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Examining the Stomach Structure and Function in Mudskipper (*Periophthalmus waltoni*) by Histological and Immunohistochemical Methods

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HIGHLIGHTS

- *P. waltoni* GI system is made of the esophagus, stomach, intestines, and rectum.
- The walls of all parts include the layers of mucosa, muscularis, and serosa.
- At the apex of the gastric gland's cells, there is an oxynticopeptic cell.

Abstract: Anatomically, the digestive system of oxudercinae lacks a distinct stomach, and that is why this group of fish is classified as stomachless. Since the environment, dietary requirements, and eating habits strongly influence the anatomy of the fish's digestive system, mudskippers (*Periophthalmus waltoni*) appear to have a stomach due to their carnivorous nature. The present study was undertaken to confirm the presence of stomach in *P. waltoni* and for this purpose, histological and immunohistochemical methods were used in this study. The results of this study showed that despite absence of an anatomical and distinct stomach, histological point of view the digestive system of this species was divided into 4 distinct parts; esophagus, stomach, intestine, and rectum. The stomach consisted of tubular glands in which the oxynticopeptic cells were present. In an immunohistochemical examination, the observation of the protein channels H+/K+ ATPase and Na+/K+/Cl cotransporter in the membrane of the oxynticopeptic cells confirmed the function of the stomach. In conclusion, the digestive system of *P. waltoni* is consist of the esophagus, stomach, intestines, and rectum, anatomically. Microscopic evaluation of digestive system indicated that unlike some other species, the Stomach is existed in *P. waltoni*, but has not any secretion of acid.

Keywords: Mudskippers; Histological; Histochemical; Immunohistochemistry.

INTRODUCTION

The digestive system of fish is different in terms of anatomy, histology and physiology [1,2]. These differences are related to dietary requirments and habits [3-6]. These differences include the length of gastrointestinal tract, the characteristics of cells and tissues in each part of the gastrointestinal tract, and anatomical properties [7,8]. Anatomically, the stomach is characterized by an increase in the diameter of its mouth and its wall, because it is the place where food is stored. Histologically, the stomach is characterized by the presence of gastric glands and oxynticopeptic cells, which break down and digest stored food by secreting gastric acid and pepsinogen, and creating acidic and enzymatic environment [7]. Despite extensive research on the digestive system of fish, research on *oxudercinae* is very limited [9]. *Gobiidae* is a large family whose members live in the seas and freshwaters around the world, and includes carnivores, herbivores, omnivores, and detritivores [10-12]. *Gobiidae* is one of the largest families of *Acanthomorpha*, and include more than 1,950 different species. They live in seawater, saltwater as well as freshwater in temperate and tropical regions [13-15]. Diverse eating habits as well as environmental conditions in which they live in, contribute to the development and emergence of features in the structure of their digestive system [10,16]. This family has one thing in common, as they lack a well-defined and developed stomach, and this anatomical feature of the digestive system has confused researchers, because many have considered this family of fish to have no stomach [17]. However, not all researchers agree on this issue [10,18,19]. Histological and physiological evidence suggests that not all species of *Gobiidae* family have stomach. Most of the studies that concluded the absence of stomach in these species have used anatomical research. *P. waltoni* feeding mainly on small animals such as insects, worms, crustaceans and fishes [9,10,12]. Histological data and research on the study of digestive system in *Gobiidae* are very limited. Thus, research on the presence or absence of stomach in *P. waltoni* seemed interesting and necessary. Because ecosystems are currently the most important factor in the survival of biodiversity, they are exposed to many biological threats due to poor management, and that is why we see the extinction of many aquatic and other animals in the wild every day. Studying fishes morphology is very valuable for several reasons, including ecological, behavioral, species conservation and water resources management and exploitation of aquatic animals. Considering the lack of sufficient information about the anatomical and histological structure of the digestive system, especially the stomach, and considering the role of this part in the nutrition and survival of this species, this investigation was undertaken in the present study. Therefore, in this study, we examined the histological study of stomach in *P. waltoni* as well as the differences in histological structure of stomach and esophagus, intestine and rectum using histological, histochemistry and immunohistochemistry methods.

