

## **Structure and ultrastructure studies of the digestive tract of the endangered Qinling lenok (*Brachymystax tsinlingensis* Li, 1966)**

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### **Abstract**

The aim of this study was to investigate the structure and ultrastructure of the digestive tract of Qinling lenok (*Brachymystax tsinlingensis* Li, 1966), a cold water Salmonidae fish, an endangered teleost species, with high potential for controlled rearing in Shaanxi Province of China, by light and electron microscopy. Morphological data of the digestive tract are important for understanding fish nutrition, pathological or physiological alterations. The histological structure of Qinling lenok consists of four layers: mucosa, submucosa, muscularis and serosa. Taste buds were found in lips and esophagus. The esophageal mucosa consists of undifferentiated mucous cells and surface epithelial cells. The U-shaped stomach was divided into cardiac, fundic and pyloric region. There are numerous gastric glands in the submucosa layer of the cardiac and fundic stomach, but none of them are present in the pyloric region. The convoluted tube-shape intestine is lined by simple columnar epithelial cells with microvilli at the apical surface, with an intestinal coefficient of 0.61. There are numerous goblet cells in the intestine. Finger-like pyloric caeca were found in the front of intestine tube, with number ranged from 42 to 88. In ultrastructural level, mucous and glandular cells in the stomach were found, the glandular cell with well-developed tubulovesicular system, a great amount of pepsinogen granules, mitochondria and Golgi apparatus. The enterocytes with abundant microvilli contained mitochondria and lysosome, and mucous granules of goblet cells were apparent in the intestine. High density of lipid droplets of pyloric caeca might be concerned with fat-absorption. The present study suggests that the digestive tract of Qinling lenok is similar to other carnivorous fishes, in relation to its feeding habits.

**Keywords:** Digestive tract, *Brachymystax tsinlingensis*, Histology, Ultrastructure, Salmonidae

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## Introduction

Qinling lenok (*Brachymystax tsinlingensis* Li, 1966) of the family Salmonidae and order Salmoniformes, a landlocked salmon fish, is one of the most significant species in Qinling Mountains of China. It is endemic to China, as a subspecies of *Brachymystax lenok* previously (*Brachymystax lenok tsinlingensis*), and only found in parts of the Taibaishan Mountains segment of the Qinling Mountains, especially in the Heihe, Shitouhe, Xushui and Taibai Rivers (Li, 1966), as a residual species migration from the north southward during glacial period. Now Qinling lenok has been considered as an independent species with Latin name *Brachymystax tsinlingensis* Li (Xing *et al.*, 2015; Meng *et al.*, 2018). As a carnivorous fish, juveniles mainly feed on aquatic invertebrate animals, and adult feed on small fishes and insects (Yue and Chen, 1998). Due to ecological environmental disruption and excessive harvest, especially associated with illegal fishing methods such as poisoning, electrification and the use of explosives, the amount of this species in the wild has declined quickly, and it was listed as class II National Protected Wild Aquatic Animal in China (Yue and Chen, 1998; Zhao and Zhang, 2009). With the development of aquaculture in Shaanxi province, as well as the important economic value of Qinling lenok, the potential for controlled rearing of this species is becoming more and more optimistic, and some scholars have done some research on artificial propagation (Zhang *et al.*, 2006; Bai *et al.*, 2007). In

addition, artificial reproduction and releasing the fry fries is beneficial to protect the endangered species. In this context, it is very necessary to obtain knowledge on digestive physiology for the potential of intensive culture in the future. Previous studies about Qinling lenok had focused on phylogenetic evolution (Froufe *et al.*, 2008; Ma *et al.*, 2009), reproduction biology and artificial propagation (Zhang *et al.*, 2006; Bai *et al.*, 2007; Mou *et al.*, 2008), subspecies differentiation or species status (Li, 1966; Xing *et al.*, 2015; Meng *et al.*, 2018), karyotype (Xu *et al.*, 2009), resource protection (Wang, 2008; Hou, 2009), diet (Nakano, 1999), early development (Zhang, 2008; Du *et al.*, 2010) and histology of digestive system (Qing *et al.*, 2012). However, little studies concerning its ultrastructure of digestive tract have been reported until now.

