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Full paper

Comprehensive morpho-histological observation of digestive system and gut content of wild-grunting toadfish, *Allenbatrachus grunniens* (Linnaeus, 1758)

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Abstract: The morphology, histology and histochemistry of the digestive system and gut content of the wild-grunting toadfish (*Allenbatrachus grunniens*) from the estuary of Pranburi River, Thailand, is investigated. The digestive system of *A. grunniens* is elongated from the oral cavity to the intestine except for the stomach of a sac-shaped type. The length of the elongated intestine has the intestine coefficient (IC) of 0.52 ± 0.1. The digestive tract is composed of four layers: mucosa, submucosa, muscularis and serosa. The longitudinal folds of the esophagus are lined by the stratified mucosal epithelium which contains several large secretory cells. The gastric rugae in both the cardiac and pyloric regions of the stomach are lined with a simple columnar epithelium. The intestine has numerous intestinal folds lined by simple columnar epithelia. Intestinal goblet cells are organised and inserted among the epithelia. The index of relative importance (IRI) suggests that the main food items are a crab group (73.64%) followed by shrimps (10.43%), fishes (8.02%) and other compositions (7.92%). The IC, morpho-histological features of the digestive system and IRI indicate that *A. grunniens* is a carnivorous fish.

Keywords: digestive tract, microanatomy, toadfish, *Allenbatrachus grunniens*, Thailand

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

INTRODUCTION

Allenbatrachus grunniens (Batrachoidiformes, Batrachoididae) has a high economic value as a food source. This wild-grunting toadfish is generally found in brackish waters of river estuaries such as northern Australia through New Guinea and the Indo-Australian Archipelago to the Gulf of Thailand [1]. Despite accumulated information about its taxonomy and distribution [1], the biological characteristics, especially histological features of its digestive system in relation to the feeding habits, are still lacking. The understanding of the digestive morphology and histology of the fish not only improves fundamental knowledge of the digestive system but also provides information of its feeding habits, leading to the development of an aquaculture programme and successful management of this fish species [2, 3].

Previous reports on toadfish have determined the microscopic structure of the digestive tract of *Halobatrachus didactylus* focusing on limited aspects such as oesophagus [4] and on glycohistochemical study of its stomach [5]. A comprehensive observation of the digestive system has not been reported for toadfish species. In order to elucidate the feeding habit of this species, the morphology, histological characteristics and histochemistry of the digestive system of *A. grunniens*, along with the gut content analysis, are investigated in this study.

MATERIALS AND METHODS

Study Site and Fish Collection

Twenty specimens of *A. grunniens* (Figure 1A) with the total length of 18.20 ± 2.67 cm (mean ± SD) and body weight of 128.25 ± 47.55 g (mean ± SD) were captured by beach seine net (mesh size 1 cm) from the estuary of Pranburi River, Thailand (N 12°24'08.5"/E 099°59'00.2") in May and September 2017. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723004).

Collection of Digestive System and Morphometric Analysis of Intestine Length

After dissection of fat and connective tissues, the complete digestive system (digestive tract together with liver, gill, gallbladder and pancreas) was quickly removed and fixed in Davidson's fixative for about 36 hr at room temperature followed by perseveration in 70% ethanol to observe the gross anatomy and histology. Additionally, we used the intestinal coefficient (IC), which is the ratio of intestinal length (L_{IT}) to body length (L_S) given by the equation $IC = L_{IT}/LS$, for evaluating the feeding behaviour of *A. grunniens*.

Morphology of Internal Surface and Histological and Histochemical Analysis

The digestive tracts were specifically dissected (from cardiac stomach to posterior intestine) and the gut content was collected. Subsequently, all organs of the digestive system were subjected to conventional histological procedures [6, 7]. The sections were cut (4-μm thickness) and stained with Delafield's hematoxylin and eosin. Some sections were histochemically treated with Masson's Trichrome, periodic acid schiff and Alcian Blue (pH 2.5) to test the smooth muscle and connective tissue and detect glycoprotein and mucopolysaccharide respectively [6, 7]. To reveal the digestive histology, all slides were photographed using a Leica 2000 light microscope (Boston Industries Inc., USA).