MATERIAL AND METHODS

Fifteen adult *P. waltoni* with average weight of 13.19 ±1.97 g and length of 11.02 ±1.17 cm were used in this study. Samples were obtained from Mahshahr city located by the Persian Gulf and After opening the abdominal cavity of the digestive system were fixed in paraformaldehyde 4% over 24 hours. After exposure to water and increasing concentrates of alcohol and xylol, they were finally framed by being placed in paraffin. Using histotechnique methods, longitudinal and transverse incisions with a thickness of 5 µm were made from different parts of their digestive system. Also, for general histology examination, the slices were painted with H&E staining, and to determine acidic mucus, Alcian Blue (AB) staining was used. Moreover, to determine neutral mucus PAS staining was used, and to determine acidic and neutral mucus AB-PAS staining was used. The slices obtained from different parts of digestive system including esophagus, stomach, intestine and rectum were examined by histological and histochemical analyzes. To study immunohistochemistry, the slices were first washed using an ultrasonic cleaner machine in an acid and alcohol solution with a concentration of 1% HCl in 70% EtOH at 60°C for 15 min and then, they were rinsed in water for 15 min followed by distilled water for another 15 min. After that, they were dried gradually at a temperature of 37°C for 24 h. After 2 days, the slices were immersed in a solution containing 245 ml of acetone and 5 ml of 3-aminoisoquinoline triethoxysilane. After placing the tissue slices on the coated slides, they were immersed in xylol and later on in a decreasing concentration of alcohol and finally, in distilled water. The slides were then boiled in 0.05% Citraconic Anhydride solution for half an hour and were cooled down in distilled water for 10 minutes and finally, they were dried in an incubator at 37°C for 1 hour. Later on, the slides were immersed in SDS solution and then TPBS solution for 5 to 10 min each, and immediately after that 75 µl of buffer blocking was added to each section. The slides were then placed in a damp room. Two different primary antibodies were added to each section. For example, we used rabbit C2 antibody simultaneously with mouse T4 antibody on one section. After doubling the primary antibody to each section, the slides were placed in a humid room and then were refrigerated overnight at 4 °C. The next morning the slides were placed inside the TPBS.

The secondary antibody was added to all sections even the control group. For this purpose, 50 µl of secondary antibody was added to each section. Blocking buffer was also used to dilute the secondary antibody. For each 500 µl of solution containing secondary antibody, 1 µl of rabbit secondary antibody and 1 µl of mouse secondary antibody were used. After adding 50 µl of secondary antibody to all sections, they were incubated for 1 h at 37°C (sections were in a humid room).

The samples were then placed in TPBS for 5 min. Then 60 ml of TPBS was mixed with 5 µl of DAPI and added to the sections. DAPI induced molecular staining of the nucleus by being attached to genes within the nucleus. Then, using Sigma Scan software, the light intensities of different colors were compared and carefully examined.

All data were represented as the mean ± standard deviation. Data distribution was controlled by the K-S test, and since the distribution of all data was normal, parametric tests were used to analyze them. The variables were analyzed by one-way analysis of variance followed by Tukey test for post hoc comparisons using SPSS version 19.0 (SPSS Inc, Chicago, Illinois, USA). The statistical significance level was set at Pvalue<0.05.

RESULTS

The digestive system of *P. waltoni* is made up of the esophagus, stomach, intestines, and rectum (Figure 1), and also the walls of all parts include the layers of mucosa, muscularis, and serosa. The mucosa layer contains the layers of epithelial tissue and lamina propria. The muscularis layer contains the inner and outer layers. The serosa layer is made up of a thin layer of connective tissue covered with epithelial tissue.

