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Research Article

Histological and histochemical studies of digestive system in the megrim, *Lepidorhombus whiffiagonis* (Teleostei: Scophthalmidae)

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Abstract: The digestive system of *Lepidorhombus whiffiagonis* was studied using histological and histochemical techniques. The wall of oesophagus and stomach fundus consisted of four histological layers: mucosa, submucosa, muscularis and the outer layer. Mucosa, the innermost layer, usually consisted of three different layers, including epithelium, lamina propria and lamina muscularis mucosae. Submucosa of connective tissue with blood vessels and nerve fibres underlined the mucosa and usually protrudes into the mucosal folds except in the gallbladder where it is completely absent. The muscular layer of the digestive system consisted of smooth muscles arranged in longitudinal, circular or oblique layers. Striated fibres were found in the oesophageal wall. The outermost layer comes as tunica adventitia in upper parts and tunica serosa in lower parts of the digestive system. Hepatocytes were surrounded by a mass of fat-storing cells. Serous acini of pancreas were scattered down the length of the stomach. Alcian blue/PAS technique revealed that alkaline and neutral mucopolysaccharides were present in oesophagus while acidic mucopoly-saccharides was found in the stomach and intestines.

Keywords: Histoarchitecture, Histochemistry, Pleuronectiformes, Adriatic Sea.

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Introduction

The megrim, *Lepidorhombus whiffiagonis* (Walbaum, 1792) (Pleuronectiformes) is a benthic flatfish, widespread in the eastern part of the Atlantic from Scandinavia and Iceland to the Western Sahara and in the Mediterranean, mainly the central and western part. It is also distributed through Adriatic Sea, except in the northernmost part, most numerous in the Middle and Southern Adriatic, inhabiting sandy and sandy-moody bottoms, between 20 to 360 meters in depth, rarely deeper, mostly 250m (Jardas 1996). Megrim is caught by bottom-travelling and it is considered as palatable and economically important food species. France and Spain are the largest consumers of this species with most of the megrim caught in British waters being exported to these nations (FAO 2018). Due to its widespread and commercial value, the megrim was an important object of several research studies concerning its age and growth (Landa & Piñeiro 2000; Šantić 2013), distribution and abundance (Sánchez et al. 1998), exploitation (Fahy & Fannon 1992), feeding habits (Šantić et al. 2009), population differentiation (Danancher & Garcia-Vazquez 2009). The spatial variations in life history characteristics of megrim were also considered, as well as differences in habitat selection of male and female individuals (Gerritsen et al. 2010).

The megrim is a predatory carnivorous fish, whose diet is dominated by fish, cephalopods and decapod crustaceans (Jardas 1996). Its prey also includes four large groups: Teleostei, Decapoda, Mysidacea and Cephalopoda. Teleosts are the most important prey of larger fishes, while Mysidacea constituted the main prey for smaller fishes. At the species level, the teleost *ntonogadus megalokynodon* and decaspods *Processa canaliculate* constitute the most frequent prey (Šantić et al. 2009).

The digestive system of fish has shown a remarkable diversity of morphological and functional features, particularly in connection with special nutritional behaviour, body shape and sex (Kapoor et al. 1975; Díaz et al. 2008). In fish, as in other vertebrates, the digestive system is a tube of variable diameter which is longitudinally divided into the oesophagus, stomach, intestines, and rectum (Treer et al. 1995). The tongue and the teeth in the oral cavity are also included as associated organs in the digestive system, as well as the extramural digestive organs: liver, gallbladder and pancreas. The salivary glands are generally missing in the oral cavity of fish (Treer et al. 1995). From the cranial end of the oesophagus to the caudal end of the rectum, the wall of the alimentary canal consists of four distinctive layers. Starting from the lumen, these layers are: mucosa, submucosa, muscularis externa and serosa or adventitia (Kierszenbaum & Tres 2012). Although the general plan of digestive system histology in fishes is very similar to those of other vertebrates, however, it shows noticeable diversity in its morphology and function related to both taxonomy and different feeding habits (Al Abdulhadi 2005). Mucosal epithelium of the alimentary tube in fishes is usually provided with mucous cells which show different histochemical features. These features depend on different content of muccopolysaccharides (MPS) secreted by these cells which is in correlation with species, age, regions of the alimentary tract and feeding habits of the fish (Park & Kim 2001, Sarasquete et al. 2001; Cao & Wang 2009). In most fishes, the intestinal mucous cells contain acidic MPS (Cataldi et al. 1987; Al-Abdulhadi, 2005; Bočina et al. 2016). It seems that the quality and quantity of the mucus from the intestinal goblet cells could also be correlated to environmental pollution (Ferrando et al. 2006).