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

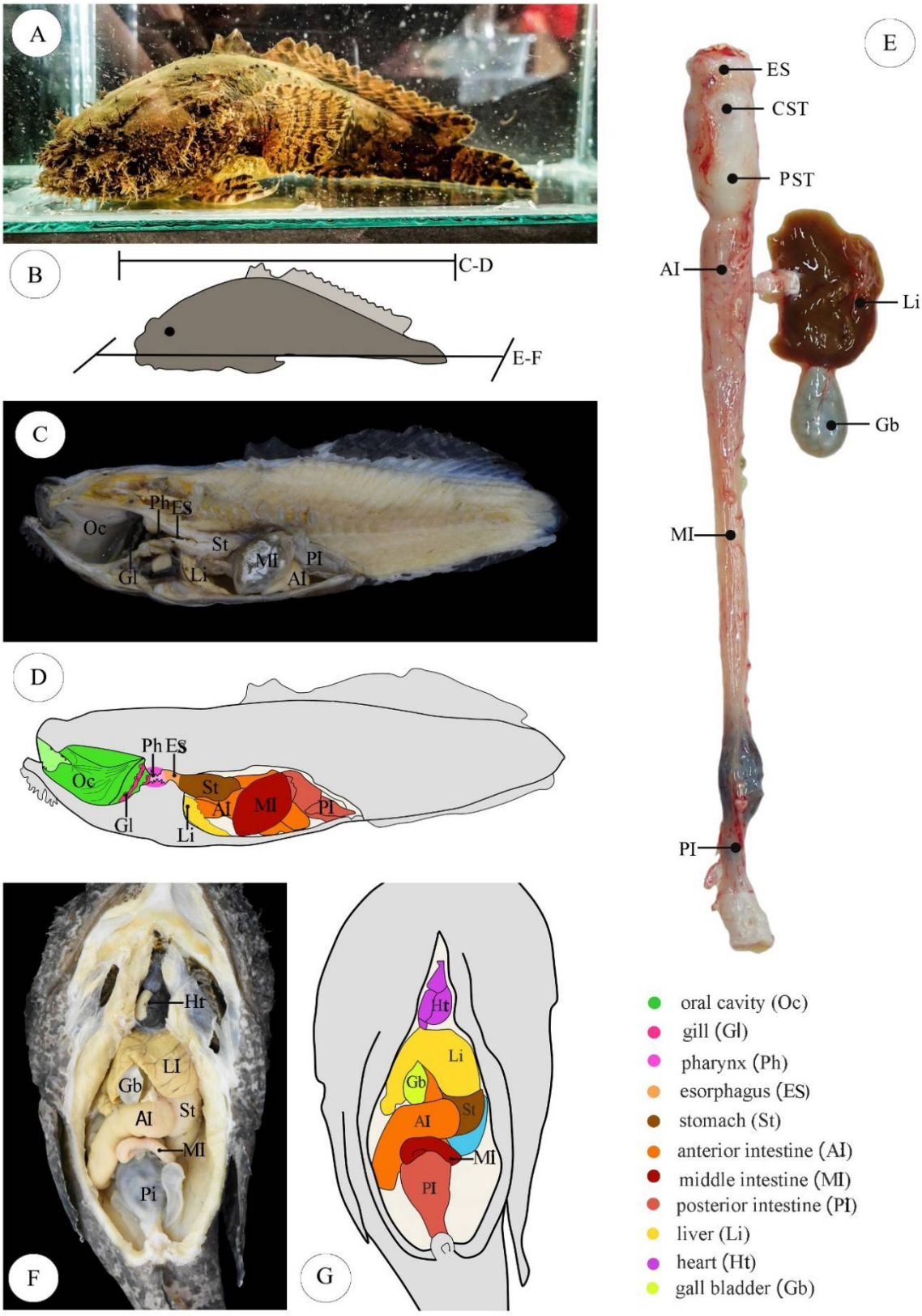


Figure 1. Gross morphology of digestive system of *A. grunniens*: (A) live *A. grunniens*; (B) locations of sections; (C) longitudinal section; (D) schematic diagram of C with several digestive organs; (E) dissected digestive system composed of oesophagus (ES), cardiac stomach (CST), pyloric stomach (PST), anterior intestine (AI), middle intestine (MI) and posterior intestine (PI) connected with liver (Li) and gall bladder (Gb); (F) sagittal section of fixed specimen; (G) schematic diagram of F. Abbreviation: GI = digestive

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

Quantification and Statistical Analyses

All measurements were performed using program LAS version 4.9. The longitudinal fold of the digestive tract from the histological sections was measured and recorded as the mean and standard deviation (SD). Additionally, the number of goblet cells was counted under the light microscope from 12 randomised villi areas (each with an area of 200 μm^2) from each intestinal region. The mean number of goblet cells of each intestine region was computed for significant difference among 3 regions using one-way ANOVA followed by Tukey-Kramer test. These statistics were calculated using the Statistical Package for the Social Sciences software (version 15.0).

Gut Content Analysis

The food in the digestive tract collected as mentioned above was identified following the guidelines on details of the lowest possible taxonomic category [8, 9]. The important mass of each consumed prey category was determined by calculating the index of relative importance (IRI) [10]. The calculation was performed by using number (N), volume (V) and frequency of occurrence (O) : IRI = %O × (%N + %V) with a slight modification in which cover area (C) was used for calculation instead of V. Thus, the final formula to express the gut content in this study is represented as IRI = %O × (%N + %C).

RESULTS AND DISCUSSION

Gross Morphology of the Digestive System

Integrated data from the longitudinal and sagittal sections clearly show the morphology of the digestive system of *A. grunniens*, which consists of the digestive tract (oral cavity, pharynx, oesophagus, stomach and intestines) (Figures 1B-1G) and accessory organs (liver and gall bladder) (Figure 1E). More specifically, the simple digestive tract appears to be a narrow tube except for the sac-shaped stomach (Figure 1E). The pyloric caecum is not observed in the gastrointestinal junction. The absence of the pyloric caecum has been associated with carnivorous feeding habit, but Faccioli et al. [11] proposed that the presence of pyloric caeca is related to phylogeny rather than feeding habit. The presence of pyloric caecum in piranha and the absence of it in walking catfish support this idea [12].

The length of the elongated intestine has the IC of 0.52 ± 0.10 (mean ± SD) (Table 1). The IC value of *A. grunniens* determined in this study is consistent with prior observations of other carnivorous fishes such as *Sparus aurata* (IC = 0.5-0.6) [13] and *Glyptosternum maculatum* (IC = 0.8) [14]. On the contrary, IC values of herbivorous fish species commonly range from 2.0 to 21.0 [15], suggesting that *A. grunniens* is a carnivorous fish. Additionally, the intestine of *A. grunniens* is divided into three regions: anterior, middle and posterior regions (Figure 1E). The liver has a dark brown reddish colour and is structurally elongated at the dorsal regions (Figures 1E-1G). The gall bladder is dark green and located at the anterior of the most posterior liver (Figure 1E).

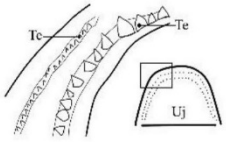
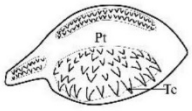
Morphological, Histological and Histochemical Observations

Mouth and oral cavity

The mouth of this toadfish has a terminal mouth position (Figure 2A) and its oral cavity is composed of lip, teeth, tongue and gill (Figures 2B-2C). The lip is lined with stratified epithelium on the connective tissue (Figure 2D). The teeth are the canine type with a sharp morphology, which

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

Table 1. Schematic diagram and morphological summary of *Allenbatrachus grunniens* collected from Pranburi River estuary on May and September 2017

	Schematic diagram		Morphometric analysis
A		Terminal mount	Esophagus (0.59±0.13 cm)
B		Two rows of teeth showing on the upper jaw	Stomach (1.94±0.52 cm)
C		Teeth embedding in pharyngeal teeth	Intestine (9.38±1.75 cm)
D		Gill raker	Intestinal coefficient 0.52
E		Sac-like shape of stomach	

is also macroscopically and histologically confirmed (Figures 2C, 2E). The teeth are found in both maxillary (upper jaw, data not shown) and mandible (lower jaw) (Figure 2C), being arranged in two rows. The developing teeth appear where the genioglossus muscle appears in the primary tongue (Figure 2C). The teeth are divided into two stages—mature and immature teeth—based on the position and histological features (Figure 2E). Mature teeth are elongated bi-directionally towards the oral cavity (Figure 2E) and are composed of the acellular dentine layer with the pulp cavity (Figure 2F). The pulp cavity is lined with odontoblast-like cells (Figure 2F). Immature teeth are observed under the oral epithelium (Figure 2E). During the maturation process, the enamel organ develops into a cup-like structure, in which the enamel epithelia produce a single-cell layer (Figure 2G). This layer is also separated into outer and inner enamel epithelia.

The dental papilla is considered to differentiate into the pulp cavity and the single-cell layer of odontoblasts (Figure 2H). Several longitudinal folds of the mucosal layer are observed in the oral cavity (Figure 2I). Under high magnification, the epithelium in the oral cavity region is lined by stratified polygonal squamous epithelium (Figures 2J-2L). Mucous cells and taste buds are prismatic and located only in the posterior region of the oral epithelium (Figures 2J-2L). Although mucous cells are observed as just an empty structure in the Masson's Trichrome stain method, these cells react positively to periodic acid-Schiff stain and alcian blue stain methods (Figures 2K-2L). These reactions indicate the presence of glycoprotein and mucopolysaccharide respectively, as reported in the oral cavity of *Trachelyopterus striatulus* [16]. The chemical secretions may be concerned with a protection against bacterial invasion [17, 18].