The morphology and mucosal histology of digestive tract of teleostean fishes shows masses of interspecies variations related to diet, growth, development and reproduction of these great events of fish life, and is of primary importance in the digestion, feed break-down and absorption of dietary nutrients (dos Santos *et al.*, 2011; Canan *et al.*, 2012), and the morphology and histology studies of digestive system are considered as one of the basic tools for understanding and exploring the physiological mechanism of ingestion, digestion and absorption for in fishes. Many studies referring to the structure and ultrastructure of the gut have been documented for plenty of fish species, either about simply

description (Lin *et al.*, 2002; Dai *et al.*, 2007; Bočina *et al.*, 2017 ) or in relation to the different feeding adaptations (Albrecht *et al.*, 2001; Abdulhadi, 2005; Xiong *et al.*, 2011; Nasruddin *et al.*, 2014).

In previous researchs, the morphology and histology of the Qinling lenok digestive tract was described roughly (Qing *et al.*, 2012), no reports concerning the ultrastructural level could be found. Knowledge of digestive physiology is helpful to understand the pathological alterations, nutritional absorption and feeding pattern (Carrassón *et al.*, 2006; Wilson and Castro, 2010; Namulawa *et al.*, 2015), so the purpose of the present study was to examine a histological and transmission electron microscopic study of the digestive tract of Qinling lenok in depth, as well as to provide a basis information regarding to digestive physiology for species conservation and artificial propagation in the future.

## **Materials and methods**

### *Animals and gross anatomy*

Ten wild specimens (5 male and 5 female), live, apparently normal and healthy were captured from Heihe River in Shanxi Province of China in April, 2011, with average body length of 191.8 mm and average body weight of 110.3 g. They were cultured temporarily two days in order to empty food before the sampling. The animals were anaesthetized with MS-222 and the abdominal cavity was dissected. The entire digestive tract was exposed by a mid-ventral incision, removed, and then divided into the lips, pharynx,

esophagus, stomach, pyloric caeca and three portions of intestine.

Meanwhile, the intestinal coefficient (IC=Li/Ls) or the relative intestine length is the ratio of intestine length to standard length of fish body, where Li is the intestine length and Ls is the standard length (mm). It was calculated from 108 samples of Qingling lenok, with standard length varying from 97 to 235 mm (Mean=166.7 mm). The number of pyloric caeca was counted from 100 adult specimens.

### *Light microscopy*

For light-microscopic study, small pieces from each part of the digestive tube were fixed in Bouin's solutions for 24h. Then the fixed specimens were preserved in 70% ethanol, dehydrated in graded ethanol solution from 70% to absolute, cleared in xylene and embedded in paraffin. Paraffin sections (5-7  $\mu$ m) of each portion of the digestive tract were prepared for staining with haematoxylin and eosin (HE) (Xiong *et al.*, 2011). All stained slides were observed under Optec BDS200 Inverted Microscope (Optec, China). The photographs were taken by TSview 7.0 software and saved in a computer.

### *Transmission electron microscopy (TEM)*

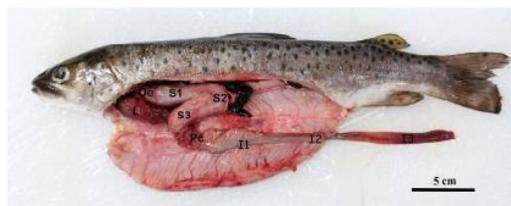
For electron-microscopic study, the fundus of the stomach, the proximal intestine and the pyloric caeca from five samples were fixed for 24 h at 4 °C in 2.5% glutaraldehyde solution, buffered at pH 7.2 with 0.1 M sodium cacodylate containing 5% sucrose. The pieces were

rinsed for 12-15 hrs in the buffer and fixed for 2 h in 1% osmium tetroxide in the same buffer. After dehydration in a graded series of ethanol, they were embedded in Epon812 (Faccioli *et al.*, 2015). These ultra-thin sections were stained with uranylacetate and lead citrate and micro-graphed with a JSM-100SX Transmission Electron Microscope (JEOL LTD, Japan).

