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Research article

Histology and Histochemistry of the Digestive Tract of Transverse-bar Barb, *Hampala macrolepidota* Kuhl & Van Hasselt, 1823

Akapon Vaniksampanna[1], Sonchai Intachai[2], Wankuson Chanasit[2], Sakorn Khongsud[2], and Akkanee Pewhom[2*]

[1] Innovative Learning Center, Srinakharinwirot University, Bangkok, 10110, Thailand
[2] Faculty of Science and Digital Innovation, Thaksin University, Phatthalung, 93210, Thailand
* Correspondence: akkanee.p@tsu.ac.th

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Abstract: *Hampala macrolepidota* is an important local fishery species; the information on its anatomy and microanatomy is scarce. Therefore, the histology and histochemistry of its digestive tract were systematically investigated. The digestive tracts of adult fish were removed and processed using the paraffin technique. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E), periodic acid-Schiff's (PAS), and alcian blue (AB), pH 2.5 and pH 1.0. Its digestive tract consisted of the oropharynx, esophagus, and intestine, with no stomach observed. The stratified epithelium of the oropharynx was weakly positive-stained with PAS and AB at pH 2.5 and pH 1.0. In contrast, in the esophagus, mucous and goblet cells were strongly positively stained with all stains, indicating the secretion of neutral mucin and carboxylated and sulfated acid mucins. The intestine can be divided into three regions: anterior, middle, and posterior. Meanwhile, the enterocytes were positively stained with PAS; however, the goblet cells were positively stained with PAS at pH 2.5 and pH 1.0. The goblet cells secreted neutral mucins, as well as carboxylated and sulfated acidic mucins. The middle intestine showed crypt-like glands and the highest histometric values, indicating that it served as the main site of digestion and nutrient absorption, despite the absence of a stomach. The muscular layer varied regionally, with skeletal muscle in the oropharynx and esophagus and smooth muscle in the intestine. The intestinal coefficient was 0.69, indicating that it was a carnivorous fish. These findings provided fundamental data relevant to aquaculture, ecology, and comparative vertebrate anatomy.

Keywords: Digestive tract; *Hampala macrolepidota*; histochemistry; microanatomy; mucopolysaccharides

1. Introduction

The digestive system is a vital organ system responsible for converting nutrients into energy to sustain animal activity. Among vertebrate digestive systems, there are many differences in anatomy, microanatomy, physiology, and function, especially in teleosts [1], depending on evolutionary processes, feeding habits [2], and taxonomy [3]. Overall, the general structure of the gastrointestinal tract is mostly similar, although variations among species can be observed [4], resulting in different functional physiology in each species [5]. Generally, the fish digestive tract comprises the pharynx, esophagus, stomach, and intestine [6]. In some teleost species, the stomach is absent, including members of the families Cyprinidae, Labridae, Gobiidae, Scaridae, some Poeciliidae [7], and such species as *Puntius stoliczkanus* [8], *Sphoeroides testudineus* [2], *Barbus altianalis* [9], *Schizothorax esocinus* [10], *Xiphophorus maculatus*, and *X. hellerii* [11].

The pharynx is the first portion of the digestive tract, connecting the oral cavity and gills to the muscular esophagus [12]. The caudal end of the esophagus connects to the stomach, which may be straight, U-shaped, or J-shaped based on the species and feeding habits [13, 14]. The intestine forms the final portion of the tract and opens to the exterior. In stomachless fish, the esophagus connects directly to the intestine, a unique characteristic [7]. Moreover, intestinal length depends on feeding habits [12], with herbivorous, carnivorous, and omnivorous fish having the longest, shortest, and intermediate lengths, respectively [15]. The intestinal coefficient (IC; the ratio of intestinal length to standard length) is widely used as a criterion for identifying feeding habits [12] or food preferences [16]. Herbivorous fish typically have IC values ranging from 0.8 – 15.0 [17], while carnivorous and omnivorous fish have values of 0.2 – 2.5 and 0.6 – 8.0, respectively [12]. In teleosts, the alimentary system has multiple functions, including food digestion and nutrient absorption [18], osmoregulation [19], hormone secretion [20], and immune defense [21]. Studies of the fish digestive tract are therefore crucial, as they contribute to many related fields. Numerous studies have examined digestive morphology and histology, including the pharynx to intestine in *B. altianalis* [9]; the esophagus and stomach in *Parupeneus forsskali*, *Papilloculiceps longiceps*, and *Acanthurus sohal* [14]; and the esophagus to intestine in *Oligosarcus hepsetus* [22], *Lates calcarifer* [23], *Sparus aurata* [6], *Larimichthys crocea* [12], *Pisodonophis boro* [24], *Hydrocynus forskahlii* [25], and *Trichomycterus bogotensis* [16]. Some studies have focused only on the stomach [26] or the intestine [11], such as in *P. stoliczkanus* [8]. Histochemical analyses have also been performed, primarily focusing on the types of mucopolysaccharides and glycoproteins secreted by cells in different regions of the tract and their functional significance [12]. Thus, histological studies of the digestive tract provide important tools for assessing gut health, digestive adaptation, environmental conditions [11], taxonomy, and feeding habits [27], with applications in feed management [12].

The transverse-bar barb, *Hampala macrolepidota*, is a member of the family Cyprinidae and is distributed across Southeast Asia, including Laos, Indonesia, and Thailand [28]. Adult *H. macrolepidota* have an elongated, laterally compressed body with dark transverse bars across the longitudinal axis. A previous study reported that *H. macrolepidota* is a carnivorous (piscivorous) species with a predatory ecological role, feeding on fish, shrimp, crabs, mollusks, and aquatic insects [29]. *H. macrolepidota* is regarded as a highly desirable species among fishermen, highly valued for consumption and economically important, which makes it one of the principal target fishes in many regions [28]. However, *H. macrolepidota* is classified as "Least Concern" [30], its populations are declining [31], and life-history traits and biological data remain scarce [32], particularly regarding anatomy, microanatomy, histochemistry, and physiology. Therefore, in this study, the histology and histochemistry of the digestive tract of *H. macrolepidota* were systematically investigated. The findings will help fill critical knowledge gaps and provide fundamental data for applications in physiology, fish health assessment, and aquaculture.

2. Materials and Methods

2.1 Fish and tissue collection

Ten healthy adult *H. macrolepidota* (five females and five males; mean standard length = 39.01 ± 0.45 cm) were wild-caught from natural freshwater habitats in Phatthalung Province, Thailand (7°46′43.9″N, 100°11′42.8″E) between July 2024 and March 2025. Fish were considered healthy based on normal external appearance and the absence of lesions and signs of disease. Standard length was measured using a measuring tape to the nearest 1 mm (0.1 cm), and each measurement was taken twice to ensure accuracy. *H. macrolepidota* were anesthetized with 100 $mg \cdot L^{-1}$ of tricaine methanesulfonate (MS-222) solution. The body was opened longitudinally from the anus to the mouth. The digestive tract was then removed, photographed, and measured with the measuring tape. Subsequently, the digestive tract was fixed in Bouin's fixative for 72 hours and then preserved in 70% ethanol. The intestinal length was used to calculate the intestinal coefficient (IC) according to the following equation [8, 12, 16, 23].

$$\mathrm{IC} = \frac{\text{Intestinal length (IL)}}{\text{Standard length (SL)}}$$

This research was approved by the Animal Ethics Screening Committee, Thaksin University (Permit number: COA TSU 2024-009; IACUC No. 0009).