The gill is not a part of the digestive system but the gill structure is also described because of its importance in fish physiology including digestion. The morphological structure of the gill can be divided into three distinct regions, i.e. gill filament, gill racker and gill ach (Figure 3A). The gill filament is histologically composed of two sub-regions, viz. primary and secondary lamellae

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

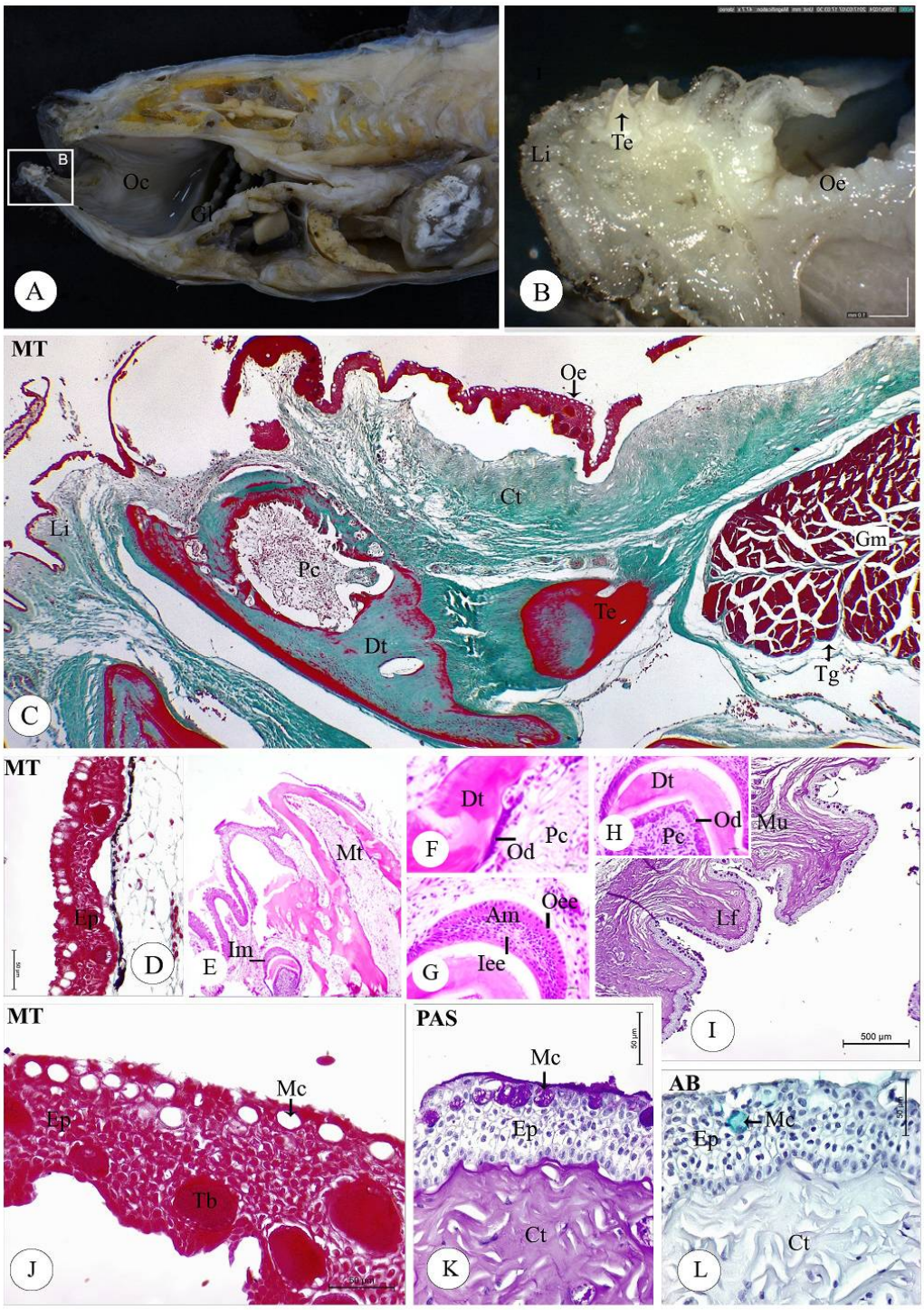


Figure 2. Mouth morphology of *A. grunniens*: (A-B) oral cavity (Oc), lip (Li) and teeth (Te); (C-D) light microphotographs of mandibular region containing tongue (Tg), mature teeth (Te) and stratified epithelium (Ep); (E-H) structure of mature teeth and immature teeth; (F) light microphotograph of longitudinal fold (Lf) in oral cavity; (G-I) epithelium (ep) of oral cavity containing various types of cells including mucous cells (Mc) and taste buds (Tb).

Abbreviations: Am=ameloblast, Ct=connective tissues, Dt=dentin, Gl=gill, Gm=genioglossus muscle, Iam=inner enamel epithelium, Mu=mucosa, Od=odentoblast, Oe=oral epithelium, Oee= outer enamel epithelium, Pc=pulp cavity.

Staining methods: AB=alcian blue stain (I); MT=Masson's Trichrome stain; PAS=periodic acid-Schiff stain.

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

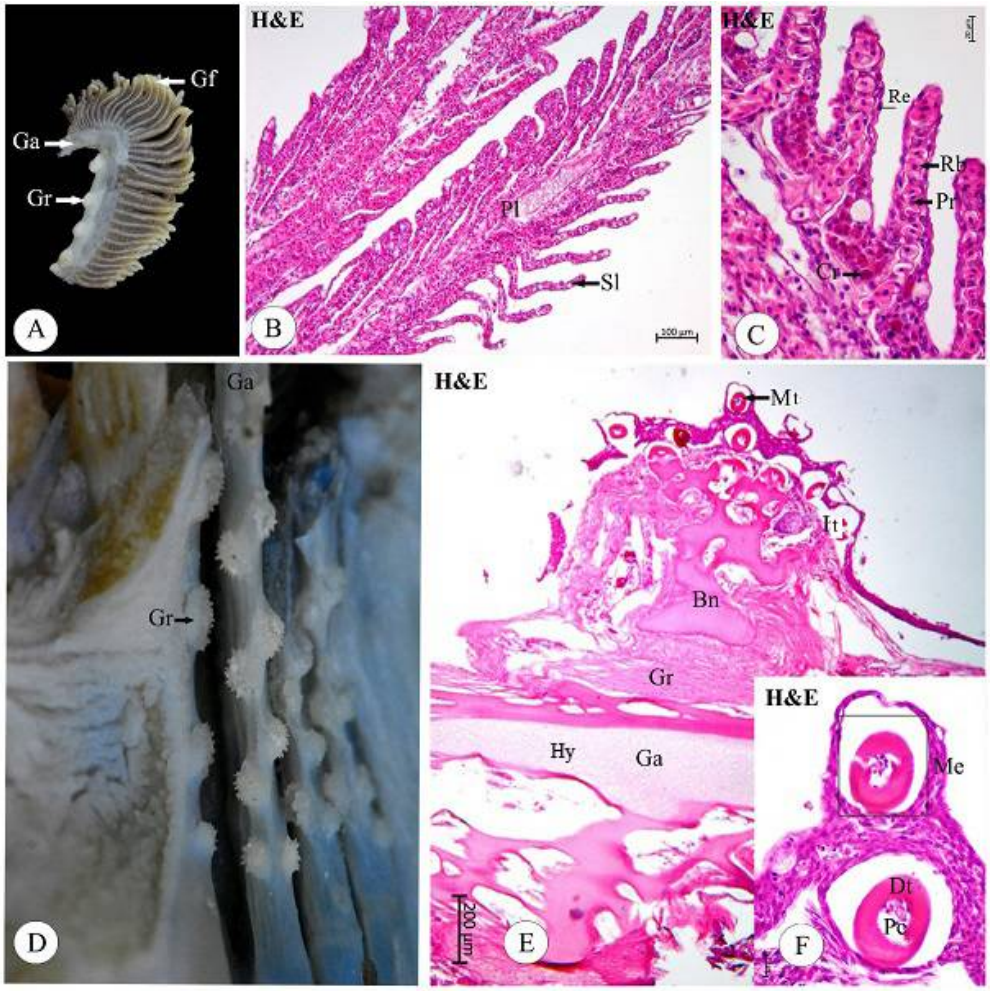


Figure 3. Structure of gill in *A. grunniens*: (A) gill consisting of gill filament (Gf), gill arch (Ga) and gill raker (Gr); (B) primary lamella (PI) and secondary lamella (SI); (C) secondary lamella containing different types of cells including respiratory cell (Rc), pillar cell (Pc) and chloride cell (Cr); (D-E) morphology (D) and histology (E) of gill raker (Gr) containing mature teeth (Mt) and immature teeth (It)

Abbreviations: Bn = bone, Dt = dentin, Hy = hyaline cartilage, Pc = pulp cavity.