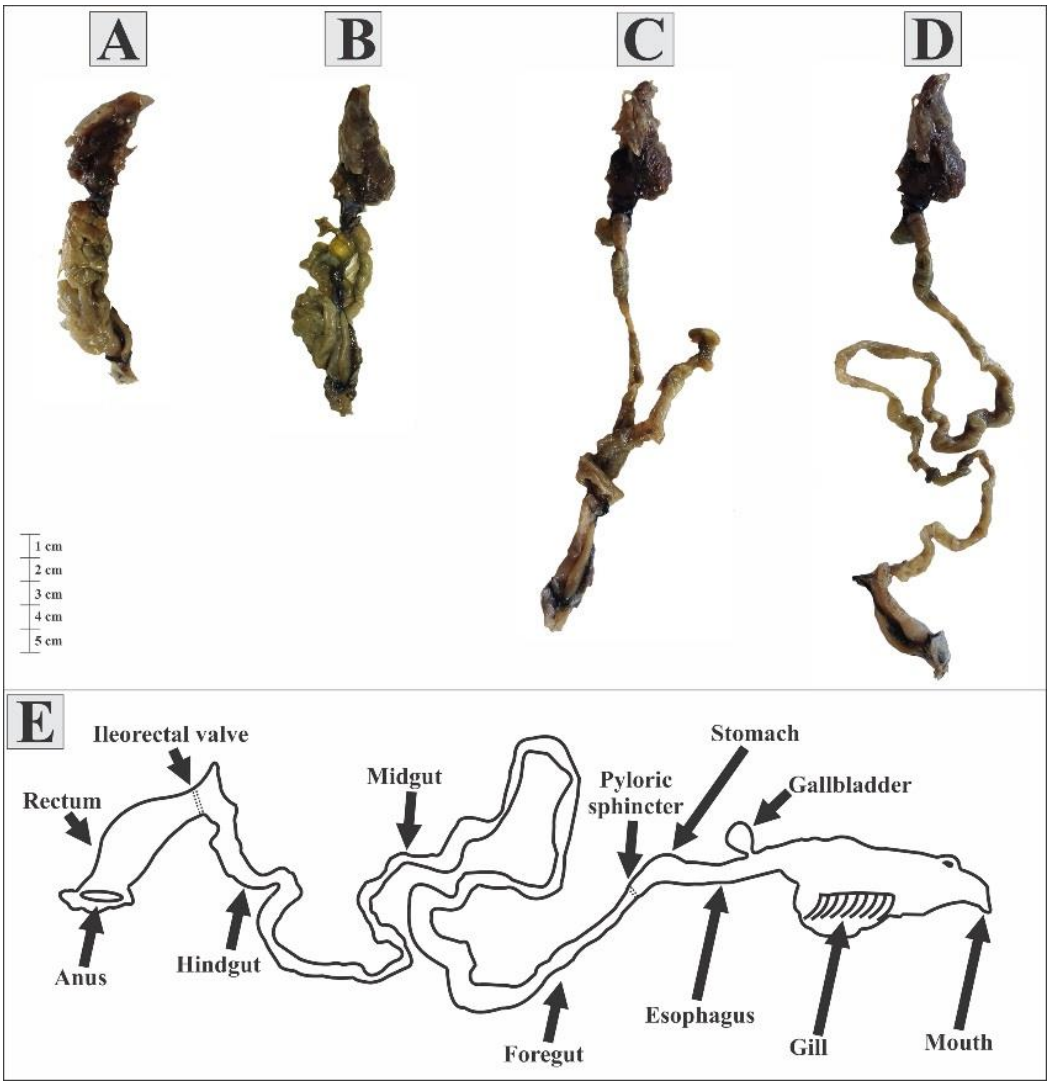

Figure 1. Image A the location of the digestive system inside the body, and Image B to D Gastrointestinal tract after the opening of the intestinal folds (lateral view). Image E Schematic view of different parts of the digestive system in *P. waltoni*.

Esophagus

The esophageal mucosa initially has fewer, shorter folds, and the squamous epithelial tissue lacks goblet cells, which gradually increase in number. The esophageal mucosa forms longitudinal folds, and the lining of the esophagus is made of stratified squamous epithelial tissue, which contains many goblet cells that has both acidic and neutral mucus. At the end of esophagus and near the stomach cell, goblet cells mainly contain neutral mucus to act against stomach acid and protect the underlying tissues. The mucus secreted by the goblet cells helps the swallowed material to move easily towards the stomach (Figure 2).

The lamina propria in the esophagus consists mainly of collagen connective tissue. The esophageal muscles have two layers of striated muscle that are located in different directions. The internal muscles are longitudinal muscles, and the external muscles are circular muscles. The muscles are separated by a thin connective tissue. The contraction of these muscles causes food to move towards the stomach.

The esophagus is connected to stomach by a transition region, which is associated with two tissue changes; lack of goblet cells and replacement of simple columnar epithelial tissue covering tissue by stratified squamous epithelial tissue lining in esophagus. The muscles of transition region are also a continuation of striated muscles of the esophagus with the difference that, they become smooth circular internal muscles and longitudinal external muscles (Table 1).