2.2 Histology and histochemistry study

The digestive tract was cut into small pieces and processed using the paraffin method. The organs were dehydrated through a graded ethanol series (70%, 80%, 95%, and absolute alcohol, two changes at each concentration). The resulting samples were then cleared in xylene (two changes) and infiltrated with Paraplast (two changes). The organs were embedded in Paraplast Plus® (Sigma-Aldrich, USA) using an embedding center to produce tissue blocks. Serial sections of 5 μm thickness were cut with a rotary microtome and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) for general tissue components, periodic acid Schiff's (PAS) for neutral mucopolysaccharides, alcian blue pH 2.5 (AB pH 2.5), and alcian blue pH 1.0 (AB pH 1.0) for carboxylated and sulfated acid mucopolysaccharides, respectively [33]. Permanent slides were examined under a compound light microscope (Olympus CX31) and photographed with a digital camera (Canon EOS 700D).

2.3 Histometric and statistical analysis

The photographs were examined, and the epithelium layer thickness, mucosal fold (villi) width, mucosal fold (villi) height, and muscular layer thickness were measured using ImageJ software. Measurements were obtained from randomly selected regions across multiple fields per section of each structure from all ten fish examined. SPSS was used to analyze the data, and Duncan's multiple-range test was employed to differentiate the parameters of the anterior, middle, and posterior intestines. The data are presented as mean ± standard deviation, and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Gross anatomy of the digestive tract

The digestive tract of *H. macrolepidota*, with a mean length of 32.49 ± 0.91 cm (Table 1), was located within the abdominal cavity, with the intestine covered by the swim bladder. Its tract consisted of the oropharyngeal cavity, esophagus, and intestine (Figures 1A – 1I), similar to those of *Belone belone* [34], *B. altianalis* [9], *X. maculatus*, and *X. hellerii* [11]. No difference in gross anatomy or histology was observed between individuals and sexes. The oropharyngeal cavity formed the oral cavity with dilated folds, whereas the esophagus was a short muscular tube (Figures 1A - 1B, 1F). The intestine was the longest portion; however, no stomach was found in this species (Figures 1A-1B). The absence of stomach was similar to that reported in *S. testudineus* [2] and *B. belone* [34]. Furthermore, the observation was a unique characteristic found in families such as Cyprinidae, Labridae, Cyprinodontidae, Gobiidae, Scaridae, and some members of Poeciliidae [7].

The oropharyngeal cavity and esophagus were short regions with a mean length of 3.78 ± 0.23 cm (Table 1). The oropharyngeal cavity possessed thick longitudinal folds and a wide dilated area (Figure 1F). Its terminal end is connected to the short tubular esophagus. The junction between two regions was marked by the narrow tubular opening of the esophagus (Figures 1A, 1C, 1F). The main difference between the oropharynx and esophagus was that the oropharynx had taller folds than the esophagus (Figure 1F). In *H. macrolepidota*, the esophagus is connected directly to the intestine, as in other stomachless fish such as *B. altianalis* [9], *S. esocinus* [10], *X. maculatus*, and *X. hellerii* [11]. At the junction between the esophagus and intestine, a constriction was observed (Figures 1A - 1C). The intestine of *H. macrolepidota* (mean length 27.01 ± 1.54 cm; Table 1) was divided into three regions: anterior, middle, and posterior (Figures 1A - 1E). The anterior intestine extended caudally before ascending to form the middle intestine, while the posterior intestine descended toward the anus (Figure 1A). These results were similar to those reported in *S. testudineus* [2]. The external wall of the intestine was attached to the abdominal wall by a thin membrane, while the internal wall contained longitudinal folds (Figures 1G - 1I).

The wall of all portions of the gastrointestinal tract contained longitudinal folds, similar to other stomachless fish such as *B. altianalis* [9], *S. esocinus* [10], *H. forskahlii* [25], and other carnivorous fish such as *Trichomycterus brasiliensis* [35]. In addition, *H. macrolepidota* lacked pyloric caeca, a condition commonly reported in carnivorous fishes. In contrast, pyloric caeca are well developed in herbivorous and omnivorous fishes, where they play an important role in prolonging food retention time, enhancing enzymatic digestion, and facilitating nutrient absorption, particularly of plant-derived materials that are structurally complex and difficult to digest [13]. Therefore, the absence of pyloric caeca in the carnivorous *H. macrolepidota* is consistent with its feeding habit and functional digestive strategy, which relies primarily on intestinal digestion of animal prey rather than extended processing of plant material [13], a condition similarly reported in other carnivorous fishes such as *T. bogotensis* [16], *P. boro* [24], *Ariopsis seemanni* [36], and *Pimelodus blochii* [37].

Table 1. Mean ± standard deviation (SD) of standard length, digestive tract length, oropharyngeal–esophageal length, intestinal length (cm), and intestinal coefficient (IC) in *H. macrolepidota* (n = 10).

	Standard length (cm)	**Digestive tract length** (cm)	**Oropharynx-esophagus length** (cm)	**Intestinal length** (cm)	**IC**
$\bar{x}$ ±SD	39.01 ± 0.45	32.49 ± 0.91	3.78 ± 0.23	27.01 ± 1.54	0.69

3.2 Histology and histochemistry

3.2.1 oropharynx

The histological characteristics of the oropharyngeal wall comprised three layers, including the mucosa (epithelium, lamina propria, and absence of muscularis mucosae), submucosa, and muscular layer (Figure 2A). The mean mucosal fold width and height were 2,613.22 ± 93.04 µm and 2,899.77 ± 29.69 µm, respectively (Table 2). The epithelium was lined by non-keratinized stratified squamous epithelium that was composed of multiple cell layers (Figures 2A – 2F), similar to those observed in *B. altianalis* [9], *X. maculatus*, and *X. hellerii* [11]. The apical cells were flat with flattened nuclei, whereas the cells in the basal and intermediate regions were cuboidal or polygonal with oval nuclei (Figures 2A – 2F). The epithelium comprised approximately 35.09 ± 1.89 cell layers and showed a mean epithelial thickness of 679.06 ± 137.79 µm (Table 2). The apical epithelial cells of the oropharynx were weakly positive for PAS (Figure 2D), AB pH 2.5 (Figure 2E), and AB pH 1.0 (Figure 2F). However, at the short junction between the oropharynx and esophagus, the sparse globular mucous cell exhibited strong positive staining with PAS (Figure 3D), AB pH 2.5 (Figure 3E), and AB pH 1.0 (Figure 3F). Beneath the epithelium, the lamina propria was observed, consisting of dense connective tissue. Blood vessels containing red blood cells and numerous fibroblasts were prominent in this layer (Figures 2A - 2B). The stratified squamous epithelium facilitated the food transport and protected the underlying connective tissue from mechanical and chemical injuries, as well as bacterial invasion [22]. Its non-keratinized characteristic additionally supported the food transport [11]. In contrast, the muscularis externa contained a longitudinal layer of skeletal muscle (Figures 2A, 3A), consistent with stomachless fish such as *B. altianalis* [9]. This skeletal muscle allowed the pharynx to reject unwanted food, and provided voluntary control of swallowing, with the mean muscular layer thickness of 1,300.34 ± 197.86 µm (Table 2).