Staining method: H &E = hematoxylin and eosin stain

(Figure 3B). The secondary lamellae are oriented perpendicular to the primary lamellae (Figure 3B) and each secondary lamella is covered by a respiratory epithelium and narrow supporting pillar cells (Figure 3C). Mucous cells and chloride cells are largely concentrated along the bases of the secondary lamella. The chloride cell is a group of cells having an eosinophilic cytoplasm (Figure 3C). The roles of the chloride cell are inward pumping of ions such as Ca^{2+} and Cl^{-} for osmoregulation [16]. Gill rakers are short with a broad base and have 6-8 pointed structures projecting in different directions (Figure 3D). This structure has also been reported in other carnivorous fishes [2, 19]. The gill raker is lined by stratified epithelium and several teeth (Figure 3E), especially mature teeth (Figure 3F), supported by loose connective tissue and bone structure (Figure 3E). The teeth are believed to have important roles in scraping food and preventing regurgitation [19]. The histological study of the gill arch shows that it principally contains a layer of hyaline cartilage (Figure 3E).

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

At the posterior side of the oral cavity, the pharyngeal teeth are observed exclusively in the pharynx region (Figure 4A) together with the pharyngeal villiform dental plate (Figure 4B). This plate is separated into two areas by the connective tissue (Figure 3B). These teeth structures are generally related to capturing and guiding the food as suggested by Rodrigues et al. [20], who studied pharyngeal teeth in *Leporinus macrocephalus*. The pharyngeal villiform dental plate contains both immature and mature teeth (Figures 4C-4E), as also reported in several fish species [18-21] such as *Conorhynchos conirostris* [2] and *Sorubim trigonocephalus* [21]. The pharyngeal teeth are similarly classified into two stages—maturely and immaturely developed teeth—based on position and histological features (Figures 4C-4E)], as observed in the teeth of the oral cavity.

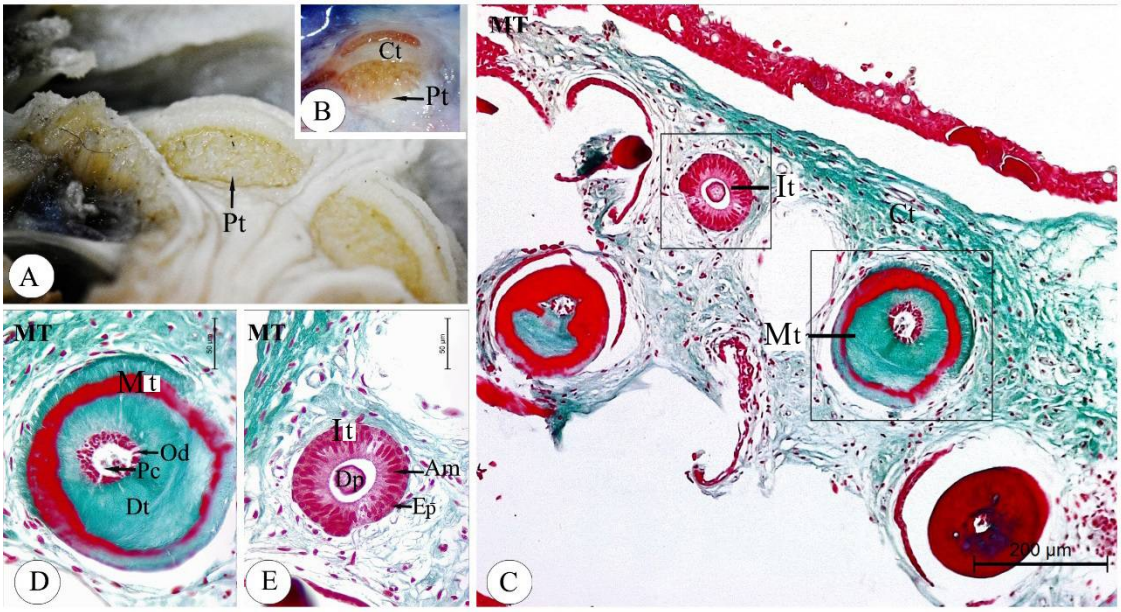


Figure 4. Structure of pharyngeal teeth of *A. grunniens*: (A-B) morphology of pharyngeal teeth (Pt) separated by connective tissue (Ct); (C) pharyngeal teeth containing mature teeth (Mt) and immature teeth (It) in connective tissue (Ct) shown as greenish colour; (D) mature teeth (Mt) with some characteristic features such as dentin (Dt), pulp cavity and odontoblast (Od); (E) immature teeth (Ic) showing dentin papilla (Dp) and ameloblast (am) lined by epithelium (Ep)
Staining method: MT = Masson's Trichrome stain

Oesophagus

Schematic diagrams and morpho-histological images of the oesophagus are shown in Figure 5A and Figures 5B-5D respectively. Variable longitudinal folds of the tunica mucosa of the oesophagus are observed under stereomicroscopic level (Figure 5B). The histological structure of the fold has four concentric tissue layers: mucosa, submucosa, muscularis and serosa (Figure 5C). Both mucosa and submucosa are a longitudinal fold protruded into the lumen (Figure 5C). The mucosa consists of two sub-layers (the epithelium and lamina propria). The mucosal epithelium is lined by a simple epithelium and large goblet cells (Figure 5D). The apex goblet cells have foamy cytoplasm and do not react with H&E (data not shown); however, a strong positive reaction is detected by the AB staining (Figure 5D), indicating the presence of mucopolysaccharides in this