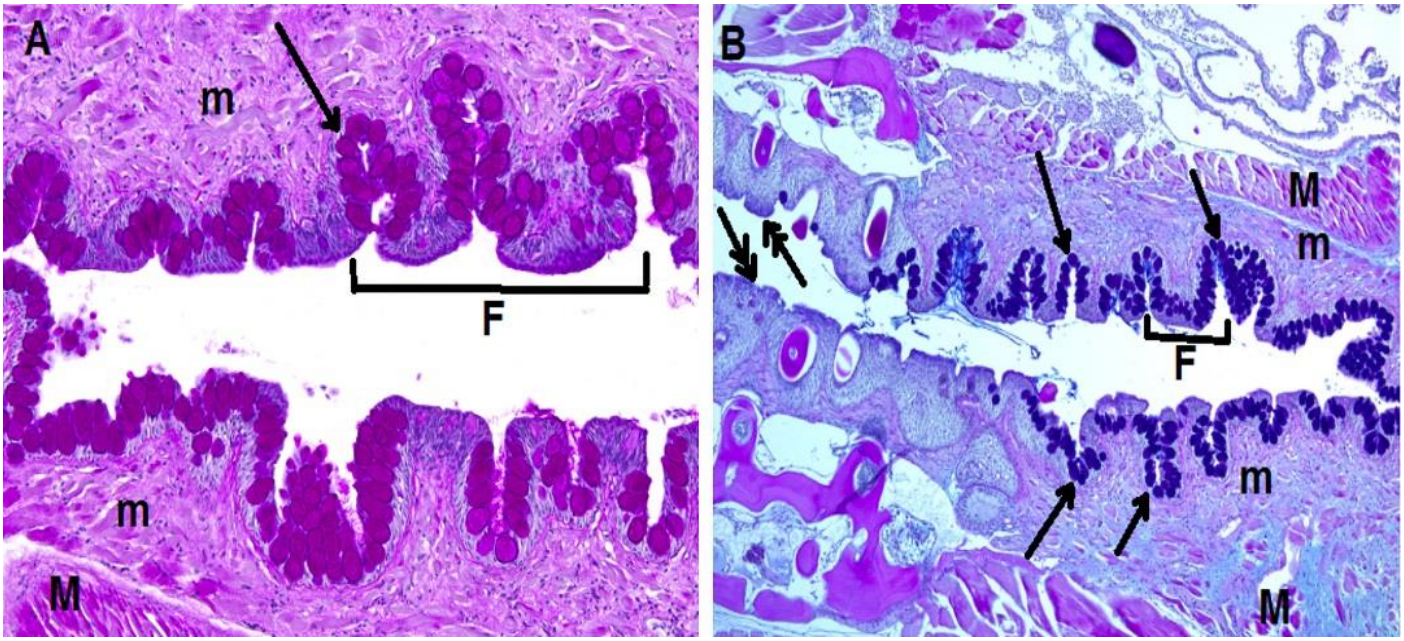

Figure 2. Image A shows the PAS staining and longitudinal cross section of esophagus, and image B shows the longitudinal cross section of esophagus, and AB + PAS staining. The mucus forms soft folds (f). Internal striated muscles (m) and external striated muscles (M). The covering tissue of the esophagus is initially made of stratified squamous epithelial tissue (double-head arrow), which lacks goblet cells, and lower down in esophagus toward stomach, goblet cells are added to it (single-head arrow). Goblet cells also contain both acidic and neutral mucus, because acidic mucus reacts positively to blue light and neutral mucus to red light.

Stomach

The stomach also forms longitudinal folds in the mucosa. The epithelial tissue in the gastric surface is composed of cylindrical cells or simple columnar epithelial tissue, and has neutral mucus in its apex that stabilizes the production of acid in the stomach. There are no goblet cells in the stomach. Lamina propria contains gastric glands that prove the existence of a functional stomach. These glands mainly contain oxynticopeptic cells. Due to the presence of glands in the lamina propria area, this area has expanded greatly. The stomach muscles are also smooth muscles, with inner layer being circular and the outer layer being longitudinal muscles. The thickness of inner muscle is also greater than the outer muscle. This muscle thickens near the intestine and forms the pyloric sphincter (Figure 3) (Table 1).

