gastric glands only peptic cells were observed (*arrows*), characterized by numerous dark cytoplasmic granules. Methylene blue-Azur II. *Bar* = 15 μm. **12** Enteroendocrine cells (Grimelius positive) were observed amongst surface epithelial cells and in the gastric pits (*GP*). In the *inset* (*lower left*) it is clear that no marking was visible after the transition point from the gastric pits to the glands (*arrow*). Grimelius silver. *Bar* = 50 μm. *Inset*: *bar* = 35 μm. **13** Semithin section of the descending "cardiac" stomach. A presumably enteroendocrine cell of the closed type (*arrow*) was observed amongst peptic cells of the deep part of gastric gland tubules. Methylene blue-Azur II. *Bar* = 15 μm

Anterior intestine

The wall of the anterior intestine also presented four layers (Figs. 5, 15). In the initial portion (often named as *duodenum*, in related species), the surface was studded with numerous mucosal folds (Fig. 15). Their epithelium was simple columnar, composed of tall absorptive cells, with a well-developed striated border in the apex, basal cells (often difficult to differentiate from occasional migrating lymphocytes) and numerous goblet cells (Figs. 16, 17). The striated border and the tall goblet cells presented a positive reaction for PAS and AB staining (Fig. 17; Table 1). Throughout the intestine, diastase-PAS staining was slightly less intense than the PAS, revealing that cells contained glycogen. There were no intestinal (Lieberkühn) glands. The *muscularis mucosae* reappeared in this intestinal portion, being particularly thick and arranged in longitudinal strands of smooth muscle. The submucosa consisted of loose connective tissue, with prominent vascularity and elements of the nervous submucosal plexus; no submucosal (Brunner) glands were observed. The *muscularis* and serosa were similar to those described for the stomach.

In the second (spiral) part of the anterior intestine (often called *ileum*, in related species) a spiral valve was present, formed by funnel-shaped folds, composed of mucosa and submucosa (Figs. 5, 18, 19). The mucosa was studded by regularly spaced mucosal folds (i.e., digit-form processes), covered by an epithelium similar to that described in the anterior intestine (Figs. 16, 17). Like in the initial portion, EC were observed in the spiral intestine: they were rare, appearing as isolated cells and