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

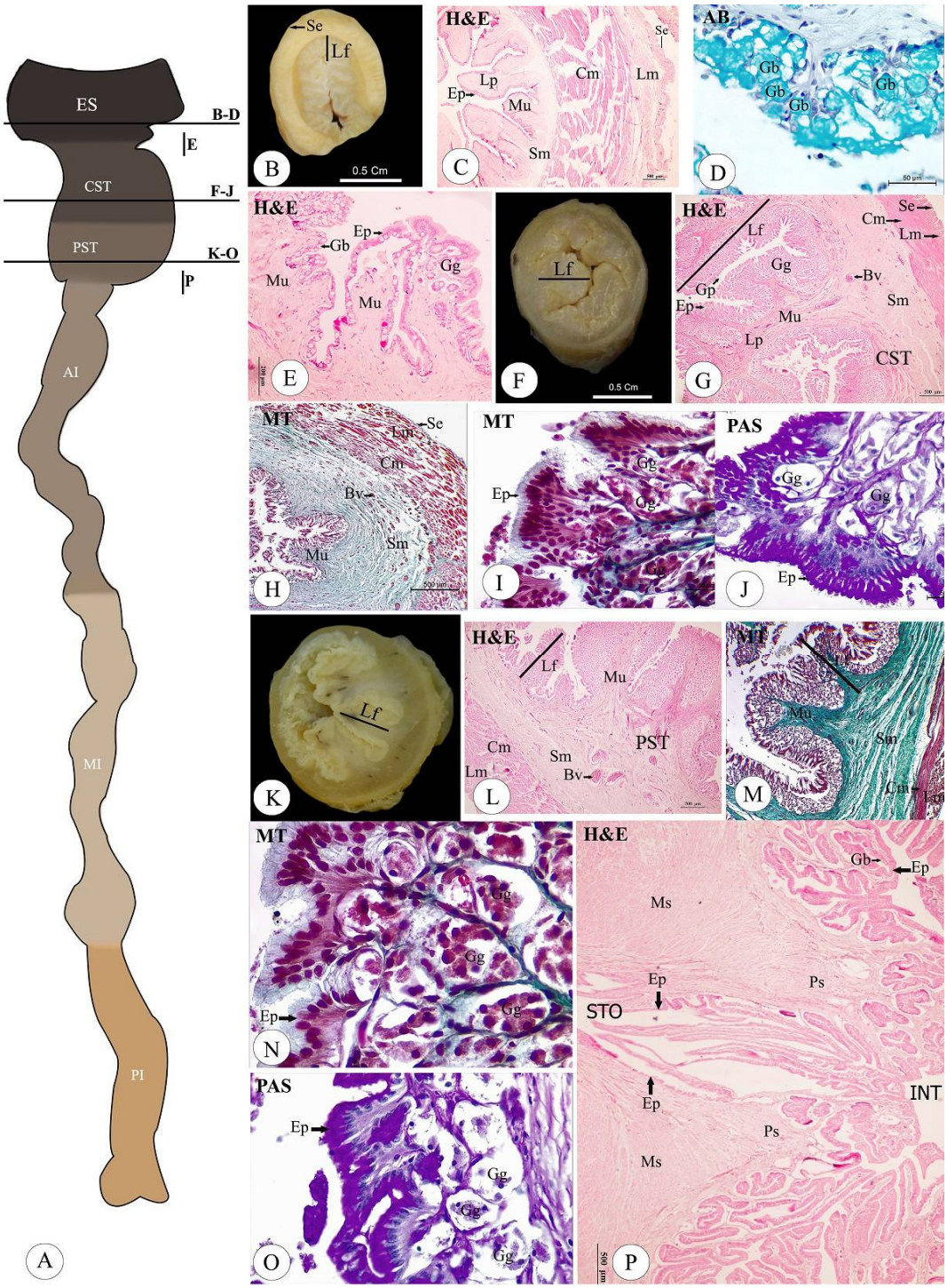


Figure 5. Morphology and structure of esophagus and stomach regions in *A. grunniens*: (A) schematic diagram of digestive tract; (B) cross-section of esophagus (ES) showing longitudinal fold (Lf); (C) oesophageal histology consisting of four layers – mucosa (Mu), submucosa (Sm), muscularis with two sub-layers of circular muscularis (Cm) and longitudinal muscularia (Lm) and serosa (Se); (D) goblet cells (Gb) stained in bluish colour; (E) gastro-oesophageal junction; (F-H) cross-section (F) and images (G-H) of cardiac stomach (CST); (I-J) mucosal layer with prominent epithelium (Ep) and gastric glands (Gg); (K-M) morphology (K) and histological structure (L-M) of pyloric stomach; (N-O) pyloric stomach (PST) with several gastric glands (Gg); (P) gastrointestinal junction containing pyloric sphincter (Ps)

Abbreviations: AI=anterior intestine, Bv=blood vessel, Gb=goblet cell, Gp=gastric pit, Lp= lamina propria, INT=intestine, MI= middle intestine, PI=posterior intestine, STO=stomach

Staining methods: AB = alcian blue stain, H&E = hematoxylin and eosin stain, PAS = periodic acid-Schiff stain, MT = Masson's Trichrome stain

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

cell. These results are similar to observations in other fishes such as *G. maculatum* [14], *A. anguilla* [22], *C. myriaster* [23], *Dentex dentex* [24] and *Anguilla bicolor* [25]. The mucous in the oesophagus acts as lubricant and is also associated with immunological response to bacterial and osmo-regulatory stimuli [25, 26]. No muscularis mucosa cells are found in the oesophagus. The submucosa layer is composed of loose connective tissue and some blood vessels, whereas the muscularis contains two sub-layers, i.e. inner longitudinal and outer circular muscles (Figure 5C). It is believed that the two features are directly related to the movement of food as observed in Anguilliforms and Siluriforms [23, 27-29].

Stomach

The stomach is connected to the oesophagus via the gastro-oesophageal junction (Figure 5E). The epithelium of the esophagus is stratified and squamous, but that of the stomach has a simple columnar shape and contains the gastric gland, as shown in the left and right sides of Figure 5E respectively.

The majority of *A. grunniens* stomach can be histologically classified as the cardiac and pyloric stomachs. Numerous thick and deep longitudinal folds are observed in the cardiac stomach (Figure 5F). The wall is thick and composed of four distinct tissue layers (from inside to outside), namely mucosa, submucosa, muscularis and serosa (Figures 5G-5H), as similarly observed in other vertebrates [30]. We confirm the presence of longitudinal folds (also called gastric rugae) in the fundic region, which are formed by the mucosa and submucosa layers invaginating into the lumen (Figure 5G). The mucosal surface is observed with several furrows, which are also called gastric pits (Figure 5G). The epithelial surface of the mucosa is lined with a simple columnar epithelium (Figures 5I-5J). Also, the surface of the epithelium is positive in PAS staining method (Figure 5J). It is confirmed that these cells produce glycoproteins, as previously demonstrated in *Pelteobagrus fulvidraco* [29]. The function of glycoproteins is likely to be the protection of the epithelial layer.

The vascularised loose connective tissue is observed with simple tubular gastric mucosa in the thick lamina propria (Figures 5I-5J). It is confirmed by the PAS staining that the gastric cells produce neutral glycoproteins and mucopolysaccharides (Figure 5J) which play a crucial role in the secretion of pepsinogen for protein digestion as reported in *A. bicolor* [25]. It is also speculated that protein secretion from the gastric gland might help nutrient absorption in the stomach in other teleosts [14, 17, 24, 31, 32]. Notably, the prominent muscularis mucosa is observed between the layers of lamina propria and submucosa (Figure 5H). Underlying the submucosa, the thick layer of muscularis is composed of two layers: inner circular and outer longitudinal smooth muscles (Figures 5H-5G). The serosa is a thin layer surrounded by the mesothelium. The posterior stomach bears a morphological/histological similarity to the cardiac stomach (Figures 5K-5O) with some exceptions. For example, the pyloric stomach has a higher number of gastric glands than that in the cardiac region (Figures 5N-5O). On the other hand, the posterior stomach has fewer longitudinal folds than those of the cardiac stomach (Figures 5L-5M).