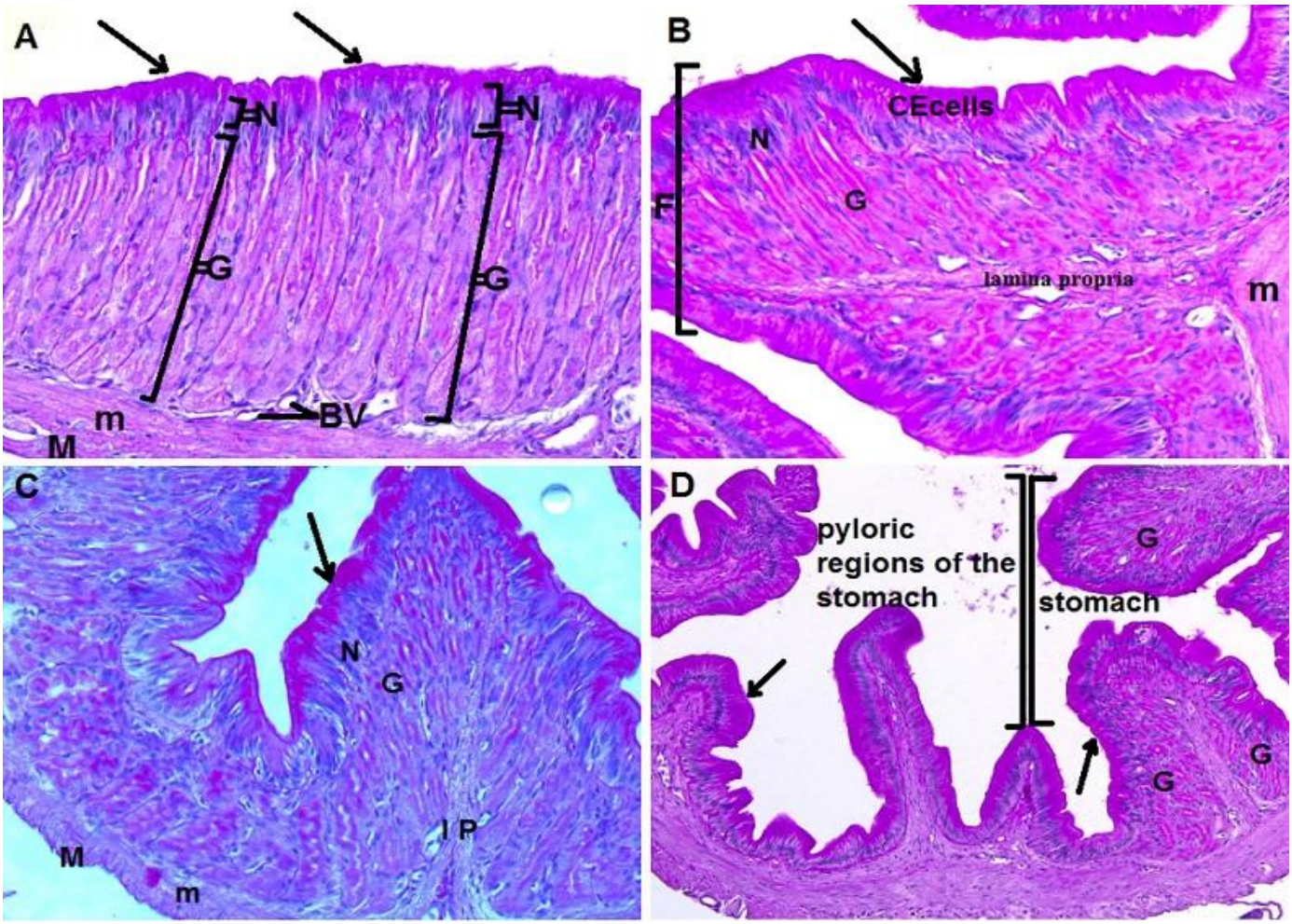

Figure 3. Image A shows the AB + PAS staining of gastric tissue - gastric glands (G), neck of gastric glands (N), simple columnar epithelial tissue is red, indicating the presence of neutral mucus to protect the stomach against gastric acid (arrow). Blood vessel in lamina propria (BV), internal muscles (m) and external muscles (M). Image B shows the H & E staining, where gastric tissue near the pyloric-mucosal region forms the folds (F), columnar epithelial cells (CEcells), glands (G), neck glands (N), lamina propria (lp), internal muscles (m). Image C shows the AB+PAS staining, where the gastric-mucosal tissue forms the folds. Columnar epithelial cells are red, indicating the presence of neutral mucus to protect the stomach against stomach acid (arrow). Glands (G), neck of the glands (N), lamina propria (lp), internal muscles (m) and external muscles (M). Image D shows the AB+PAS staining, the stomach connection to the pyloric regions of the stomach where, the glands (G) suddenly disappear. The simple cylindrical covering tissue is red, indicating the presence of neutral mucus to protect the stomach against stomach acid (arrow) forming longer and thinner mucosal folds.

Immunohistochemistry

At the top of the gastric gland's cells, there is an oxynticopeptic cell or enzyme H, K-ATPase, which pumps the gastric acid out. In immunohistochemical locating, these enzymes are visible in green. In the base and lateral parts of the oxynticopeptic cell or oxyntopeptic cell, there is another enzyme called $Na^+/K^+/Cl$ cotransporter, which is involved in the production of acid and provides Cl for acid cells. In immunohistochemical locating, these enzymes are visible in red.

As a result, in addition to the existence of a histologically well-defined stomach, the existence of a functional stomach that produces acid is quite evident.

The connection of stomach to pyloric sphincter is made by the pyloric regions of the stomach, in which the gastric glands suddenly disappear, and the thickness of muscle layer as well as the number of mucosal folds increase. The folds also become taller and thinner.

In the pyloric sphincter area, the mucosal folds become longer and thinner, creating a considerable thick muscle layer. The covering tissue is a simple columnar epithelial tissue, and the cylindrical cells of the mucosa at their apex contain neutral mucus (Figure 4).