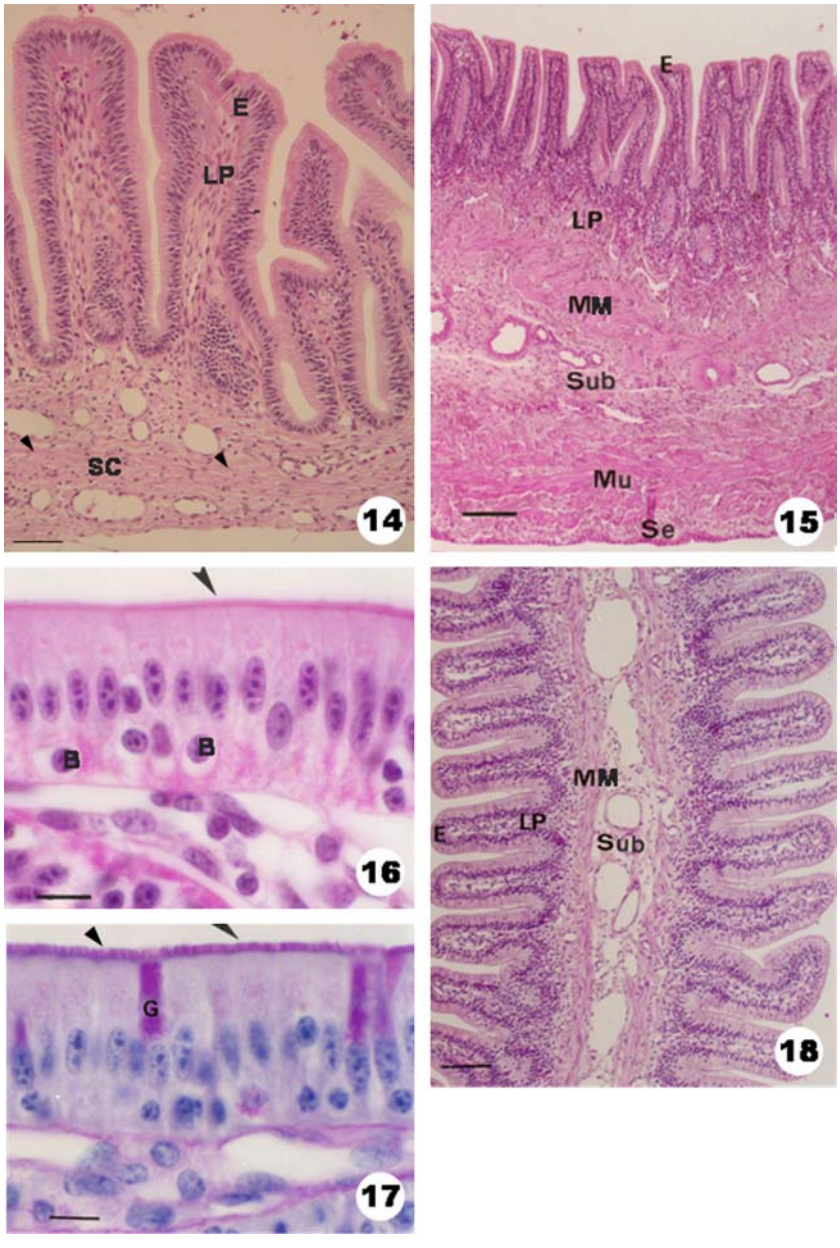

Fig. 14-18 14 Cross-section of the ascending "pyloric" stomach, revealing the absence of gastric glands, the highly cellular *lamina propria* (*LP*) and the thin *stratum compactum* (*SC*) (the upper limit of the SC is marked by *arrowheads*). *E* epithelium. Hematoxylin and eosin. *Bar* = 40 μm. **15** Cross-section of the initial anterior intestine. Numerous mucosal folds were visible. *E* epithelium, *LP lamina propria*, *MM muscularis mucosae*, *Sub* submucosa, *Mu muscularis*, *Se* serosa. Hematoxylin and eosin. *Bar* = 100 μm. **16** Detail of the simple columnar epithelium of the anterior intestine, revealing the well-developed striated border (*arrowhead*). *B* basal cells. Hematoxylin and eosin. *Bar* = 25 μm. **17** In the anterior intestine the striated border (*arrowhead*) and goblet cells (*G*) were PAS positive. PAS and hematoxylin. *Bar* = 25 μm. **18** Cross-section of the spiral intestine, showing the spiral valve in detail—this was formed by mucosa and submucosa (*Sub*). *E* epithelium, *LP lamina propria*, *MM muscularis mucosae*. Hematoxylin and eosin. *Bar* = 50 μm

mainly located in the deepest parts of the mucosal folds. These cells were cone-shaped, with long cytoplasmic processes that reached the lumen (Figs. 19, 20). The submucosa axis of the spiral folds was rich in blood and lymph-like vessels, with many scattered lymphocytes. No submucosal (Brunner) glands were observed in this segment. The other histological features were similar to those described above. The spiral valve ended at the place where the simple surface epithelium abruptly changed to stratified columnar; this marked the transition to the posterior intestine (Fig. 21).

Posterior intestine

The wall of the posterior intestine consisted of three layers: mucosa, *muscularis* and serosa (Figs. 5, 21). The epithelial lining was stratified columnar (Fig. 22). Whereas EC were extremely rare, goblet cells were more numerous than in the anterior intestine, being tall columnar or globular shaped and positive for PAS and AB (Fig. 23; Table 1). No *muscularis mucosae* were observed. The *muscularis* was thicker in this intestinal segment, being composed of three distinct layers of smooth muscle: an inner oblique, a middle circular and an outer longitudinal. The posterior intestine ended in a cloaca, which presented similar histological and histochemical features, except for the epithelial lining (stratified cuboidal) and the somewhat irregular arrangement of the *muscularis* (Fig. 24).