Intestines

The transition between the stomach and the small intestine is histologically identified as the gastrointestinal junction (Figure 5P). Goblet cells and mucosal folds are not observed in the intestine side (Figure 5P). The main part of this junction is composed of smooth muscle (Figure 5P) which controls the flow of partially digested food from the stomach to the small intestine [18].

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

The intestine is histologically classified into three regions, viz. anterior, middle and posterior intestines (Figures 6A-6N). Numerous folds are observed on the internal surface of the anterior intestine (Figure 6B) and their lengths are significantly different among the three regions (Table 2; $P < 0.05$). The mucosal layer of the anterior intestine protrudes into the longitudinal folds of the lumen (Figures 6B-6D) and is lined by a simple columnar epithelium without distinct villi (Figure 6E). These characteristics have been observed in most non-mammalian and non-avian vertebrates [30, 33]. On the other hand, the mucosa with villi has been reported in reptiles including *Xerobates agassizii* [34] and *Kinosternon scorpioides* [35], but the reason for this difference remains unclear. The mucosal epithelial layer contains columnar absorptive enterocytes and goblet cells (Figures 6E-6F). Interestingly, goblet cells are negatively stained in the MT staining (Figure 6E) but they positively react in PAS and AB staining (Figures 6F-6G), similar to previous observations in *A. anguilla* [22], *C. myriaster* [23] and *A. bicolor* [25]. Goblet cells present in the intestine at high density might serve as a lubrication support for defecation in fishes [14, 17, 22, 24, 31]. In addition, goblet cells have been documented to play a vital role in absorption and transportation, as well as providing enzymatic cofactors and defence against bacteria in several fish species including *Soleasene galensis* [36] and *Tilapia* spp. [37].

The lamina propria contain loose connective tissues and blood vessels (Figure 6E), which are normally present in vertebrate stomachs [28, 34]. The inner circular and outer longitudinal muscle layers are observed (Figure 6C). The histological observation of the middle intestine is the same as that of the previous region (Figures 6H-6J); however, goblet cells in the epithelial mucosa are more abundant than in the anterior intestinal region (Figure 6J, Table 2). The morpho-histology of the posterior intestine is also similar to that of the anterior and middle intestines (Figures 6K-6L) However, the number of goblet cells in the posterior region is significantly higher ($P < 0.05$) than that in the anterior and middle regions (Figures 6M-6N, Table 2). These results are consistent with other reports of carnivorous fishes such as *G. maculatum* [14], *Rastrelliger brachysoma* [17], *Anguilla bicolor* [25], *Monopterus albus* [38] and *Pseudoplatystoma fasciatum* [39]. The high density of goblet cells in the last portion of the intestine would be beneficial not only for lubrication and fecal expulsion, but also for protecting the epithelium [40-42]. The muscularis layer is supported by an outer longitudinal and an inner circular layer and the serosa layer is also observed in this part (Figure 6L).

Histological and Histochemical Observations of Accessory Organs

Liver

The encapsulation of the liver lobes is surrounded by loose connective tissue and the hepatic parenchyma is comprised of hepatic sinusoids and hepatocytes (Figure 7A). At high magnification, each granular hepatocyte has a polygonal shape and a spherical nucleus in the centre of the cell (Figure 7A). A strongly positive reaction to PAS is observed in hepatocytes (Figure 7B), indicating the presence of glycoprotein or glycogen as similarly noted in former reports [22, 43, 44]. The sinusoid is irregularly disposed and varies in size (Figure 7A) and a simple line of the endothelial cell is observed on the basement membrane.

Gall bladder

The gall bladder is composed of a gland and cystic ducts (Figure 7C) and its thin wall has four distinct layers (Figure 7D). A simple cuboidal epithelium, mucous cells and enteroendocrine

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

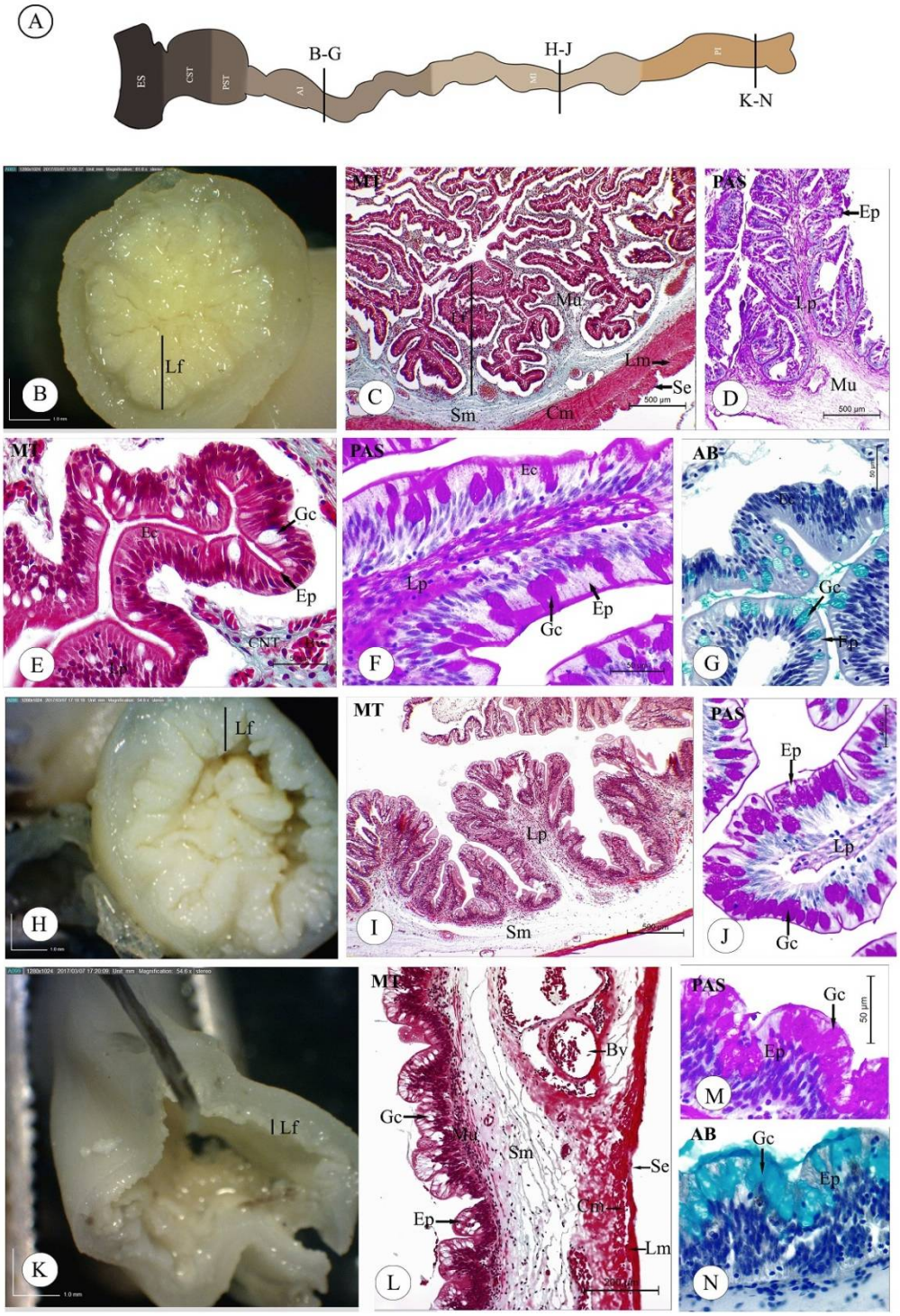


Figure 6. Morphology and structure of intestine of *A. grunniens*: (A) schematic diagram of intestinal regions; (B-D) morphology (B) and images showing longitudinal fold (Lf) and several histological walls of anterior intestine composed of four layers viz. mucosa (Mu), submucosa (Sm), muscularis with two sub-layers (circular muscularis (Cm) and longitudinal muscularis (Lm)) and serosa (Se); (E-G) mucosa in anterior intestine lined with epithelium (Ep); (H-J) morphology (H) of longitudinal fold (Lf) and images of middle intestine with mucosal layer (mu), epithelium (Ep) and goblet cells (Gc); (K-N) morphology (K) and images of posterior intestine showing marked accumulation of goblet cells (Gc)