Furthermore, at the transitional junction between the oropharynx and cranial esophagus, the epithelium gradually changed from stratified squamous to stratified squamous/cuboidal epithelium with a mean cell layer of 10.33 ± 2.00 cell layers (Figures 3A - 3B). This short transitional segment is composed of apical squamous/cuboidal cells, intermediate polygonal cells, and sparse globular mucous cells (Figures 3B - 3F). In addition, near the base of the epithelium, club cells (alarm cells) were observed, which were large, oval to round with a prominent nucleus (Figures 3B – 3F). The cytoplasm was stained with eosin (Figures 3B - 3C), meanwhile displayed weak staining with PAS (Figure 3D), similarly to *B. altianalis* [9], *T. bogotensis* [16], *T. brasiliensis* [35], *A. seemanni* [36] and *Pimelodus pictus* [38]. Whereas these cells were consistently negative with staining AB pH 2.5 (Figure 3E) and AB pH 1.0 (Figure 3F). In contrast, the club cells had rarely been reported in fishes, possibly because most digestive tract studies began at the esophagus rather than the oropharynx [12, 22, 24]. These findings suggested that club cells functioned in producing and secreting substances, including alarm pheromones released on the approach of predators [39]. However, the presence of club cells in the epidermis of fish suggests that club cells in the esophagus might be associated with the secretion of substances that defend against parasites and pathogens, as well as play roles in immune responses and healing [40, 41]. Future studies are seriously explored to clarify the precise function of these cells. In the present work, however, the oropharynx of *H. macrolepidota* lacked taste buds (Figures 3A - 3F).

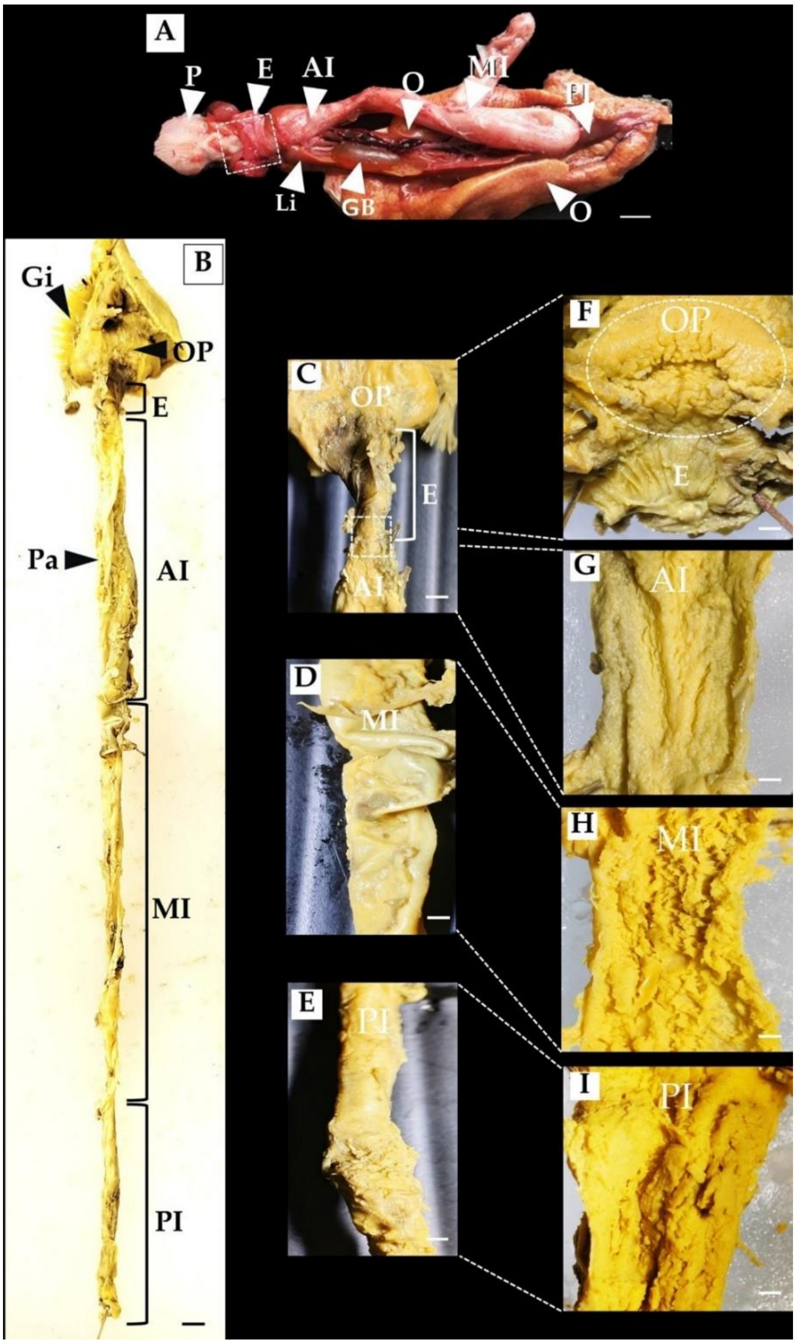

Figure 1. (1A) The fresh digestive tract in its true position among the reproductive system of *H. macrolepidota*. (1B) The fixed and extended digestive tract, consisting of oropharynx (OP), pharynx (P), esophagus (E), anterior intestine (AI), middle intestine (MI), and posterior intestine (PI). (1C) The enlarged view of the oropharynx, esophagus, and anterior intestine, where the square dotted line frame represents the constricting junction between the esophagus and anterior intestine. (1D - 1E) The enlarged view of the middle and posterior intestines, respectively. (1F) The interior wall of the oropharynx (the oval dotted line frame) and esophagus. (1G, 1H and 1I) The interior wall of the anterior, middle, and posterior intestines, respectively. Gall bladder (GB), gill (Gi), liver (Li), ovary (O), and pancreas (Pa).