## Results

### Gross morphology

The digestive tract of Qinling lenok included lips, oral-pharynx cavity, esophagus, obvious stomach, pyloric caeca and short intestine with few loops (Fig. 1). The buccopharyngeal cavity was relative large and there were about 46 minute teeth on the upper and 28 on the lower jaw. The esophagus was short located close to the liver. Behind the stubby esophagus, the classic U-shaped stomach was divided into three parts: cardiac, fundic and pyloric section. The intestine was short, slightly coiled, and it was divided to anterior, middle and posterior intestine, named I1, I2 and I3 as shown in Fig. 1. There are numerous, distinct, tubular (finger-like) pyloric caeca arranged, located behind the pylorus of stomach and opening independently in the anterior of intestine. The amount of multi-finger type pyloric caeca was ranged from 42 to 88, with 62 as average number (n=100). The intestinal coefficient (IC value) was estimated as  $0.61 \pm 0.08$  based on 108 individuals (Mean $\pm$ SD, n=108).

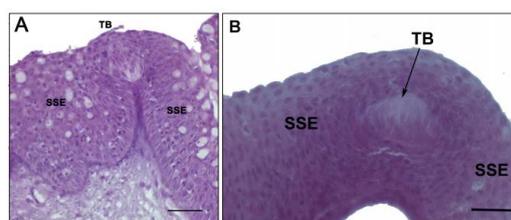


**Figure 1: Picture of the general structure of the digestive tract of Qinling lenok. Oe, Oesophagus; L, liver; S1, cardiac stomach; S2, fundic stomach; S3, pyloric stomach; Pc, pyloric caeca; I1, anterior intestine; I2, middle intestine; I3, posterior intestine.**

### Histology features

The digestive tract of Qinling lenok had a classic four-layered structure: mucosa, submucosa, muscularis and serosa, like many other teleostean fishes. The muscularis mucosa was present in esophagus and stomach epithelium, but absent in the mucosa of intestine and pyloric caeca.

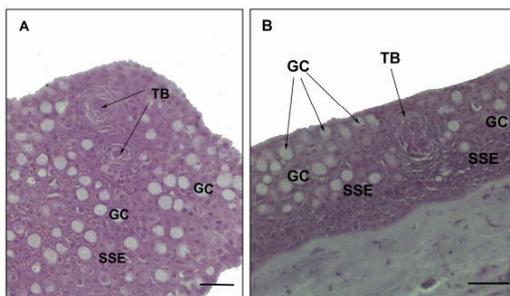
The upper and lower lips were consists of stratified squamous epithelium with taste buds on the external surface (Fig. 2A and 2B). No goblet cells were found in this area.



**Figure 2: Light photomicrograph of lips, H/E. (A) The upper lip, showing taste bud (TB). (B) The lower lip, showing taste bud (TB). GC, goblet cells; SSE, stratified squamous epithelium; TB, taste bud. Scale bars: A and B=50 $\mu$ m.**

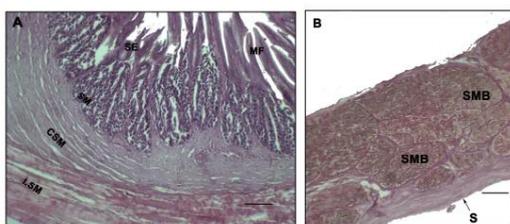
The upper and lower wall of buccopharynx mucosa was composed of stratified epithelium with many goblet cells and some taste buds (Fig. 3A and 3B), and the goblet cells were

observed in this part.