Nevertheless, the morphological studies could help to better understand the relationship between physiological and biochemical functions and molecular mechanisms (Kalhoro et al. 2018). Even in aquaculture, anatomical and histological characteristic of the fish digestive system could help to elucidate the development of pathological conditions, to improve nutritional formulations and thus promote the feed management (Kalhoro et al. 2018).

In view of the paucity of knowledge on the histophysiology of the digestive system of *L. whiffiagonis* in relation to their food and feeding habits, the aim of the present study was to elucidate the histological structure of the digestive system as well as the histochemical nature of the mucins secreted by the epithelial mucous cells in the gut of this fish.

Materials and Methods

A total of five adult *L. whiffiagonis* individuals of average length 20.9cm and average weight 73,574 g were sampled to study the histology and histochemical characterization of the alimentary tract. The specimens were collected in the southern part of the Adriatic Sea, in May 2017. Immediately after collecting the parts of digestive tube (oesophagus, stomach, intestines, liver, pancreas and gallbladder) were fixed in 10% formalin.

Histological study: After two days of fixation, the tissues samples for histological study (oesophagus, stomach, intestines, liver, pancreas and gallbladder) were dehydrated in an ascending series of ethanol, starting from 70% ethanol. Tissue samples were then cleared with xylene and embedded in paraffin. Tissues sections were cut transversally using Microm microtome at 6μm and mounted on glass slides. The sections were deparaffinised with xylene and stained with haematoxylin-eosin staining to present the main morphological features of the mentioned organs of the megrim's digestive system. The sections were observed using an Axioskop Zeiss light microscope

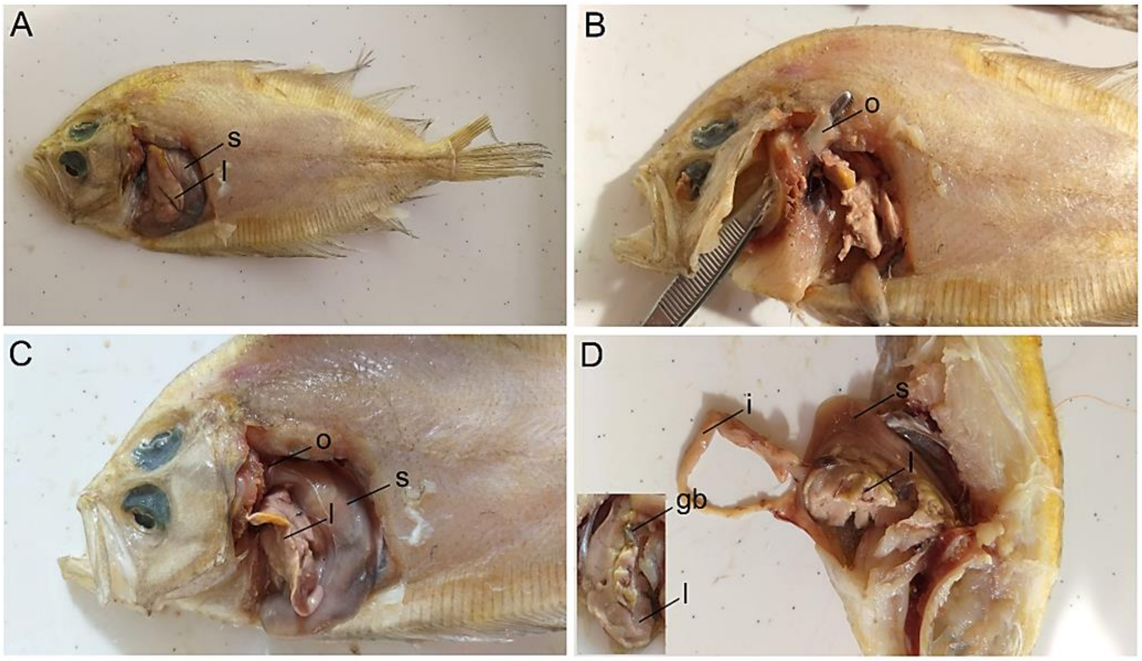

Fig.1. Anatomy of the megrim's digestive system. (A) s, stomach; l, liver, (B) o, oesophagus, (C) o, oesophagus; s, stomach; l, liver. and (D) s, stomach; l, liver; i, intestines; gb, gallbladder.

and photos were taken under Sony DSC-S75 digital camera (Sheehan & Hrapchak 1980).