Discussion

The digestive tract of fishes has been extensively studied, but most of the gathered information refers to teleosts—a much scarce number of studies focused their attention on cartilaginous fishes (Holmgren and Nilsson 1999). These basic studies are important for comparative anatomy and physiology analyses and also for species preservation and/or production purposes. Here we studied the histology of the digestive tract of *H. signifier*.

In Icthyology, the comparative perspective of morphological studies has been often hampered by a less precise nomenclature, when compared with higher vertebrates, both at the anatomical and histological levels. Indeed, indiscriminate direct use of human medical nomenclature is often not advisable, because it can be misleading. For instance, in the stomach the most used terms for the two main regions, cardiac and pyloric (Holmgren and Nilsson 1999) or corpus and pyloric (Tagliafierro et al. 1985), can be misleading by implying that both these regions are glandular or somehow similar to a mammalian model; for these regions, the terms we used (descending and ascending) seem preferable. The same is true for the intestine, in which the anterior and posterior intestines have been called small intestine (or *duodenum* or *ileum*) and large intestine (or *rectum*), respectively (Yokote 1982). Again, these terms can be misleading when used in elasmobranches, as the anterior portion is actually larger (in diameter) than the posterior part (often called "large intestine") (Reifel and Travill 1979; Holmgren and Nilsson 1999); the term *duodenum* also seems inappropriate to designate a portion which generally does not have either true villi (true in a sense that the mucosal projections do not have central lacteals) (Reifel and Travill 1979) or true Lieberkühn crypts or submucosal (Brunner) glands (Andrew and Hickman 1974; Holmgren and Nilsson 1999).

Regarding the mouth and pharynx in *H. signifer*, the small oral opening correlates with the reported diet for this species (Wongrat 1998) and the flat molariform teeth are typical of the Dasyatidae and Rajidae families (Moss 1972). The epithelial lining of the pharynx (stratified cuboidal with numerous mucous cells) was similar to that of dogfish (Andrew and Hickman 1974), but different from most teleosts which bear a stratified squamous type (Yokote 1982; Albrecht et al. 2001). The mucous cells are common in the epithelium of this region in fishes, serving to lubricate the wall surface. Regarding the muscular, it presented an arrangement similar to that of most teleosts (Yokote 1982).

The epithelial lining of the esophagus (stratified columnar epithelium without cilia) is similar to that of the thornback ray, *Raja clavata* (Holmgren and Nilsson 1999), but different from that of the dogfish *Squalus acanthias*, which has cilia (Leake 1975), and that of most teleosts, which have a multi-layered squamous epithe-

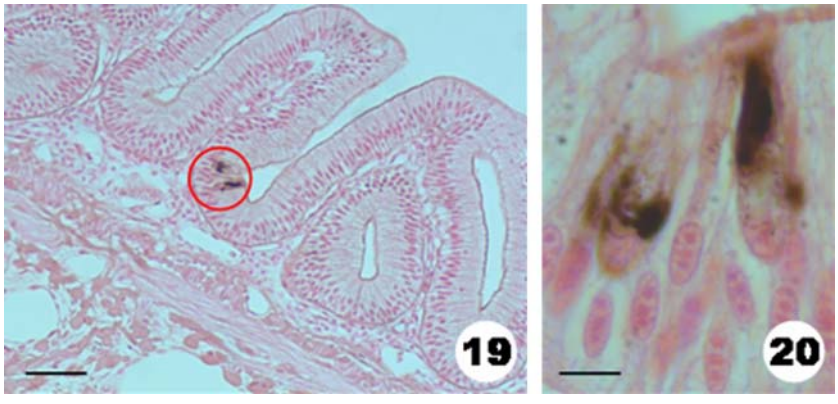

Fig. 19-20 In the anterior intestine enteroendocrine cells (Grimelius positive) were rare (*circle*). Grimelius silver. *Bar* = 50 μm. **20** Detail of two enteroendocrine cells (Grimelius positive) of the open type. *Bar* = 35 μm