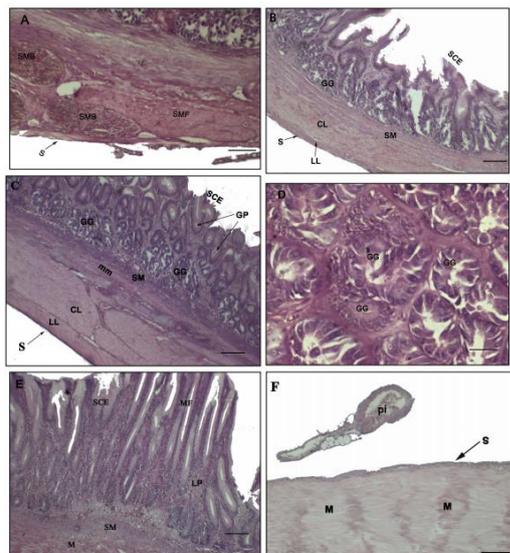
**Figure 3:** The light photomicrograph of buccopharynx. (A) The upper wall of buccopharynx, showing goblet cells (GC) and taste bud (TB). (B) The lower wall of buccopharynx, showing goblet cells (GC) and taste bud (TB). GC, goblet cells; SSE, stratified squamous epithelium; TB, taste bud. Scale bars: A and B = 50  $\mu$ m.

The esophagus was lined with stratified epithelium and a high amount of goblet cells (Fig. 4A). The tunica muscularis consisted of two layers of striated muscle, an inner longitudinal and outer thicker circular (Fig. 4A), with extending into the submucosa. The striated muscular bundles were observed in the esophagus (Fig. 4B).



**Figure 4:** The light photomicrograph of oesophagus. (A) Histological structure of oesophagus. (B) Striated muscular in the oesophagus. CSM, circular layers of striated muscle; GC, goblet cells; LSM, longitudinal layers of striated muscle; LP, lamina propria; MF, mucosal fold; S, serosa; SE, stratified epithelium; SM, submucosa; SMB, striated muscular bundle. Scale bars: A and B=100  $\mu$ m.

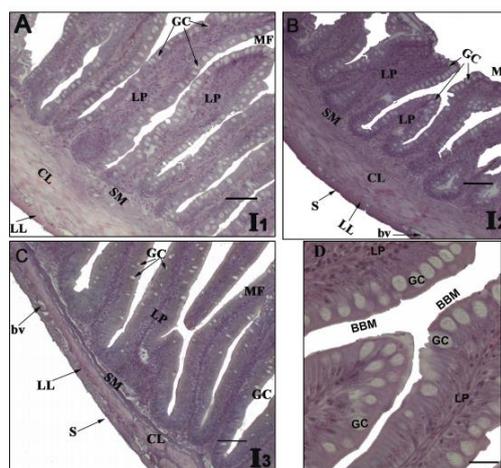
The transition from the esophagus to the stomach was clearly discernible through the change of the epithelium into a simple columnar epithelium and secretory cells gradually disappear, the occurrence of numerous gastric glands, and the transit of striated muscle by smooth muscle layers (Fig. 5A and 5B). Smooth muscular fibers gradually substitute the striated ones of esophagus and the longitudinal muscularis appeared on the external wall (Fig. 5A). There are no goblet cells in the stomach epithelium, which always occurred in that of buccopharyngeal cavity, esophagus and intestine. The lamina propria is well developed. In the cardiac and fundic stomach, there were a great amount of gastric glands between the epithelium and lamina propria (Fig. 5B–5D), whereas no gastric glands were found in the pyloric stomach closing to the intestine (Fig. 5E). The transition between the fundic and pyloric portions is marked by the abrupt disappearance of gastric glands. Pancreas has observed nearby pyloric stomach, especially pancreas islet is clear (Fig. 5F).



**Figure 5:** The light photomicrograph of stomach, H/E. (A) The transition of muscle from oesophagus to stomach. (B) The cardiac portion of the stomach, showing gastric glands. (C, D) The fundic portion of the stomach, showing gastric glands. (E) The pyloric stomach without gastric glands. (F) The pancreas nearby pyloric stomach. CL, inner circular layer of smooth muscle; GG, gastric glands; GP, gastric pits; LP, lamina propria; LL, outer longitudinal layer of smooth muscle; M, muscularis; MF, mucosal fold; mm, muscularis mucosa; P, pancreas; pi, pancreas islet; S, serosa; SCE, simple columnar epithelium; SM, submucosa; SMB, stratified muscular bundle; SMF, smooth muscle fibers; Scale bars: A, B, C, E and F=100  $\mu$ m, D= 30  $\mu$ m.