3.2.2 Esophagus

The esophageal wall consisted of four layers: mucosa, submucosa, muscularis, and serosa. The mucosa formed thick longitudinal folds projecting into the lumen (Figures 3A, 4A), with mean fold width and height of 409.39 ± 59.99 µm and 609.09 ± 96.41 µm, respectively (Table 2). The mucosal layer of the esophagus was lined with the non-keratinized stratified squamous epithelium, containing 5 - 7 layers of epithelial cells (Figures 4C - 4F), which were fewer than those observed in the oropharynx. The mean epithelial thickness was 71.47 ± 6.36 µm (Table 2). The stratified squamous epithelium was consistent with that reported in *S. testudineus* [2], *B. altianalis* [9], *X. maculatus*, X. *hellerii* [11], *L. crocea* [12], *Serrasalmus nattereri* [13], *P. forsskali*, *P. longiceps* [14], *O. hepsetus* [22], *B. belone* [34], and *Scorpaena porcus* [42]. However, in *S. testudineus* [2], the esophagus was divided into two regions: a straight tube and an abdominal pouch, owing to its pouch-like appearance, whereas in *H. macrolepidota*, the esophagus showed the same structure throughout the tube. The epithelium displayed five cell types: apical squamous cells, mucous cells, non-mucous cells, goblet cells, and basal cells (Figures 4C - 4F). The apical squamous cells were flattened and located near the lumen, whereas the mucous cells were oval or globular, with flattened or ovoid nuclei at the cell base. The goblet cells exhibited a characteristic goblet shape, with nuclei similar to those observed in mucous cells. The basal cells were flat or cuboidal, whereas non-mucous cells were cuboidal or polygonal, with round or oval nuclei (Figures 4C - 4F). With H&E staining, the cytoplasm of mucous and goblet cells appeared clear (Figure 4C). PAS staining revealed magenta (Figure 4D), while staining with AB at pH 2.5 (Figure 4E) and pH 1.0 (Figure 4F) resulted in intense blue. These results indicated that the mucous and goblet cells secreted neutral and acidic mucins, consistent with the previous reports in *S. aurata* [6], *L. crocea* [12], *S. nattereri* [13], *O. hepsetus* [22], *L. calcarifer* [23], *P. boro* [24], *B. belone* [34], and *T. brasiliensis* [35]. In contrast, the cytoplasm of apical squamous, basal, and non-mucous cells was negatively stained with PAS (Figure 4D), AB pH 2.5 (Figure 4E), and AB pH 1.0 (Figure 4F). Moreover, the esophagus exhibited a greater number of goblet and mucous cells than the oropharynx, consistent with the observations in other stomachless fishes, including *B. altianalis* [9], *X. maculatus*, and *X. hellerii* [11]. These phenomena indicated that these mucous and goblet cells were equivalent to the esophageal glands of higher vertebrates [12].

The esophagus and oropharynx of *H. macrolepidota* were lined with the stratified squamous epithelium, which protected the luminal epithelium from mechanical injury and abrasion [43]. In addition, the multilayered cells served as a defensive barrier against pathogens and parasites. Besides, taste buds were globular in the esophageal epithelium, consisting of columnar taste cells with oval nuclei, while the basal cells were located near the base of each bud (Figures 4C – 4D). The observations were comparable to those described in *B. altianalis* [9], *G. maculatum* [17], *T. brasiliensis* [35], and *Pristolepis fasciata* [44]. The results indicated that the esophagus of *H. macrolepidota* played a primary role in the food conduction and a secondary role in the taste reception. The taste buds functioned as chemoreceptors, enabling fish to select food and reject unsuitable items [45], reflecting the predatory behavior of *H. macrolepidota*. However, in some fish, such as *L. crocea* [12], their taste buds were absent. Furthermore, esophageal glands were absent in *H. macrolepidota*, unlike in *S. testudineus* [2] and *O. hepsetus* [22]. However, the esophageal glands of *S. testudineus* were globular unicellular mucous glands rather than multicellular ones. Thus, the unicellular glands of *S. testudineus* might be functionally similar to the mucous cells of *H. macrolepidota*. The absence of esophageal glands was a common characteristic observed in teleosts. The glycoconjugates secreted by mucous and goblet cells might compensate for the absence of salivary and esophageal glands and perform functions equivalent to those of mammalian salivary glands [46, 47]. Other studies have suggested that neutral mucins produced by these cells might aid in food digestion within the esophagus [22]. Although the digestive role of the esophagus remained unclear, the present study observed the secretion of acidic mucins, which could create a favorable environment for digestion, consistent with the studies of Ghosh and Chakrabarti [27] and de Oliveira Ribeiro and Fanta [35]. Furthermore, the mucins might play an important role in lubricating the esophageal mucosa during the passage of food from the oropharynx to the anterior intestine [35, 48] and in protecting the epithelium [11]. In addition, the mucins protected the mucosa from mechanical injury (solid food) and harmful microorganisms [2, 49]. Beneath the epithelium, the lamina propria consisted of dense connective tissue without a muscularis mucosae; consequently, the boundary between the lamina propria and submucosa was indistinct (Figure 4A).

This feature was reported in several fish species, including *L. crocea* [12], *O. hepsetus* [22], *T. brasiliensis* [35], and *S. porcus* [42], indicating that it represented a common histological characteristic among fish [50]. The dense connective tissue likely strengthened, expanded, and supported the esophagus, reflecting *H. macrolepidota*'s carnivorous habit, and similar findings were reported in *S. testudineus* [2] and *S. porcus* [42]. Deeper within the esophageal wall, beneath the submucosa, two sublayers of muscle were observed in the esophagus of *H. macrolepidota*: an inner longitudinal and an outer circular layer (Figures 4A - 4B). The mean muscular thickness was 809.36 ± 29.42 µm (Table 2). These two arrangements of skeletal muscle are similar to those described in *S. aurata* [6], *L. crocea* [12], *O. hepsetus* [22], and *P. boro* [24]. However, this appearance differed from *B. belone* [34], which exhibited two longitudinal skeletal muscle layers separated by thin connective tissue, as well as from *L. calcarifer* [23] and *H. forskahlii* [25], which showed an inner circular and outer longitudinal arrangement. In contrast, species such as *P. forsskali* [14] and *Lepidorhombus whiffiagonis* [51] possessed only a single longitudinal muscle layer. The presence of skeletal muscle in the esophagus indicated that *H. macrolepidota* could reject unwanted food or prey and assist in swallowing. Although the oropharynx and esophagus were relatively short, the longitudinal folds and skeletal muscle in these regions suggested that the lumen could expand and distend during swallowing [52]. Externally, the serosa was composed of connective tissue, nerve fibers, blood vessels, and a mesothelium.

Table 2. Histometric measurements (mean ± standard deviation, µm) of epithelial layer thickness, mucosal fold width, mucosal fold height, and muscular layer thickness of the oropharynx and esophagus in *H. macrolepidota* (n = 10).

Histometric parameter	Oropharynx (µm)	Esophagus (µm)
epithelial layer thickness	679.06 ± 137.79	71.47 ± 6.36
mucosal fold width	2,613.22 ± 93.04	409.39 ± 59.99
mucosal fold height	2,899.77 ± 29.69	609.09 ± 96.41
muscular layer thickness	1,300.34 ± 197.86	809.36 ± 29.42