Histochemical study: Alcian blue/ PAS staining was used to establish the histochemical nature of mucins secreted by the epithelial mucous cells in megrim's alimentary canal. The sections were deparaffinised first and then were brought to distilled water through downgraded ethanol series and oxidized in 1% aqueous periodic acid solution for 10-15 minutes at room temperature. After washing in distilled water, the sections were immersed in Schiff's reagent for 45 min and washed again in water. The sections were then immersed in 1% AB in 3% acetic acid (pH 2.5) for 45 min. Then the slides were finally washed in running tap water, dehydrated through ascending series of ethanol, cleared in xylene and mounted in Neomount. The sections were dehydrated, cleared and mounted on glass slides (Sheehan & Hrapchak 1980).

Results

Oesophagus: The oesophagus of the megrim *L. whiffiagonis* is a straight tube of relative length value of 1.7cm (Fig. 1B). Four distinctive layers could be distinguished in the oesophageal wall of megrim: mucosa (tunica mucosa), submucosa (tunica submucosa), muscular layer (tunica muscularis) and outer layer (tunica adventitia). The mucosa is innermost, thin layer, which formed high folds deeply protruding into the oesophageal lumen (Fig. 2A). It is lined with a simple columnar epithelium containing microvilli (Fig. 2B). The epithelial cells were high with centrally positioned nuclei. The numerous, big, bubble-shaped goblet cells were scattered among columnar epithelial cells (Fig. 2A and 2B). When treated with PAS/AB, apical parts of epithelial cells stained purple, indicating the presence of alkaline and neutral mucopolysaccharide (Fig. 2C). Just beneath the epithelium, there was lamina propria, a thin layer of loose connective tissue containing blood vessels which separates mucosa from submucosa. Lamina muscularis mucosae has not been noticed. The submucosa is the widest layer of the oesophageal wall. It is formed of dense connective tissue containing blood vessels and nerve fibres. The muscular layer consisted of circularly arranged striated muscle fibres (Fig. 2D). Tunica adventitia is the outermost layer, formed of