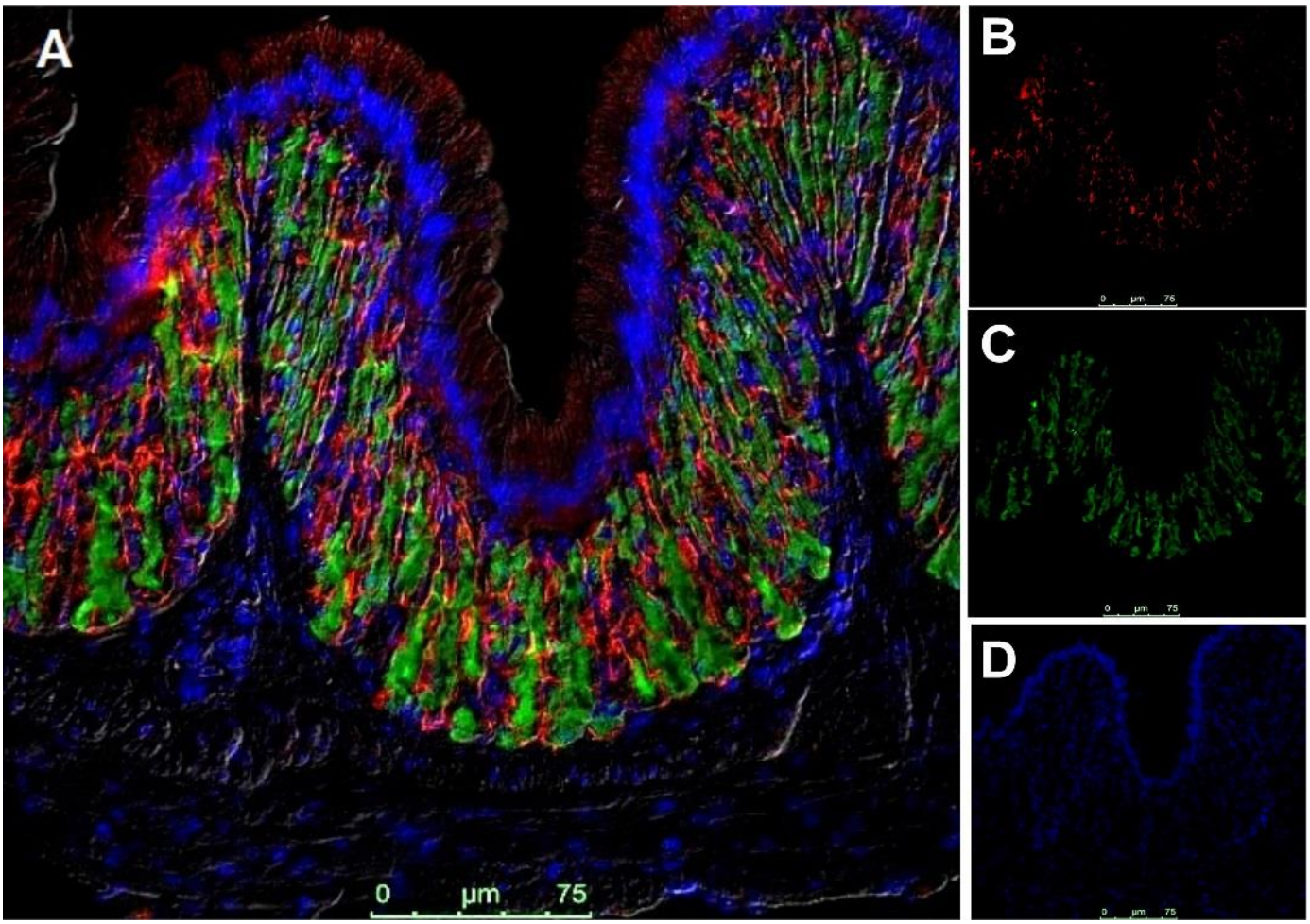

Figure 4. Image A is a fusion image of immunofluorescence localization (H^+/K^+ ATPase) in color (green) at the apex of gastric gland cells and $Na^+/ K^+/Cl$ cotransporter in color (red) at the base and sides of the cells. The nucleus turns blue with DAPI. Image B shows the $Na^+/ K^+/Cl$ cotransporter in color (red) at the base and next to the cells. Image C is the immunofluorescence localization image (H^+/K^+ ATPase) in green at the apex of gastric gland cells. Image D is the image of nucleus that turn blue by DAPI.

Intestine

Intestine forms the thin and long folds in its mucosa. The covering tissue of the intestinal mucosa is the simple columnar epithelial tissue lining. There are villi hairs on the surface of these cells to increase the level of absorption in the intestine. The mucosal epithelial tissue is covered with simple cylindrical cells whose nucleus is located at the base of the cell. In between these cells, there are goblet cells that have a mixture of acidic mucus, neutral mucus or a combination of acidic and neutral mucus. As the intestine extends into the rectum, the mucus of the goblet cells becomes more acidic. Lamina propria has the characteristics of collagen connective tissue and, unlike stomach, is smaller. The muscular layer has two layers, with the inner circular layer being much wider than the outer longitudinal layer. Finally, there is a serous layer that is thin (Figure 5) (Table 1).

Rectum

Like the intestine, rectum has folds that are shorter than the intestinal folds, and the longer the rectum extends toward the anus, the shorter the length of these folds become. The mucosal epithelial tissue is covered with simple cylindrical cells whose nucleus is at the base of them, where they have villi hairs. In between these cylindrical mucosal cells, there are goblet cells, which contain acidic mucus. Lamina propria also has the characteristics of collagen connective tissue, but its collagen fibers are less. The serous layer is also present as the outermost layer with a small thickness. Finally, the rectal mucosa near the anus turns into stratified squamous epithelial tissue, which then attaches to the stratified squamous epithelial tissue of the fish's skin (Figure 5) (Table 1).