There were no obvious difference between the three portions of intestine lumen in histological structure, except the height of mucosa folds were decreased gradually and the muscularis became thin gradually from the front intestine to posterior part (Fig. 6A). The mucosal folds were abundant and deeply folded, and many goblet cells were observed. The layer of submucosa and lamina propria was thin, and the dividing line between lamina propria

and submucosa was blurry. No muscularis mucosa was detected. Two thin layers of smooth muscle with an inner circular and an outer longitudinal layer were detected (Fig. 6B). The intestine lumen is lined by a simple columnar epithelium with a well developed brush border interspersed by numerous goblet cells (Fig. 6C and 6D).

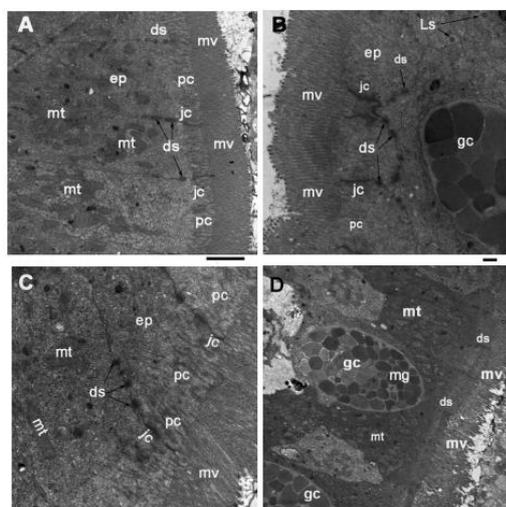


**Figure 6:** The Light photomicrograph of intestine, H/E.(A) The anterior part of intestine (I1). (B) The middle part of intestine (I2). (C) The posterior part of intestine (I3). (D) Goblet cells and brush border of intestine epithelium. BBM, brush border microvillus; bv, blood vessel; CL, inner circular layer of smooth muscle; GC, goblet cell; I1, anterior intestine; I2, middle intestine; I3, posterior intestine; LL, outer longitudinal layer of smooth muscle; LP, lamina propria; MF, mucosal fold; SM, submucosa; S, serosa. Scale bars: A, B and C= 100  $\mu$ m, D= 30  $\mu$ m.

The organization of cell layers in the pyloric caeca and intestine was similar to that in other vertebrates. The surface of the pyloric caeca mucosa is covered with villi but there was less folding than intestine (Fig. 7A). Many lipid vacuoles were found in the pyloric caeca (Fig. 7A and 7B), so that the



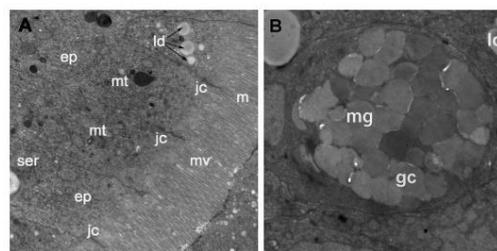
including the evident desmosomes ((Fig. 9A and 9B). The nuclei are situated toward the base of the cells.



**Figure 9: Ultrastructure of intestine.** (A, C) Electron micrograph of the enterocytes. (B, D) The intestine epithelium with goblet cell. ds, desmosomes; ep, epithelial cell; gc, goblet cell; jc, junctional complexes; Ls, lysosome; mg, mucous granules; mt, mitochondria; mv, microvilli; pc, pinocytotic channel. Scale bars: A= 2  $\mu$ m, B= 0.5  $\mu$ m, C=0.5  $\mu$ m, D=2  $\mu$ m.

The ultra-structure of pyloric caeca was similar to intestinal enterocyte, well-developed brush border microvilli towards the lumen but a smaller number of goblet cells. The dominant feature between the intestinal mucosa and pyloric caeca epithelium was the latter filled with high density of lipid droplets (Fig. 10A and 10B). The main type of cell junctions was junctional complex in the pyloric caeca (Fig. 10B), but desmosomes were evident between the intestinal enterocytes (Fig. 9B). Number of goblet cells in the mucosa of pyloric caeca were less than that of intestine, which were filled with

granules mucous (Fig. 10B).