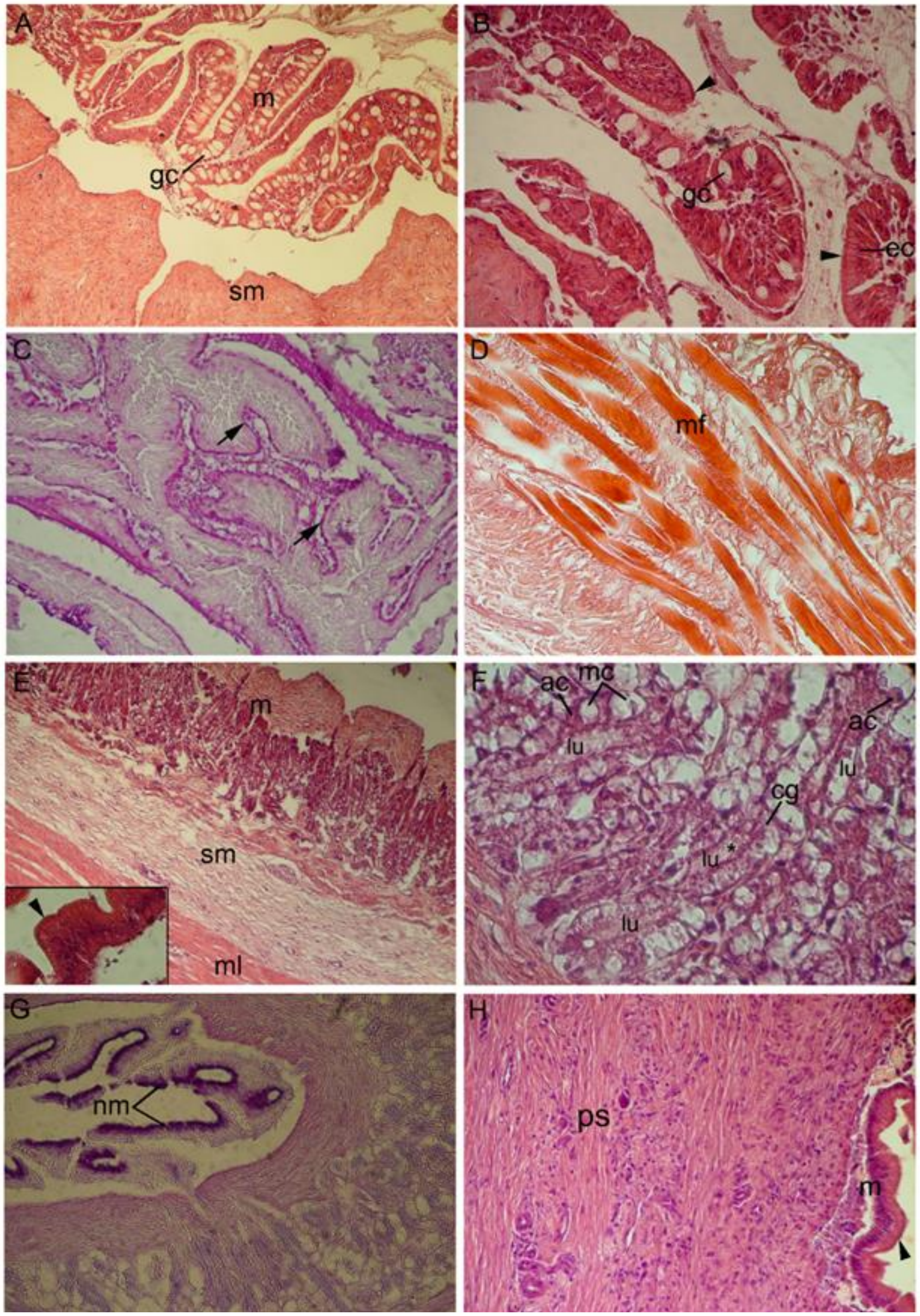

Fig.2. Transverse section through the upper part of the megrim's digestive system: (A) Section through the oesophagus: m, mucosa; sm, submucosa; gc, goblet cell. Hematoxylin-eosin, 200X, (B) Oesophageal epithelium: gc, goblet cell, (arrowhead), microvilli; ec, epithelial columnar cells. Hematoxylin-eosin, 400X, (C) Section through the oesophageal mucosa: (↑) – PAS positive cells to neutral mucin. AB/ PAS, 400X, (D) Section through the oesophageal muscular layer: mf, striated muscle fibers. Hematoxylin-eosin, 400X, (E) Section through the stomach: m, mucosa; sm, submucosa; ml, muscular layer. Hematoxylin-eosin, 200X. Inset: Microvilli in stomach epithelium (arrowhead). Hematoxylin-eosin, 400X, (F) Section through the tubular gastric glands: lu, lumen; mc, mucous (goblet) cells; ac, acidophilic cells; cg, cells with granulated cytoplasm; (asterix), secreted granules. Hematoxylin-eosin, 1000X, (G) Section through the stomach mucosa: nm, PAS positive cells to neutral mucin. AB/PAS, 400X, and (H) Section through the pyloric region: m, mucosa; ps, pyloric sphincter; (arrowhead), microvilli. Hematoxylin-eosin, 400X.

connective tissue containing blood vessels.

Stomach: The stomach of megrim is sac-like organ placed between the oesophagus and the intestines (Fig. 1A, C, D). Its relative length was 4.5cm. The fundic wall of the stomach consisted of four layers: mucosa, submucosa, muscular layer and outer layer. The mucosa forms deep folds and it is lined by a simple columnar epithelium. The nuclei of epithelial cells are placed at the basal domain of the cell, while the apical domain of the cell contains microvilli (Fig. 2E). Lamina propria is the broadest layer of stomach wall, which contains parallel, long, tubular gastric glands and blood capillaries scattered among gastric glands. The gastric glands consisted of different cell types: the most numerous mucous cells, the acidophilic cells and cells with granulated cytoplasm. The secreted granules could also be seen in the lumen of the glands (Fig. 2F). When treated with AB/PAS apical parts of epithelial cells stained purple indicating the presence of neutral mucin (Fig. 2G). The submucosa is a thick layer of connective tissue with blood vessels and nerves. Muscular layer consisted of two layers of smooth muscles separated by narrow layer of connective tissue. The muscle fibres of the inner layer were oblique, while those of the outer layer were circular. The outer surface of the stomach is lined with a thin layer of mesothelial cells.