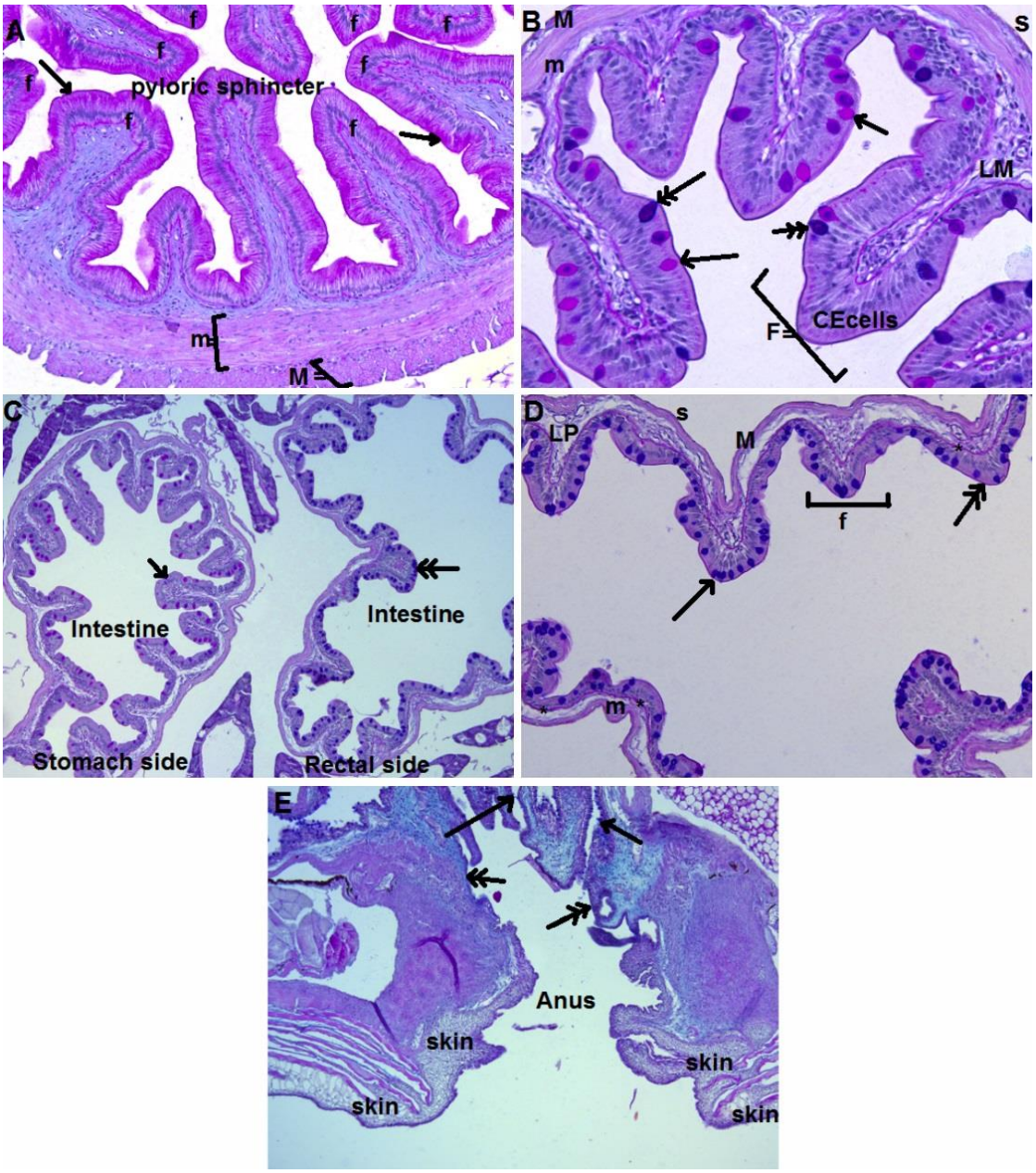

Figure 5. A, Cross section of pyloric sphincter, folds (f). Simple columnar epithelial tissue, neutral mucus (arrow) internal muscles (m) and external muscles (M). PAS staining. B, Cross section of the intestinal folds (f). Columnar epithelial cells (CEcells). Goblet cells also contain both acidic mucus (double-headed arrow), neutral mucus (single-headed arrow). Lamina propria (LM), internal striated muscle (m), external striated muscle (M), serous (S). AB + PAS staining. C, Cross section of the intestine. AB+PAS staining. D, Cross section of rectum. Internal striated muscle (m), external striated muscle (M), serous (S). AB+PAS staining. E, Longitudinal cross section of the rectum and anus. AB + PAS staining.

Table 1. Histomorphometrical results of different parts of the digestive system in *P. waltoni*.

Region		Mucosa		Mascularis		Serosa (µm)
		Epithelium (µm)	Lamina propria (µm)	Inner layer (µm)	Outer layer (µm)	
Esophagus		30.95 ± 2.61^{a}	115.55 ± 6.14^{a}	70.29 ± 3.49^{a}	81.66 ± 4.28^{a}	2.14 ± 0.10^{a}
Stomach		27.13 ± 2.74^{a}	102.24 ± 5.64^{b}	19.64 ± 3.81^{b}	17.80 ± 2.97^{b}	2.84 ± 0.19^{b}
Intestine	Foregut	29.25 ± 3.06^{a}	23.22 ± 2.78^{c}	19.57 ± 3.13^{b}	15.61 ± 2.78^{b}	2.50 ± 0.21^{b}
	Midgut	30.49 ± 4.11^{a}	21.49 ± 1.88^{c}	20.35 ± 2.98^{b}	14.17 ± 2.13^{b}	2.16 ± 0.18^{a}
	Hindgut	29.87 ± 3.45^{a}	22.36 ± 3.05^{c}	18.21 ± 3.07^{b}	15.99 ± 1.99^{b}	2.66 ± 0. 23^{b}
Rectum		24.82 ±2.97^{b}	21.81 ± 2.80^{c}	20.91 ± 2.42^{b}	29.82 ± 3.19^{c}	2.79 ± 0.16^{b}

Dissimilar letters indicate significant differences between the groups ($p < 0.05$).