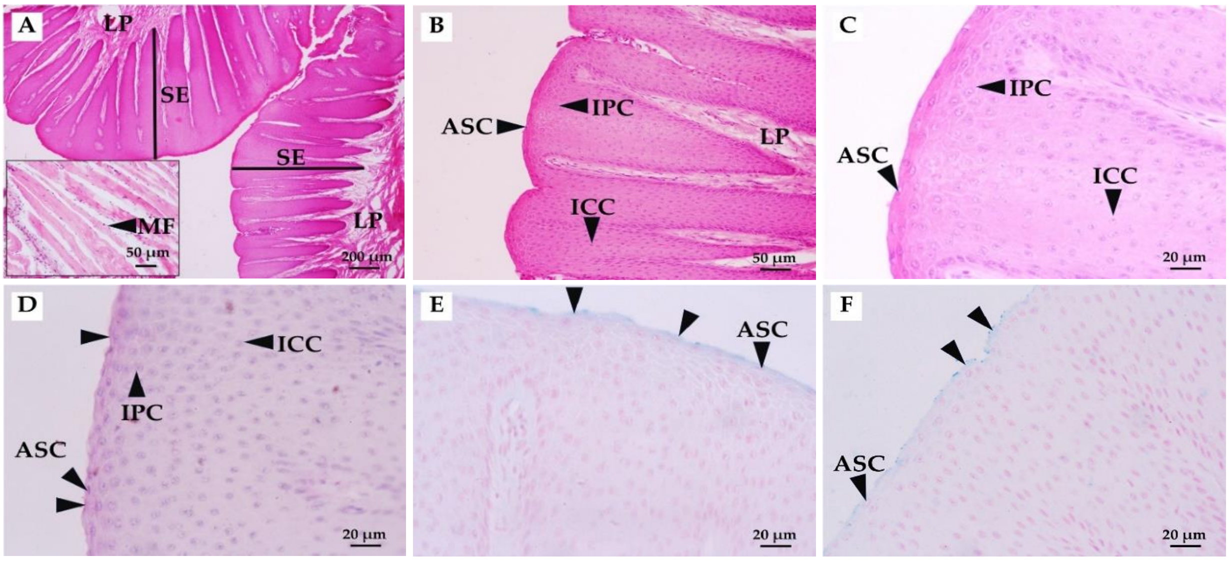

Figure 2. (2A) – (2F) The oropharynx of *H. macrolepidota*. (2A) The mucosal layers, including stratified epithelium (SE) of the mucosal fold, lamina propria (LP), and the box highlighting the muscular layer, consisting of muscular fiber (MF). (2B - 2C). The enlarged image of stratified epithelium of the oropharynx depicts the apical squamous cell (ASC), the intermediate polygonal cell (IPC), and the intermediate cuboidal cell (ICC). (2D - 2F) The apical squamous cell of the oropharynx contains neutral mucins, carboxylated and sulfated acid mucins, respectively. The arrowheads display the positive staining with the respective stains. (2A – 2C, and the box in 2A: H&E), (2D: PAS), (2E: AB pH 2.5), (2F: AB pH 1.0).

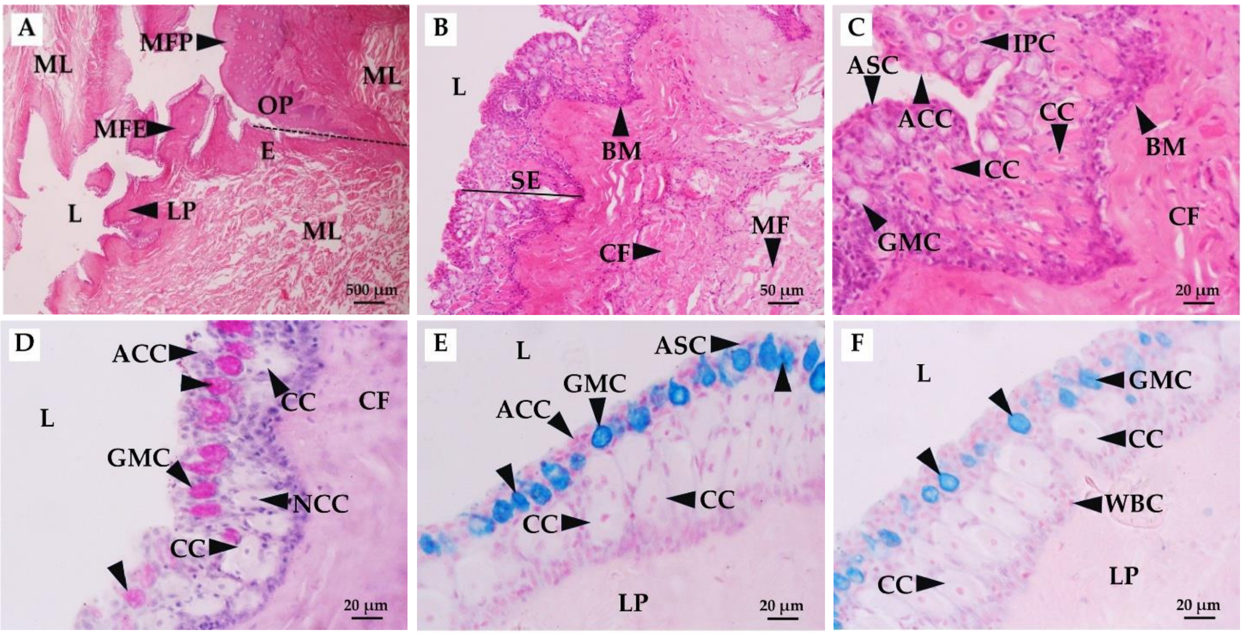

Figure 3. (3A) – (3F) The junction between the oropharynx (OP) and cranial part of esophagus (E) of *H. macrolepidota*. (3A) The dotted line shows the junction between the oropharynx and cranial region of the esophagus and distinct layers, including mucosa and muscular layer (ML). (3B) The stratified epithelium (SE) is located upon the basement membrane (BM) of the mucosal fold. (3C) The epithelium of the cranial region of the esophagus consists of apical squamous cells (ASC), apical cuboidal cells (ACC), intermediate polygonal cells (IPC), globular mucous cells (GMC), and club cells (CC). (2D - 2F) The arrowheads depict the globular mucous cell of the cranial part of the esophagus, containing neutral mucins, carboxylated and sulfated acid mucins, respectively. In contrast, the apical squamous and apical cuboidal cells exhibit negative staining. Collagen fiber (CF), lumen (L), mucosal fold of esophagus (MFE), mucosal fold of oropharynx (MFP), nucleus of club cell (NCC), lamina propria (LP), muscle fiber (MF), white blood cell (WBC). (3A – 3C: H&E), (3D: PAS), (3E: AB pH 2.5), (3F: AB pH 1.0).