The wall of the stomach pyloric region is mostly occupied by a strong circular muscle made of smooth muscle tissue forming pyloric sphincter. The mucosa of pyloric wall is lined by a simple columnar epithelium containing microvilli. When using AB/PAS, apical parts of epithelial cells stained purple due to the presence of neutral mucopolysaccharide inside them. The epithelium lies on a thin layer of connective tissue. The submucosa has not been observed (Fig. 2H).

Intestines: The relative length value of intestines in megrim measured in the present study was 3.5cm (Fig. 1D). Intestines of megrim could be distinguished in two parts: the anterior and the posterior intestines. Despite the differences in their diameter, these two parts showed certain similarities in their histological structure. The wall of the intestines consisted of: mucosa, submucosa, muscular layer and the outer layer.

The mucosa of the anterior intestines is lined by a simple columnar epithelium containing microvilli. Large, bubble-shaped goblet cells are inserted between the epithelial cells (Fig. 3A). These cells stained purple with PAS/AB indicating the presence of neutral mucin (Fig. 3B). Dense connective tissue of lamina propria deeply protruded into the folds of the mucosa bringing blood vessels. The absorptive area of the intestine is increased by two intestinal caeca located at the juncture of the stomach and the intestines. Submucosa is a layer of connective tissue underlying mucosa and protruding into the mucosal folds. Two muscular layers, the inner longitudinal and the outer circular layer underlined submucosa. The muscular layer is lined by an outer layer consisting of a loose connective tissue (Fig. 3A).

The wall of the posterior intestines is thicker than those of anterior intestine. The mucosa of the posterior intestines is also lined by simple columnar epithelium, but without microvilli. Lamina propria is made of dense connective tissue with blood vessels. The muscular layer consisted of two layers of muscles, inner circular and outer longitudinal layer showing the reverse distribution when compared to the muscular layer in the anterior intestines (Fig. 3C). Staining with Alcian blue/PAS shows the same as in the anterior intestine (Fig. 3D).

Liver: The liver in megrim seemed to be very fatty. It consists of one lobe and its parenchyma contained radially arranged plates of hepatocytes surrounding the central vein. These irregular plates were separated from each other by sinusoidal capillaries which opened into the central vein. Hepatocytes contained a large, spherical nucleus and cytoplasm filled with lipid droplets (Fig. 3E).

Pancreas: Pancreatic tissue is scattered down the length of the stomach wall. It consists of exocrine portions in a form of densely packed circular clusters of cells (serous acini). The basal parts of pyramidal acinar cells contain nuclei and stained basophilic