DISCUSSION

This study confirms the hypothesis that mudskippers have stomach, and provides histological and physiological evidence on the existence of stomach. Although the stomach was not anatomically distinct, it was histologically evident. These results challenge the common view that claims oxudercine gobies have no functional stomach. Histological examination showed that the digestive system of *P. waltoni* consisted of esophagus, stomach, intestines and rectum. The sections were clearly separated by sphincters. The sphincters included the pyloric sphincter between the stomach and intestines, and also the ileorectal valve between the intestines and rectum [20]. This is also found in other species of the *Gobiinae* family, including *Mesogobius Batrachocephalus*, *Neogobius Ophiocephalus*, and *Neogobius Gymnotrachelus* [16,17].

Sphincters control the transport of food between different parts of the gastrointestinal tract and divide it into different histological and physiological parts. In the *Pleuronectes Americanus* and *Pleuronectes Ferruginea* species, the transition between esophagus and stomach changes abruptly from a stratified squamous epithelium to a simple cylindrical epithelium [21]. In species such as *Mystus Gulio* and *Clarias Gariepinus* [22], the change in the epithelium is not obvious and is gradual. Due to the lack of a clear boundary between the esophagus and stomach, there are many differences in the classification or title of this area. Many authors, especially in the field of marine fish, describe this area as part of the esophagus. In *P. waltoni*, the stomach has its histological features including; a) the presence of well-developed gastric glands, b) the presence of protein channels that play an active role in the production of gastric acid, and c) the presence of neutral mucus in the apical area of the epithelial cells of the gastric surface. The gastric glands in fish are tubular and alveolar [9], and their growth and distribution depend on the type and amount of food consumed [1,4,23]. The gastric glands in *P. waltoni* were also tubular and densely distributed in the lamina propria, but were absent in the pyloric regions of the stomach and pyloric sphincter. Among *Gobiidae*, the gastric glands are well developed in the carnivorous species such as *Rhinogobius Giurinus*, *P. semilunaris* and *Mesogobius Batrachocephalus*, and these glands are alveolar, less compact, and distributed in a smaller area [16,24,25]. The gastric glands in *P. barbarus* are composed of oxyntopeptic cells, similar to those found in other fish, *amphibians*, reptiles, and birds [25]. The cells that make up the glands in *P. waltoni* are also called oxyntopeptic cells. The role of neutral mucus on the surface cells of the gastric mucosa is to protect the gastric mucosa against stomach acid. The presence of neutral mucus on the surface of mucosa cells is one of the indicators of the existence of a functional stomach. Neutral mucus was present in the lumen of *P. Semilunaris* stomach, which was also observed in *B. Gymnotrachelus*, so we can conclude that neutral mucus is the result of the secretory activity of cells that make up the gastric glands, because some glands' cells responded positively to PAS staining [17].

In the present study, there was no evidence of mucus in the gland's cells and in the lumen of the gastric glands in *P. waltoni*. In histological examinations of the digestive system of *Periophthalmus Genera*, there was no mucus to protect the mucosa against stomach acid. This led to the conclusion that these fish do not produce stomach acid [16,17].

Also, in the histological examination of *P. semilunaris*, the results showed that in this species, neutral mucus is produced by gastric epithelial cells, so it was concluded that the gastric glands produce hydrochloric acid.26 The mucus produced by the esophagus and stomach may play an important role in protecting the mucosa against mechanical damage caused by passing food, in addition to creating favorable conditions for the absorption of short-chain fatty acids [21,26-28].

The striated muscles of the esophagus move food from the esophagus towards stomach [16,17,25].

In other species of the *Gobiidae* family, the esophagus has goblet cells among its epithelial cells [16,24,25]. In goblet cells of the esophagus a mixture of acidic and neutral mucus is produced that covers the surface of the mucosa and facilitates the swallowing of solid foods. Mucus also protects the mucosa against microorganisms and mechanical damage. Also, in other species of the *Gobiidae* family, there are many goblet cells in the intestinal epithelium. The intestine in *P. waltoni* is divided into two parts; the part that is close to stomach and the part that is close to rectum. The goblet cells have both acidic and neutral mucus in the first part and in the second part, it only has acidic mucus [16,24].

In conclusion, the digestive system of *P. waltoni* is consist of the esophagus, stomach, intestines, and rectum, anatomically. Microscopic evaluation of digestive system indicated that unlike some other species, the Stomach is existed in *P. waltoni*, but has not any secretion of acid. Also, Hisatologically, the walls of all parts include the layers of mucosa, muscularis, and serosa. The mucosa layer contains the layers of epithelial tissue and lamina propria. The muscularis layer contains the inner and outer layers. The serosa layer is made up of a thin layer of connective tissue covered with epithelial tissue.

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