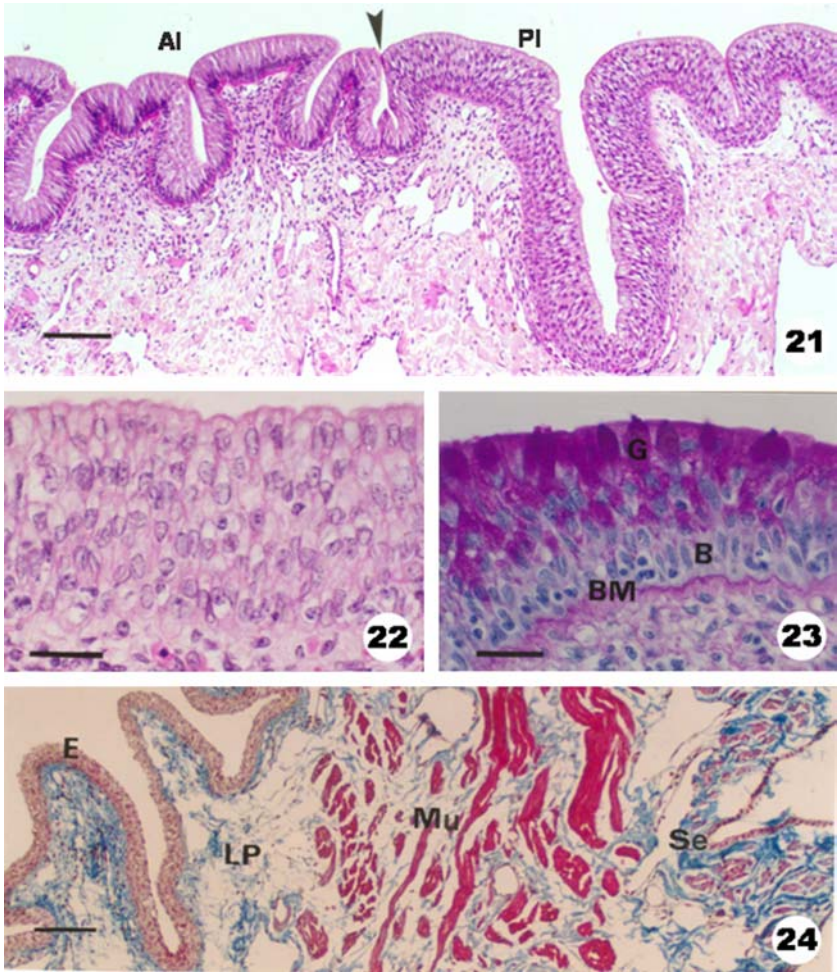

Fig. 21-24 **21** Longitudinal section of the anterior–posterior intestine junction (*AI* versus *PI*), revealing the abrupt change of epithelium (*arrowhead*), from simple columnar (at *left*) to stratified columnar (at *right*). Hematoxylin and eosin. *Bar* = 100 μm. **22** Detail of the stratified columnar epithelium of the posterior intestine. Hematoxylin and eosin. *Bar* = 25 μm. **23** Detail of the stratified columnar epithelium of the posterior intestine, strongly positive to PAS. *G* goblet cells, *B* basal cells, *BM* basal membrane. PAS and hematoxylin. *Bar* = 25 μm. **24** Cross-section of the cloaca, presenting three distinct layers. *E* epithelium, *LP lamina propria*, *Mu muscularis*, *Se* serosa. Masson trichrome. *Bar* = 100 μm

lium (Harder 1975). In fishes, the particularly abundant mucous cells in the esophagus make a slippery wall, helping the food transit to the stomach (Harder 1975; Cataldi et al. 1987; Grau et al. 1992); those cells are equivalent to the esophageal glands found in the *lamina propria* or in the submucosa of higher vertebrates (Andrew and Hickman 1974). Like in other elasmobranches, the mucosal layer of *H. signifier* forms folds, which allow distention during swallowing (Holmgren and Nilsson 1999). The existence of taste buds in the esophagus has been reported in several fishes (Grau et al. 1992), indicating a gustatory function for this segment. Hirji (1976) suggested that food selection takes place in this region; this is also probably the case in *H. signifer*, because in this species, besides the taste buds located rostrally, the esophagic striated circular musculature is well developed, making food rejection still possible in case of need. As expected, *H. signifer* had an organ of Leydig, typically seen in the deep *lamina propria* (submucosa for some authors) of the esophagus in many cartilaginous fishes (Fange and Grove 1979; Estecondo et al. 1988).