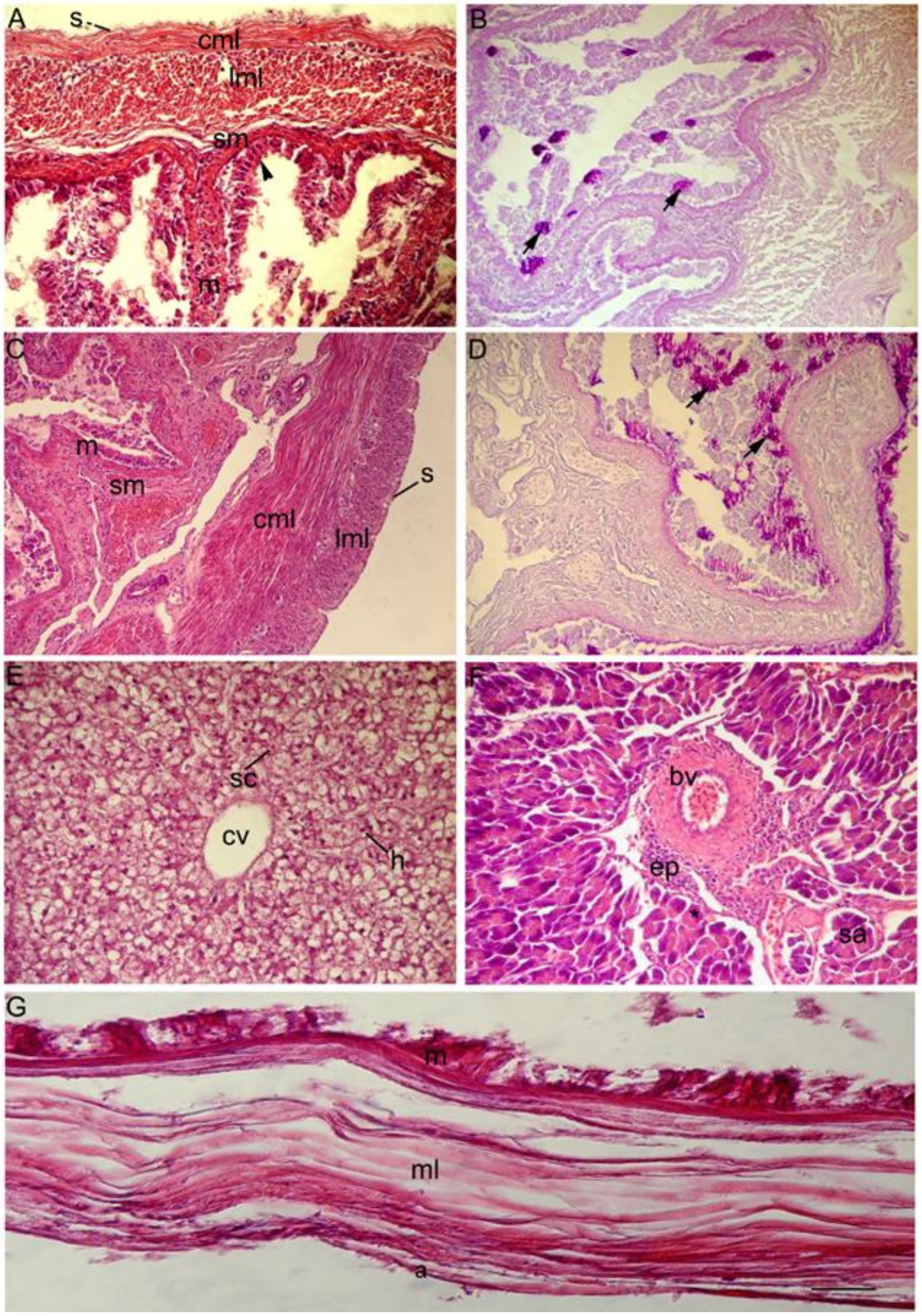

Fig.3. Transverse section through the lower part of the megrim's digestive system and organs associated with the digestive system: (A) Section through the anterior intestines: m, mucosa; (arrowhead), microvilli; sm, submucosa; lml, longitudinal muscular layer; cml, circular muscular layers; s, serosa. Hematoxylin-eosin, 400X, (B) (↑), goblet cells PAS positive to neutral mucin. AB/PAS, 400X, (C) Section through the posterior intestines: m, mucosa; sm, submucosa; cml, circular muscular layer; lml, longitudinal muscular layer; s, serosa. Hematoxylin-eosin, 200X, (D) (↑) PAS positive cells to neutral mucin. AB/PAS, 400X, (E) Section through the megrim's liver: h, hepatocytes with lipid droplets; sc, sinusoidal capillaries; cv, central vein. Hematoxylin-eosin, 400X, (F) Section through the megrim's pancreas: sa, serous acinus; ep, endocrine portion; bv, blood vessel; (asterix), serous cells with granules in the apical part. Hematoxylin-eosin, 400X, and (G) Section through the gallbladder: m, mucosa; ml, muscular layer; a, adventitia. Hematoxylin-eosin, 400X.

while the apical part contains numerous zymogene granules stained eosinophilic. Numerous blood vessels could be seen throughout the pancreatic tissue usually distributed among the serous acini. The endocrine portions of pancreas are also seen in the pancreas sections usually distributed around blood vessels (Fig. 3F).

Gallbladder: The gallbladder wall is relatively thick, consisted of the mucosa, the muscular and the outer layer. Since submucosal layer is missing the mucosa is attached directly to the underlying muscular layer consisting of a single columnar epithelium and lamina propria. The outer layer is abundant tunica adventitia (Fig. 3G).