3.2.3 Intestine

The transition from the esophagus to the intestine was marked by a muscular sphincter that functioned as a protective barrier against food reflux and contributed to the regulation of food transport. Furthermore, the epithelium changed from stratified squamous in the esophagus to simple columnar in the intestine. The intestine of *H. macrolepidota* could be distinguished into three portions: anterior, middle, and posterior, based on histological characteristics and histometric analyses (Figures 5A – 5L). This three-portion division was according to other fish species such as *S. aurata* [6], *B. altianalis* [9], *L. crocea* [12], *L. calcarifer* [23], *P. boro* [24], *H. forskahlii* [25], and *B. belone* [34], in contrast to those of *X. maculatus*, *X. hellerii* [11], and *O. hepsetus* [22], in which the intestine was subdivided into two portions, anterior and posterior portions. The intestinal wall of all portions comprises four layers: mucosa, submucosa, muscularis, and serosa (Figures 5A, 5F). All portions were filled with villi and lined with simple columnar epithelium (Figures 5A – 5L), corresponding to the works in *P. stoliczkanus* [8], *B. altianalis* [9], *X. maculatus*, *X. hellerii* [11], *O. hepsetus* [22], *P. boro* [24], and *S. porcus* [42]. In addition, the histomorphology of *H. macrolepidota* resembled other carnivorous fish with various features, including blood and lymph vessels in the lamina propria, loose connective tissue in the submucosa, absence of glands and muscularis mucosae, and two muscle sub-layers (inner circular and outer longitudinal layers) (Figures 5A – 5L) [2, 12, 16, 34, 35]. The anterior intestine was lined by simple columnar epithelium, composed of tall cells arranged in a single layer, with a mean epithelial thickness of 46.36 ± 8.27 µm (Table 3). Two cell types were found: enterocytes and goblet cells, among which the enterocytes showed centrally located oval nuclei with apical microvilli (brush border) (Figures 5B – 5E). The goblet cells lacked brush borders, with basally located nuclei and cytoplasm containing clear mucins on H&E staining (Figure 2B), consistent with studies in *X. maculatus* and *X. hellerii* [11]. In some regions, intraepithelial leukocytes with basophilic, round nuclei were observed near the basement membrane (Figures 5B–5D). The mucosa formed thin folds (villi) with a lamina propria core, some of which branched into secondary folds and showed a slender, sharp-tipped appearance (Figure 5A). The mean villi width and height were 146.20 ± 24.80 μm and 866.40 ± 10.86 μm, respectively (Table 3). Enterocytes were weakly PAS-positive, especially at the base of the brush border (Figure 5C), but were negatively stained with AB at pH 2.5 (Figure 5D) and pH 1.0 (Figure 5E). The cytoplasm of goblet cells was positively stained with PAS, showing magenta (Figure 5C), and with AB at pH 2.5 (Figure 5D) and pH 1.0 (Figure 5E), appearing blue. The lamina propria forming the villus core contained collagen fibers, blood and lymph vessels, and fibroblasts, with more blood vessels near the villus tips (Figures 5B – 5E). Because the muscularis mucosae was absent, the boundary between the lamina propria and submucosa was indistinct. The muscularis consisted of two smooth muscle layers: an inner circular and an outer longitudinal (Figure 5A), with a mean thickness of 202.99 ± 39.15 μm (Table 3). The myenteric nerve plexus was observed between these layers (Figure 5B), corresponding to the observations in *B. altianalis* [9] and *O. hepsetus* [22]. The outermost serosa was lined by mesothelium and contained blood vessels that were embedded in the connective tissue (Figure 5A).

While the middle intestine displayed histological features similar to those of the anterior intestine, except that the epithelium became invaginated, forming crypt-like glands (Figure 5F). This appearance was consistent with that described in other carnivorous fish such as *T. bogotensis* [16]. Ortiz-Ruiz et al. [16], which identified these structures as Lieberkühn’s crypts. The mean epithelial thickness of the crypts was 52.22 ± 3.83 μm (Table 3). The epithelium comprised enterocytes and goblet cells (Figures 5F - 5G), with the goblet cells staining positive for PAS (Figure 5G), AB pH 2.5 (Figure 5H), and AB pH 1.0 (Figure 5I). Compared with the anterior intestine, the middle intestine showed a greater number of goblet cells (Figures 5G – 5I). The mean villi width and height were 162.74 ± 14.27 μm and 885.28 ± 70.94 μm, respectively (Table 3). The submucosa, muscularis, and serosa were similar to those of the anterior intestine (Figure 5F). The mean muscular layer thickness was 241.81 ± 28.59 μm (Table 3). Besides, the posterior intestine displayed the histological and histochemical features comparable to those of the anterior and middle intestines (Figures 5J – 5L). The mean epithelial thickness was 37.85 ± 2.50 μm (Table 3), while the mean villi width and height were 87.08 ± 14.24 μm and 609.43 ± 64.14 μm, respectively (Table 3). The goblet cells interspersed among enterocytes positively stained with PAS (Figure 5J), AB pH 2.5 (Figure 5K), and AB pH 1.0 (Figure 5L), and were more numerous than those in the anterior portions (Figures 5J – 5L), with abundant lymphocyte aggregates in the submucosa (Figure 5J). The muscularis and serosa resembled those of the anterior and middle intestines; however, the muscularis of the posterior intestine was the thinnest among the three portions (Figure 5J). The mean muscular layer thickness was 160.39 ± 20.34 μm (Table 3).

Enterocytes in all regions exhibited apical microvilli, whereas the goblet cells were less frequent in the anterior intestine, corresponding to the reports in *X. maculatus*, *X. hellerii* [11], and *O. hepsetus* [22]. The numerous brush borders enhanced the surface area for absorption and interaction with the gut microbiota [49]. The findings suggested that the intestine was a site of enzymatic digestion, especially in the middle intestine, where crypt-like glands were observed. The goblet cells in the anterior, middle, and posterior intestines produced neutral mucins, as well as carboxylated and sulfated acid mucins, according to those observed in *B. altianalis* [9], *L. crocea* [12], *S. nattereri* [13], *O. hepsetus* [22], *L. calcarifer* [23], *P. boro* [24], *T. brasiliensis* [35], and *L. whiffiagonis* [51]. Based on the positive PAS and AB (pH 2.5 and 1.0) staining of globular mucous cells and their mucin secretion, the esophagus, anterior intestine, and middle intestine were considered sites of digestion. These characteristics were similar to those of other agastric fish such as *S. testudineus* [2] and *B. belone* [34]. Consistent with other studies, the goblet cells played a key role in absorption, transport, and the provision of enzymatic cofactors [53]. In addition, the neutral mucins released by the goblet cells of *H. macrolepidota* might also act as the cofactors in enzymatic digestion [12]. Therefore, various mucopolysaccharides may play a crucial role in digestive mechanisms. However, the exact mechanism of digestion in stomachless carnivorous fish has remained unclear [54]. Moreover, despite the evolutionary loss of the stomach, the digestion was not problematic in agastric herbivorous or carnivorous fish [34].

The highest mean epithelial thickness, villi height, and villi width were observed in the middle, anterior, and posterior intestines, respectively, along with the numerous goblet cells and complex crypts of the middle intestine, indicating that the middle intestine played the key roles in the main site for digestion, absorption, and possibly food storage. This observation was similar to that of stomachless fish such as *B. altianalis* [9] and was consistent with Canan et al. [55], who explained that nutrient absorption typically occurs in intestinal regions with complex mucosal structures. Meanwhile, *H. macrolepidota* fed on various invertebrates and fish; therefore, the goblet cells could protect the mucosa from damage caused by large prey, consistent with their secretion of multiple mucin types [53]. The acid mucins secreted by the goblet cells were thought to shield the intestinal mucosa from glycosidase degradation [56]. Furthermore, numerous goblet cells and the smallest mucosal folds were observed in the posterior intestine, consistent with the observations in *P. stoliczkanus* [8], *X. maculatus*, *X. hellerii* [11], *L. crocea* [12], *O. hepsetus* [22], *L. calcarifer* [23], *P. boro* [24], and *H. forskahlii* [25]. These results indicated that the posterior intestine functioned as the site for transporting undigested food. Consistently, the mucins produced by the goblet cells could protect the mucosa during fecal transport. Other studies have reported that acid mucopolysaccharides are important for defense against pathogenic bacteria, immune activation, and protein digestion, and have further noted that goblet cells influence the composition and activity of gut microorganisms [57]. The goblet cells secreted both neutral and acidic mucins, which contained diverse carbohydrates and could serve as a nutrient source for mucin-degrading bacteria [58]. Further studies are required to clarify the correlation between goblet cell number and intestinal function.