Despite the basic structure of the gastric wall of *H. signifer* being globally similar to that of other fishes, some details deserve attention. The glands in the anterior portion presented three types of cells: oxyntic, peptic and intermediate cells (these corresponding to oxyntico-peptic cells). Although it has been referred that, like teleosts, elasmobranches have only one gland cell type in gastric glands (Holmgren and Nilsson 1999), three cell types have also been described in *D. sabina* (Smolka et al. 1994) and in *Raja asterias* (Faraldi et al. 1984). The absence of glands in the ascending stomach also happens in other elasmobranches and most teleosts (Yokote 1982; Holmgren and Nilsson 1999). In *H. signifer*, that ascending region was particular for displaying a thin *stratum compactum*, instead of a *muscularis mucosae*, and also for having an extensive submucosa. This resembles the "*pars pylorica*" of the stripped bass (Groman 1982). As elasmobranches have long gastric emptying times (Holmgren and Nilsson 1999), it is possible that this region stores food while it is being chemically digested. This is supported by the higher mucous content of the epithelium and by the presence of a long, probably able to distend, submucosa (Albrecht et al. 2001), as we found in *H. signifer*. The *stratum compactum* can be observed in the stomach and intestine of teleosts (Yokote 1982), but in *H. signifer* it was only observed in the ascending stomach. As this region probably stores food, it is possible that the *stratum compactum* has a protective role against perforation by

the exoskeleton of the small prawn and other invertebrates that constitute the diet of this stingray (Wongrat 1998). The protective role of this layer has been suggested in carnivorous fishes (Kapoor et al. 1975) and carnivorous mammals (protecting against perforation from sharp bone edges) (Frappier 1998).

The spiral fold is the most appealing feature of stingray intestine. As in other elasmobranches, *H. signifer* has a relatively short intestine compared to other fishes (Holmgren and Nilsson 1999). The increase of inner surface area is achieved by the presence of the folds along the length of the spiral intestine (Wischnitzer 1972). The number of turns (of the spiral) is related to the diet, as they delay the digestion and provide an increased surface for absorption (Holmgren and Nilsson 1999). The small number of turns in *H. signifer* is likely related to the carnivorous diet, typical of bottom feeder elasmobranches (Bertin 1958). In this species, like in all elasmobranches and teleosts (except in Gadidae), no crypts of Lieberkühn were observed (Harder 1975). Facing this absence, it has been suggested that teleosts have lost the ability to secrete NaCl and fluid in the intestine, whereas elasmobranches retained this secretory ability via rectal gland (Loretz 1987). This has been contradicted by a recent in vitro and in vivo study (Marshall et al. 2002), which showed that teleost intestine is capable of salt and fluid secretion, by the action of a subpopulation of enterocytes interspersed among absorptive cells. Since no rectal gland is observed in *H. signifer*, it is possible that in this species the intestine presents a secretory role. This could be important to purge toxic intestinal bacterial flora, a protective role that has been proposed for teleosts living in estuarine habitats (Marshall et al. 2002).

In the posterior intestine, the epithelium of *H. signifier* (stratified columnar with goblet cells) is similar to that reported in *R. clavata* (Holmgren and Nilsson 1999) and in *S. acanthias* (Leake 1975), but contrasts with that of some other elasmobranches, which have microvilli (Holmgren and Nilsson 1999). Probably, in this species the posterior intestine does not have strong absorptive properties. The increased number of goblet cells we found (in comparison to the anterior intestine) is a common feature in teleosts, serving as a lubrication aid to defecation (Kapoor et al. 1975). For this purpose, a thicker muscular coat (Grau et al. 1992), with a three-layered arrangement, is probably also important. This three-layered *muscularis* was appealing; to the best of our knowledge, that has never been described and clearly contrasts with the usual bi-layered *muscularis* reported in elasmobranches (Holmgren and Nilsson 1999) and most teleosts (Reifel and Travill 1979; Yokote 1982).