Discussion

The histological structure of the digestive system in fishes is usually associated with their diet, as was studied in numerous species (e.g., Al-Hussaini 1949; Clarke & Witcomb 1980; Cataldi et al. 1987; Albrecht et al. 2001; Nazlić et al. 2014; Bočina et al. 2016; Bočina et al. 2017). The main plan of the digestive system is very similar in all vertebrates (Andrew 1959). It consists of the alimentary canal and ancillary organs such as liver, gallbladder and pancreas (Stevens & Hume 1995). The alimentary canal of fishes is usually divided into the oesophagus, the stomach, the intestine and the rectum (Treer et al. 1995), although the rectum in megrim was not histologically distinguishable from the posterior intestines. The structure of intestines is usually consistent among vertebrates, but stomach morphology shows remarkable diversity (Smith et al. 2000). In some freshwater and marine fishes such as the Cyprinidae, Labridae and Gobiidae, the stomach has been evolutionary lost (Al-Hussaini 1949). It is difficult to understand how stomach-less fishes digest food, especially the carnivorous ones with great uptake in their diet (Day et al. 2011), but it seems that this loss does not influence the digestion, as the stomach-less fish can be either herbivorous or carnivorous. The herbivorous fishes usually have relatively long guts, whereas in carnivorous fishes the guts are generally shorter than their body length (Buddington et al. 1997; Horn et al. 2006; German et al. 2010).

The megrim is predatory fish, and predominantly feeds with fishes, cephalopods and crustaceans. Accordingly, the digestive system begins with a large mouth set upward. In the megrim, the alimentary tube opens with a big mouth containing thin and peaked teeth arranged in several rows (Jardas 1996). Since the megrim is a carnivorous fish, its oesophagus is short, muscular tube conducting the food to the stomach. The oesophagus of different teleost fish is usually lined by stratified squamous epithelium and cells that secrete mucus (Cataldi et al., 1987; Al Abdulhadi 2005; Nazlić et al. 2014; Bočina et al. 2016). In spite of this, columnar epithelium has been recorded in some marine and euryhaline fish as well as freshwater fish (Stevens & Hume, 1995; Chatchavalvanich et al. 2006).

The oesophagus of the megrim, as shown in the present study, is also lined by simple columnar epithelium all along its length. It seems that these columnar cells contained microvilli at their apical domain. The epithelium of megrim's oesophagus is also provided with numerous goblet cells. The secretion of goblet cells probably facilitates the transport of the food particles through the oesophagus. In some fish, such of genus *Gobio* and *Cyprinus*, the selection of food is based largely on taste; hence they are supplied with taste buds from lips to oesophagus. On the other hand, some of them augment the taste by sight and taste buds are accordingly restricted in distribution and less abundant (Al-Hussaini 1949). The taste buds were not noticed in the oesophagus of megrim as well as lamina muscularis mucosae which is usually missing in fish (Kozarić 2001; Nazlić et al. 2014). Although in many fishes, oesophageal muscular layer is usually double-layered and it contains striated muscles (Clarke & Witcomb 1980; Nazlić et al. 2014; Bočina et al. 2016, 2017), it seems that in the megrim the muscular layer is single-layered: it contained one circular layer of striated muscles. The complex morphology and histochemistry of the oesophagus may indicate additional functions as it was shown in bream, *Mylio cuvieri* whose oesophagus exhibits a diversity of morphologically and histochemical recognizable type of mucous cells (Al Abdulhadi 2005). According to the results of AB/PAS, we reported that oesophageal epithelial cells in the megrim contain alkaline and neutral mucopolysaccharide.

In some fish stomach is absent (Bočina et al. 2017). The gastric glands are the main part of the stomach mucosa in vertebrates (Kierszenbaum & Tres 2012), and also in fish, both carnivorous and herbivorous (Al Abdulhadi 2005). The stomach of the sea bream *Sparus aurata* has a single-layered columnar epithelium, under which, in the cardiac and fundic portion, gastric glands are placed (Cataldi et al. 1987). The stomach of megrim is also covered by single-layered columnar epithelium and it seems that these epithelial cells contained microvilli and they were PAS/AB positive to neutral mucin. In the stomach of *S. senegalensis*, gastric glands are numerous in the fundic and pyloric region, while in the cardiac region are absent (Arellano et al. 2001). The gastric glands are usually placed in lamina propria of the stomach mucosa (Nazlić et al. 2014; Bočina et al. 2016). In the megrim's stomach, the gastric glands were found only in the lamina propria of the fundic portion but not in the pyloric one. These glands were tubular in structure and several types of cells could be distinguished inside the glands: the mucous cells, the acidophilic cells and granulated cells, respectively. In pylorus of common eel, the gastric glands are also absent (Clarke & Witcomb 1980). In humans, gastric glands found in stomach cardia and fundus are different from those found in pylorus (Kierszenbaum & Tres 2012). The main characteristic of the pyloric region in most species and megrim is the presence of pyloric sphincter, a strong circular muscle enabling food particles to move towards the intestine (Albrecht et al. 2001; Chatchavalvanich et al. 2006; Kierszenbaum & Tres 2012).