Moreover, all intestinal portions lacked muscularis mucosae and glands, consistent with *P. stoliczkanus* [8], *X. maculatus*, *X. hellerii* [11], *L. crocea* [12], and *L. calcarifer* [23]. The mucosa and submucosa were surrounded by two smooth muscle sublayers: the inner circular and the longitudinal layers, as reported in other carnivorous fish [2, 9, 11, 12, 22, 24, 25]. These two layers enhanced the contraction efficiency for mixing food with digestive enzymes and mucus, promoted propulsion, and likely facilitated nutrient absorption and peristalsis [11]. Although fish in the family Cyprinidae are generally regarded as herbivorous or omnivorous, trophic strategies within this family are highly variable and species-specific, ranging from herbivory and omnivory to carnivory depending on ecological niche, prey availability, and morphological adaptations. Accordingly, family-level classification may not accurately reflect the feeding ecology of individual species. In this context, available ecological and dietary evidence suggests that *H. macrolepidota* is more appropriately classified as a carnivorous species despite its placement within a predominantly omnivorous family. The IC of *H. macrolepidota* in the present study was 0.69 (Table 1), which is lower than 1.0 [16] and falls within the range of 0.2–2.5 reported for carnivorous fishes [12], indicating a relatively short intestine adapted for a protein-rich diet. This interpretation is consistent with previous stomach content analyses [29], which reported that *H. macrolepidota* primarily consumes animal prey, including microinvertebrates and small animals such as fish, shrimp, crabs, mollusks, and aquatic insects. In addition, histological characteristics observed in this study, such as the presence of skeletal muscle in the esophagus and diverse mucin secretions along the esophagus and intestines, particularly in the middle intestine, further support the classification of *H. macrolepidota* as a carnivorous species. Furthermore, the IC of *H. macrolepidota* fell within the reported range for carnivorous fish similar to *T. bogotensis* (IC = 0.59) [16], *P. blochii* (IC = 0.93) [37], and *Salminus affinis* (IC = 0.58) [59].

Table 3. Histometric measurements (mean ± standard deviation, µm) of epithelial layer thickness, villi width, villi height, and muscular layer thickness in the anterior, middle, and posterior intestines of *H. macrolepidota* (n = 10). Different superscript letters indicate significant differences among intestinal regions ($p < 0.05$, Duncan's test).

Histometric parameter	Anterior intestine (µm)	Middle intestine (µm)	Posterior intestine (µm)
epithelial layer thickness	46.36 ± 8.27^{b}	52.22 ± 3.83^{a}	37.85 ± 2.50^{c}
villi width	146.20 ± 24.80^{b}	162.74 ± 14.27^{a}	87.08 ± 14.24^{c}
villi height	866.40 ± 10.86^{b}	885.28 ± 70.94^{a}	609.43 ± 64.14^{c}
muscular layer thickness	202.99 ± 39.15^{b}	241.81 ± 28.59^{a}	160.39 ± 20.34^{c}

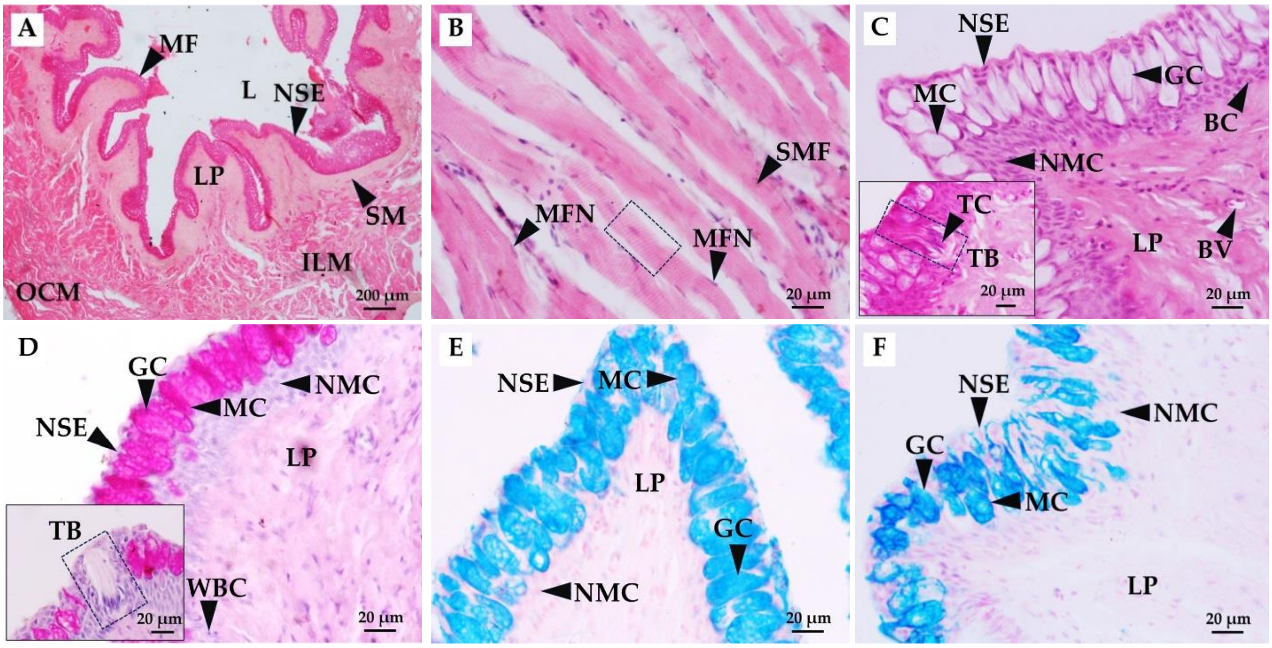

Figure 4. (4A) – (4F) The esophagus of *H. macrolepidota*. (4A): the distinct layers, including non-keratinized stratified epithelium (NSE) of the mucosal fold (MF), lamina propria (LP), submucosa (SM), and muscular layer, where the muscular layer contains two sub-layers of skeletal muscle, namely inner longitudinal muscle (ILM) and outer circular muscle (OCM). (4B) The enlarged picture of the skeletal muscular layer exhibiting the skeletal muscular fiber (SMF), muscular fiber nuclei (MFN). The square-dotted line frame displays the striation of the muscle cell. (4C): the enlarged picture of epithelium, depicting the non-keratinized stratified epithelium cell (NSE), goblet cell (GC), mucous cell (MC), non-mucous cell (NMC), and basal cell (BC). The box in 4C highlights the taste bud (TB), which is embedded in the epithelium and consists of taste cells (TC). (2D - 2F) The goblet cell and mucous cell, containing neutral mucins, carboxylated and sulfated acid mucins, respectively. In contrast, the non-mucous and club cells exhibit negative staining. The box in 4D highlights negative PAS staining of the taste bud. Blood vessel (BV), lumen (L), white blood cell (WBC). (4A – 4C, and the box in 4C: H&E), (4D and the box in 4D: PAS), (4E: AB pH 2.5), (4F: AB pH 1.0).