Abbreviations: AI=anterior intestine, Bv=blood vessel (Bv), CST=cardiac stomach, Ec= enterocytes, Es=esophagus, Lp=lamina propria, MI=middle intestine, PST=pyloric stomach

Staining methods: AB = alcian blue stain, MT = Masson's Trichrome stain, PAS = periodic acid-Schiff stain

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

Table 2. Major histological variation among oesophagus, stomach and intestines and goblet cell counts from different intestinal regions of *A. grunniens* collected from Pranburi River estuary in May and September 2017. Significant differences in the means are indicated by * ($P < 0.05$).

Digestive organ	Mucosal epithelium	Goblet cell	Gastric gland	Longitudinal fold	Submucosa
Oesophagus	SSE	Present	Absent	Present (1.15±0.21)	Present
Anterior stomach	SCE	Absent	Present	Present (2.36±0.25)	Present
Posterior stomach	SCE	Absent	Present	Present (1.76±0.17)	Present
Anterior intestine	SCE	Present (9.00±1.87)*	Absent	Present (2.19±0.36)	Present
Middle intestine	SCE	Present (15.80±2.28)*	Absent	Present (1.15±0.13)	Present
Posterior intestine	SCE	Present (31.40±8.11)*	Absent	Present (0.22±0.06)	Present

Note: No significant differences are observed in longitudinal folds. SSE= simple squamous epithelium SCE= simple columnar epithelium

cells overlie the lamina propria (Figure 7E). The cystic duct proper continues from the gall bladder wall (Figure 7C). This duct is lined with a simple cuboidal epithelium and covered with the submucosa and muscularis (Figure 7F).

Pancreas

The pancreas is located near the mesentery in the anterior intestine (Figure 7G). Histologically, the bilobed pancreas contains two cell types: exocrine and endocrine. The exocrine pancreatic cells have acini and cluster features, being surrounded by the connective tissue (Figure 7H). Each exocrine pancreatic cell has a pyramidal shape and an ellipsoid nucleus located in the basal region (Figure 7H). Several eosinophilic zymogen granules are found in the cytoplasm of the acinar cells (Figure 7I). They are also positive to PAS stain (Figure 7I). These granules are known to accumulate pancreatic enzymes including trypsinogen, elastase and amylase as well as produce hormones [44, 45].

Gut Content Analysis

Out of the 111 specimens of *A. grunniens* collected in the present study, 50 samples were used for the gut content analysis. The IRI is highest for crab (73.64%), followed by shrimps (10.43%), fishes (8.02%) and other components (7.92%) (Table 3), indicating that crabs are the most important food of *A. grunniens*. These findings are in agreement with a previous observation in *Opsanus tau* [46], which shows that two crabs (*Panopeusherbstii* and *Eurypanopeus depressus*) are the main food for this toadfish [47, 48]. Similarly, a toadfish *H. didactylus* has been identified as a carnivorous fish [48]. Meanwhile, sea urchin has also been found in the gut content of some toadfishes (*Amphichthys cryptocentrus* and *Sanopus barbatus*). It was speculated that sea urchin is the most preferred food of toadfishes and when it is not available, they switch to crabs [49]. Therefore, the gut content varies based on the site of collection, habitat and food availability [50].