The intestines of megrim could be distinguished in proximal and distal part, which is consistent with the published data (Al Abdulhadi 2005; Chatchavalvanich et al. 2006; Dai et al. 2007). The megrim intestines consist of four layers: mucosa, submucosa, muscular layer and serosa. Although these layers are continuously present in total length of the intestine, two previously mentioned intestine parts differs in the wall thickness and the morphology of mucosal folds and muscular layer. The anterior intestine has a largest diameter and deeper folds than those in the posterior intestine and the muscle layers were in reverse distribution in the tunica muscularis. The goblet cells are usually found in fish intestines (Al Abdulhadi 2005; Nazlić et al. 2014; Bočina et al. 2016). The same was confirmed by present study in megrim's intestine. Ferrando et al. (2006) mentioned that the quality and quantity of the mucous from the intestinal goblet cells is correlated to environmental pollution. In different fish species, liver consists of different number of lobes. Based on the results, the liver of megrim consists of one lobe as was described in garfish, *Belone belone*, while the liver of European hake, *Merluccius merluccius* and black scorpionfish *Scorpaena porcus* is formed of three lobes (Nazlić et al. 2014; Bočina et al.2016, 2017). The pancreas in vertebrates is separate organ with its exocrine and endocrine function (Kierszenbaum & Tres 2012). In megrim, the pancreatic tissue is usually spread along the wall of digestive system. The exocrine pancreas usually consists of serous acini. Endocrine portions of the pancreas are usually found near blood vessels (Bishop & Odense 1966; Clarke & Witcomb 1980) as described in megrim.

Conclusions

The present study provides first description of the megrim's *L. whiffiagonis* alimentary canal histology and histochemistry. The data showed that megrim has a short alimentary canal as a carnivorous fish. Similar to other carnivorous fish, it contains mucosal, submucosal, muscular and outer layer. Neutral mucopolysaccharides were mostly present in the megrim's digestive system. The histology and histochemical composition of the megrim's alimentary canal seem to be similar to other carnivorous fishes.

Acknowledgements

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مقاله پژوهشی

مطالعه بافت‌شناسی و هیستوشیمی دستگاه گوارش ماهی مگریم، *Lepidorhombus whiffiagonis* (ماهیان استخوانی حقیقیی: خانواده **Scophthalmidae**)

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چکیده: دستگاه گوارش ماهی *Lepidorhombus whiffiagonis* با استفاده از تکنیک‌های بافت‌شناسی و هیستوشیمی مطالعه شد. دیواره مری و قاعده معده شامل چهار لایه بافتی بودند: مخاطی، زیرمخاطی، ماهیچه‌ای و لایه بیرونی. موکوزا، داخلی‌ترین لایه، معمولاً شامل سه لایه مختلف شامل پوششی، آستر مخاطی و لایه ماهیچه‌ای می‌باشد. زیرمخاطی از بافت پیوندی به همراه رگ‌های خونی و رشته‌های عصبی در زیر لایه مخاطی قرار دارد و معمولاً از برجستگی‌های مخاطی بیرون می‌زند به‌جز در ناحیه کیسه صفرا جائی‌که کلاً یافت نمی‌شود. لایه ماهیچه‌ای دستگاه گوارش شامل ماهیچه‌های نرم که به‌صورت لایه‌های طولی، حلقوی و یا مورب آرایش یافته‌اند، می‌باشد. رشته‌های طبقه طبقه در دیوار مری یافت می‌شود. بیرون‌ترین لایه به‌صورت بافت همبند پوششی در بخش‌های بالایی و به‌صورت غشای سروزی در بخش‌های پایین دستگاه گوارش می‌باشند. هپاتوسیت به‌وسیله یک توده از سلول‌های ذخیره کننده چربی احاطه شده بودند. غده‌های سروزی پانکراس در امتداد زیرین معده پراکنده بودند. تکنیک رنگ‌آمیزی آلسیان بلو/ PAS آشکار کرد که موکوپلی‌ساکاریدهای قلیایی و خنثی در مری حضور دارند. درحالی‌که موکوپلی‌ساکاریدهای اسیدی در روده و معده‌ها یافت می‌شوند

کلمات‌کلیدی: ساختار بافتی، هیستوشیمی، ماهیان پهن، دریای آدریاتیک.