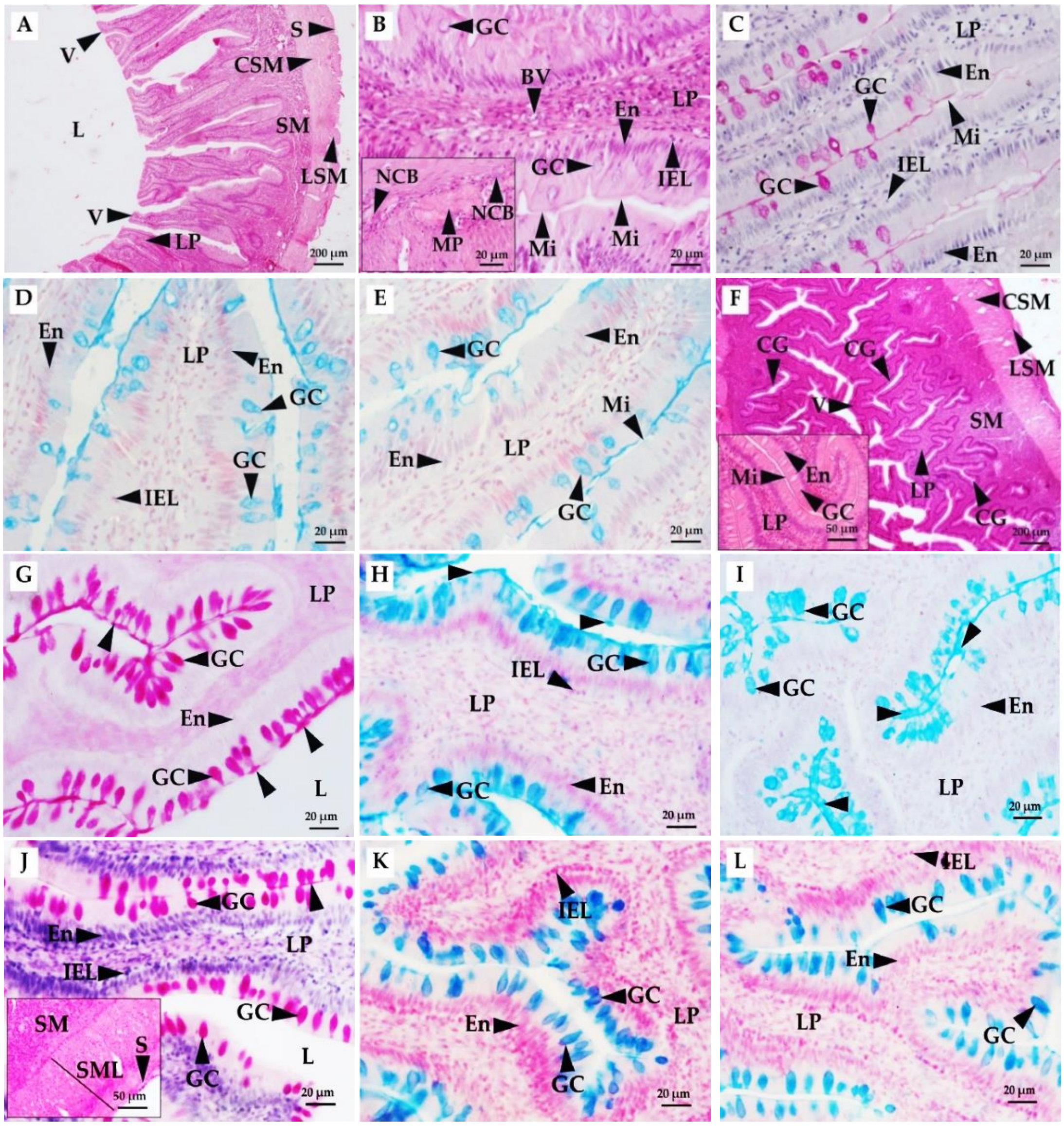

Figure 5. (5A) - (5E) The anterior intestine. (5F) - (5I) The middle intestine. (5J) - (5L) The posterior intestine of *H. macrolepidota*. (5A) The distinct layers of intestine, consisting of mucosa formed by villi (V), submucosa (SM), muscular layer, and serosa (S). The muscular layer is subdivided into two layers: circular smooth muscle (CSM) and longitudinal smooth muscle (LSM). (5B) The villi of the anterior intestine, comprising enterocyte (En), goblet cell (GC), and intraepithelial leukocyte (IEL). The blood vessel (BV) is located in the lamina propria (LP). The box in 5B highlights the myenteric plexus (MP), comprising nerve cell bodies (NCB). (5C) The base of microvilli (Mi) of the enterocyte and goblet cell of the anterior intestine is PAS-positive. (5D) and (5E) AB pH 2.5 and AB pH 1.0 positive goblet cells, respectively. (5F) The distinct layers and villi of the middle intestine, and the box illustrating the enlarged view of the villi. The villi form the crypt-like glands (CG), which display invagination of structures. (5G) The base of microvilli of the enterocyte and goblet cell of the middle intestine with PAS-positive staining. (5H) - (5I) AB pH 2.5 and AB pH 1.0-positive goblet cells, respectively. (5J) The villi of the posterior intestine and the box illustrating the enlarged view of the submucosa, muscular layer, and serosa. The goblet cells are PAS-positively stained. (5K) and (5L) AB pH 2.5 and AB pH 1.0 positive goblet cells, respectively. Lumen (L), smooth muscle layer (SML). The arrowheads demonstrate positive staining of secretion. (5A, 5B, 5F and the box in 5B, 5F and 5J: H&E), (5C, 5G, 5J: PAS), (5D, 5H, 5K: AB pH 2.5), (5E, 5I, 5L: AB pH 1.0).

4. Conclusions

This study provided the first report on the histology and histochemistry of the digestive tract of *H. macrolepidota*. The digestive tract consisted of the oropharynx, esophagus, and intestine, while the stomach was absent, indicating that *H. macrolepidota* was an agastric fish. The oropharyngeal and esophageal epithelia were lined with non-keratinized stratified squamous/cuboidal epithelium, differing in the number of epithelial cell layers. The oropharyngeal epithelial cells were weakly positive-stained for neutral mucopolysaccharides, carboxylated and sulfated acid mucopolysaccharides, whereas the esophageal epithelial cells, especially mucous cells and goblet cells, were strongly positive-stained with all stains. The club cells were observed at the transition junction between the oropharynx and the esophagus, whereas the taste buds were present in the esophagus. In addition, of those portions, the mucosa was surrounded by skeletal muscle. The intestine was divided into three portions, with the mucosa in all regions lined by the simple columnar epithelium containing enterocytes and goblet cells. Enterocytes were PAS-positive and exhibited a brush border, while the goblet cells were positively stained for neutral mucopolysaccharides, carboxylated, and sulfated acid mucopolysaccharides. Comparison among three portions of the intestine showed that the highest values of epithelial layer thickness, villi height and width, and muscular layer thickness were observed in the middle, anterior, and posterior intestines, respectively. Whereas, the mucosa of the middle intestine formed crypt-like glands. Therefore, based on histological and histochemical observations and histometric measurements, although *H. macrolepidota* lacked a stomach, the esophagus and the anterior and middle intestines were identified as the primary sites of digestion, with digestion and nutrient absorption occurring mainly in the middle intestine. Furthermore, the IC value supported a carnivorous feeding habit in *H. macrolepidota*, consistent with the histological and histochemical characteristics and with previous studies on feeding habits and gut contents. The findings enhanced the understanding of the anatomy, microanatomy, and histochemistry of *H. macrolepidota* in relation to its physiology and feeding habits. Future studies may employ additional techniques, such as electron microscopy and immunohistochemistry, to provide deeper insights.

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