Mucins are known to play an important role in protecting the mucosa against bacterial attack and physical/chemical damage. Inter- and intraspecific differences in the content of mucosubstances in the digestive tract have been related to different feeding habits and digestive processes in teleosts (Kapoor et al. 1975; Sarasquete et al. 1993). Neutral mucins have been associated with the absorption of easily digestive substances (Kapoor et al. 1975; Grau et al. 1992), whilst acid mucins have been more related to secretion of food lubricants and mucosal lubricants (that keep the internal mucosa moist) (Sinha and Chakravorty 1982). The presence of both neutral and acid mucins in the epithelium of the mouth, pharynx and esophagus has been reported in most teleosts (Reifel and Travill 1977; Sis et al. 1979; Hirji 1983; Grau et al. 1992); *H. signifer* seems particular for having more neutral mucins in those regions than acidic mucins. In such regions the epithelium seems not to be absorptive (stratified columnar), and so the mucins are probably not related to absorption, but act more as food lubricants, for helping in the movement of hard and rough food (Wongrat 1998), and also likely as regulators of the pH of the stomach (Reifel and Travill 1977; Hirji 1983). On the contrary, the presence of neutral mucins associated with the simple columnar epithelium with microvilli in the stomach suggests an absorption role, which has been reported in teleosts (Ezeasor and Stokoe 1980; Grau et al. 1992), but to our knowledge, not in elasmobranches. As in this segment the highest content of proteins was observed, it can be hypothesized that during the long permanence of food in the stomach, some protein absorption takes place. Additionally, the stomach neutral mucins may also play a protective role, by forming a physical coat for the epithelium and by assisting in the control of local pH (Clarke and Witcomb 1980). In the intestine, both neutral and acid mucins are present in the goblet cells, but the intensity of sulfated acid mucin staining in goblet cells of rectum and cloaca was stronger than that found in the anterior intestine; these mucosubstances may thus assist in lubricating the undigested material for defecation.

It has been known for long that the gastrointestinal tract is not only a digestive organ, but also a large and complex endocrine organ. Enteroendocrine cells have been extensively studied in mammals and in teleosts (Pan et al. 2000; Buddington and Krogdahl 2004), but the information on these cells in cartilaginous fishes is still scarce (Hakanson et al. 1986; Tagliafierro et al. 1985, 1988, 1989; Falkmer 1993). In our study, by using a Grimelius silver reaction, we detected a part of the EC population, namely those cells rich in serotonin (Hould 1994). The highest number of EC present in the stomach of *H. signifer* is in accordance to previous studies in nurse-hound shark, *Scyliorhinus stellaris* (Tagliafierro et al. 1985) and *R. asterias* (Tagliafierro et al. 1989). Most of the EC were of the open type, meaning that hormones are transported to the gut lumen by long cytoplasm processes (Pan et al. 2000). The rare triangular-shaped EC observed in the gastric glands probably corresponds to glucagon-secreting cells (Tagliafierro et al. 1989). In some occasions these cells resembled EC of the closed type, typical of more developed vertebrates (Falkmer 1993); however, we think this resemblance was probably due to the angle of sectioning, as reported by Pan et al. (2000).

In conclusion, *H. signifier* presents the typical features of the digestive tract of elasmobranches, with some particularities, like a stomach with three cell types in the glands, a thin *stratum compactum* and with absorptive characteristics or a three-layered *muscularis* in the posterior intestine. The data gathered here offer a baseline for future developments at the ultrastructural level and for further detailed immunohistochemical studies, whereas serving as a reference for histopathological evaluations and for comparative physiological approaches.

Acknowledgements We thank Maria Helena Galante for the technical support and efficiency in making the histochemical preparations.

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