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

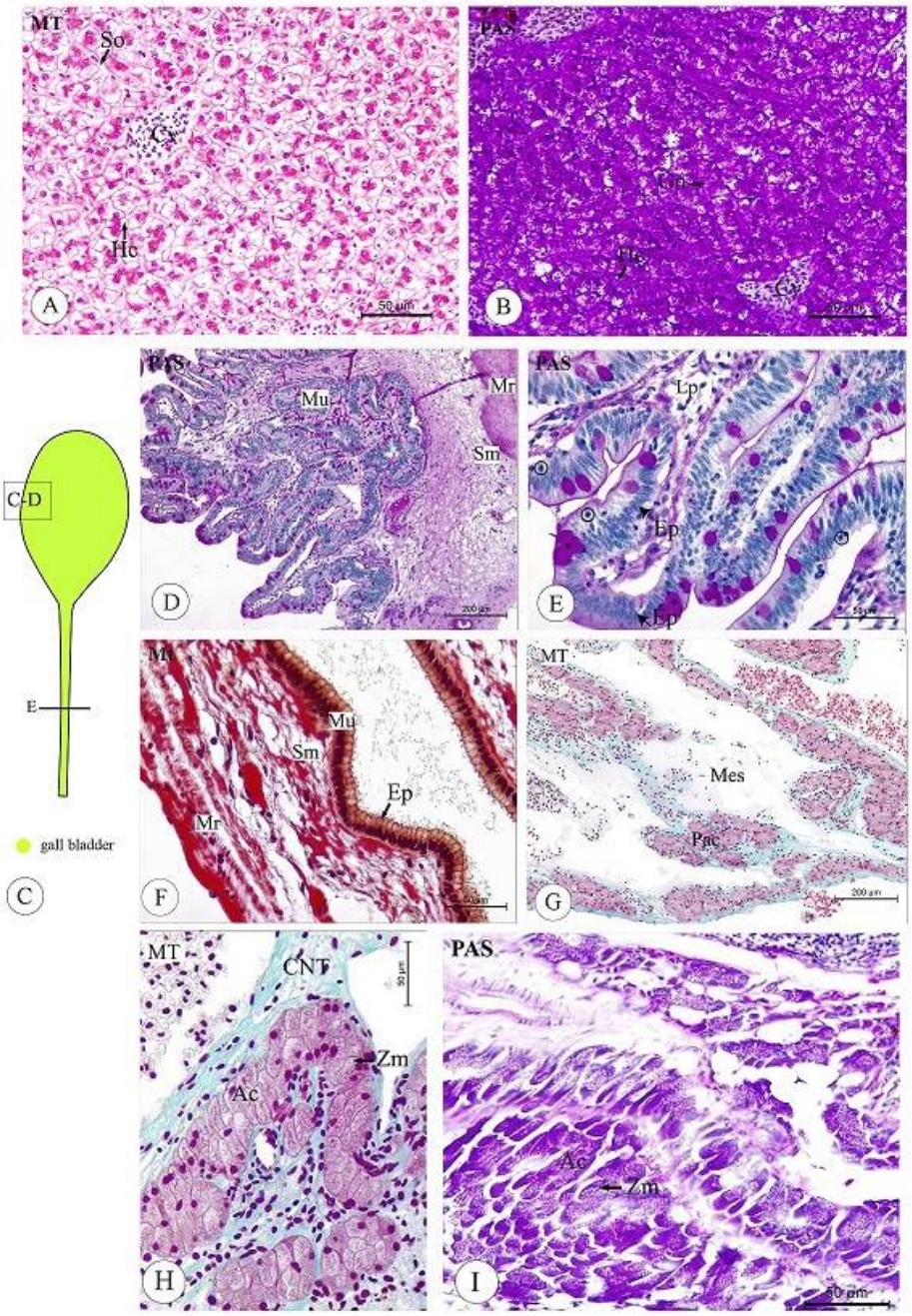


Figure 7. Structure of accessory organs in *A. grunniens*: (A-B) histological structure of liver composed of hepatocytes (Hc) and sinusoids (So); (C) schematic diagram of gall bladder; (D) wall of gland in gall bladder containing three layers, viz. mucosa (mu), submucosa (sm) and muscularis (Mr); (E) mucosa containing epithelium (Ep), mucous cell (*) and enteroendocrine cell (circles); (F) wall of cystic duct consisting of three layers [mucosa (Mu), submucosa (Sm) and muscularis (Mr); (G) distribution of pancreas (pac) among mesentery (Mes); (H-I) pancreatic acini (Ac) and zymogen granule (Zm)

Abbreviations: CNT = connective tissue, Cv = central vein, Lp = lamina propria, glycogen (Gn)

Staining method: MT = Masson's Trichrome, PAS = periodic acid-Schiff stain

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

Table 3. Summary of prey items found in gut content of *A. grunniens* collected from Pranburi River estuary in May and September 2017

Food type	N	V	F	%N	%V	%O	IRI	%IRI
Crabs	40	140.5	25	46.51	59.66	50.00	5308.60	73.64
Shrimp	13	32.5	13	15.12	13.80	26.00	751.83	10.43
Isopod	1	1	1	1.16	0.42	2.00	3.17	0.04
Insect larvae	2	0.5	1	2.33	0.21	2.00	5.08	0.07
Fishes	12	29	11	13.95	12.31	22.00	577.89	8.02
Gastropod	3	6	3	3.49	2.55	6.00	36.22	0.50
Detritus	12	19.5	11	13.95	8.28	22.00	489.14	6.79
Unidentified	3	6.5	3	3.49	2.76	6.00	37.49	0.52
Total	86	235.5	50	100.00	100.00	100.00	7209.42	100.00

Note: IRI = index of relative importance, N = number of items in all guts; V = volume, O = frequency of items

CONCLUSIONS

This study newly provides the histological and histochemical properties of the digestive system of *Allenbatrachus grunniens*. These results comprise important basic histological knowledge as summarised in Figure 8, which will be useful for future development of aquaculture of this species. Based on IRI, crabs are identified as the major food of *A. grunniens*, followed by shrimps and other animals. The morpho-histological observation and gut content analysis consistently indicate that *A. grunniens* is a carnivorous toadfish.

Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

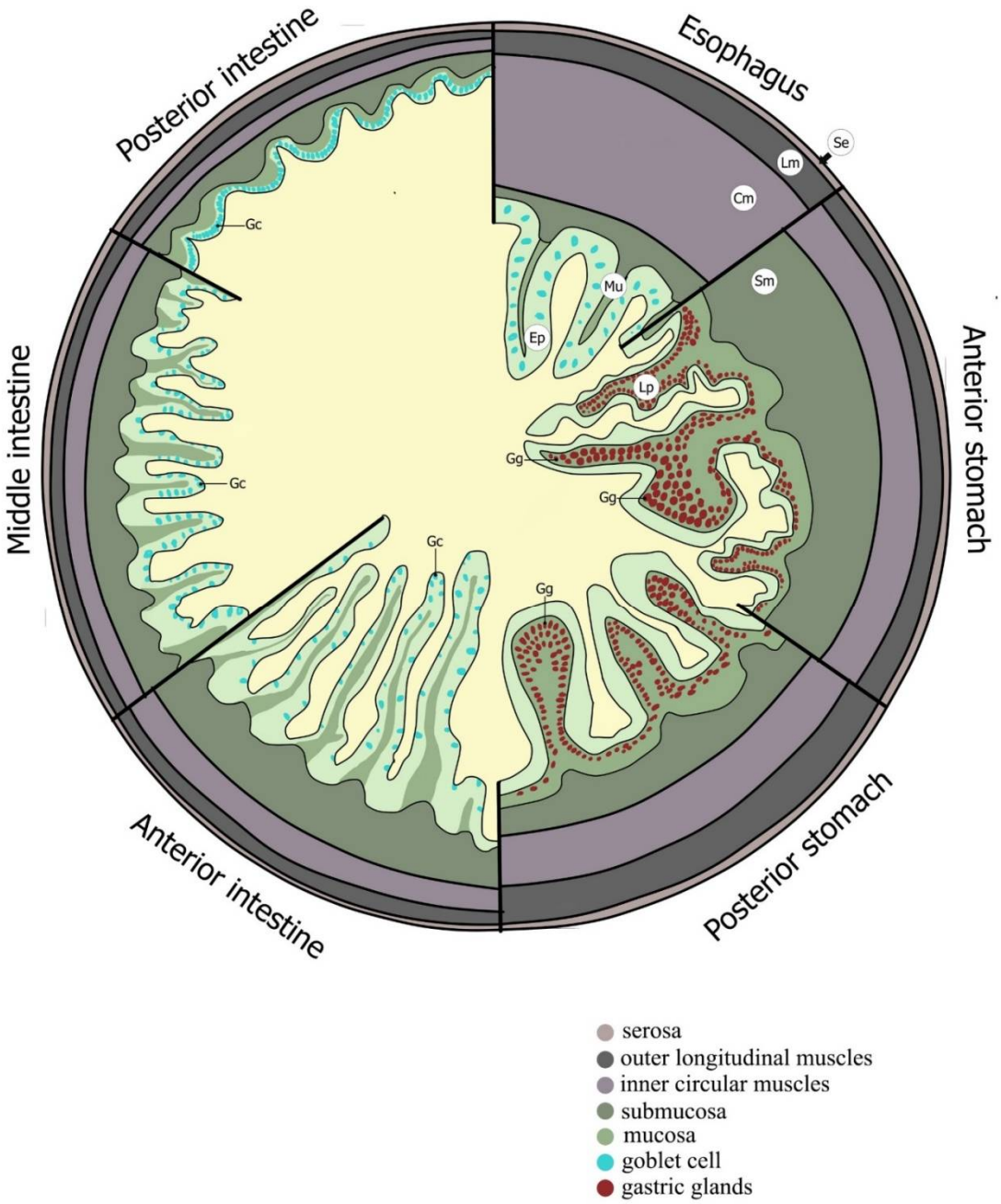


Figure 8. Schematic diagrams of different regions of digestive tract of *Allenbatrachus grunniens*. All structures are appropriately drawn to scale in terms of thickness and abundance. Abbreviations: Cm = circular muscularis Ep = epithelium, Gc = goblet cells, Gg = gastric glands, Lm = longitudinal muscularis, Lp = lamina propria, Mu = mucosa, Se = serosa, Sm = submucosa

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Maejo Int. J. Sci. Technol. **2021**, *15*(03), 222-241

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