**Figure 10: Ultrastructure of pyloric caeca.** (A) Electron micrograph of the simple columnar epithelium. (B) The intestine epithelium with goblet cell and lipid droplets. ep, epithelial cell; gc, goblet cell; jc, junctional complexes; ld, lipid droplets; mg, granules mucous; mt, mitochondria; mv, microvilli; ser, smooth endoplasmic reticulum. Scale bars: A= 2  $\mu$ m, B= 2  $\mu$ m.

## Discussion

Taste buds were observed in the lips and buccopharynx mucosa of Qinling lenok. Albrecht *et al.* (2001) pointed out those taste buds on the external surface of the lips of two Anostomidae fishes might indicate that feed is selected before ingestion, which was also documented by Xiong *et al.* (2011). Then the presence of taste buds in the lips and buccopharynx cavity in present study possibly indicate feed selection within the buccopharyngeal cavity.

One of the most remarkable features in the esophageal region of the Qinling lenok was the existence of a thick striated muscle layer, which may protect this organ against abrasions. Several cell layers, arranged in a continuous order and the mucous secretion in the esophageal epithelium of Qinling lenok, are important for rapid and even lubrication of feed particles along the whole esophagus during swallowing, as well as the

striated muscle fibers allow expansion of the pharynx and esophagus for ingesting feed (Albrecht *et al.*, 2001; Abdulhadi, 2005; Dai *et al.*, 2007). And feed selection may also take place in this region, because well-developed striated circular musculature in the esophagus making food rejection possible (Nasruddin *et al.*, 2014; Chatchavalvanich *et al.*, 2006). Also, it allows the fish to ingest routinely items such as scales and fin rays of other fish, chitinized fragments of insect, in lined with Qinling lenok mainly feed on worms, crustaceans, insect larvae and small fishes, reflects its carnivorous feeding type (Yue and Chen, 1998).

The U-shaped or sac-like stomach of Qinling lenok is observed easily, especially when it is full of feed. The same feature is present in some classic carnivorous species, such as *Dentex dentex* (Carrassón *et al.*, 2006), *Micropogonias furnieri* (Diaz *et al.*, 2008) and *Anguilla bicolor bicolor* (Nasruddin *et al.*, 2014). These authors thought that this type of stomach allows these fishes to ingest a large content of feed. The classification of the three regions of the stomach as cardiac, fundus and pyloric in this work, is based upon the presence of gastric glands in the anterior portion of the stomach and their absence in the posterior region (Diaz *et al.*, 2008). Presence of the gastric glands in the stomach fundus and cardiac part indicates that Qinling lenok could produce gastric juice containing pepsin like the mammals.

The intestinal coefficient (IC), as an important morphometric parameter, has

been considered as an index to classify species into nutrition categories, and in fact it correlated with feeding habits, age and degree of development phase (Marcos *et al.*, 2004; Wang *et al.*, 2006). Generally, the herbivorous species have very long intestine with a complex convoluted tube referring to high IC values group, but the intestines of carnivorous or piscivorous fishes are short and straight with low IC value, whereas intermediate IC values are associated with omnivores (German *et al.*, 2010). The carnivorous fish tend to have shorter intestines than the herbivorous fish due to lower percentage of plant materials in the diet, and the long intestines of herbivores prolongs the retention time of food and more extensive exposure for the slowly digested cellulose components of food. Pan *et al.* (1996) described the IC values of six kinds of carnivorous fishes ranged from 0.40 to 0.82. Two kinds of fishes belonging family Anostomidae are classified as omnivores with their IC values 1.25 and 1.14, respectively (Albrecht *et al.*, 2001). Ni and Hong (1963) reported the intestinal coefficient of the celebrated herbivores grass carp *Ctenopharyngodon idellus* was above 2.0. However, Kapoor *et al.* (1975) concluded that the intestinal length was influenced by the changes in feeding conditions and the development phase. Cao *et al.* (2011) also pointed out that the intestinal coefficient could only be a criterion-referenced factor for classification of fish feeding habits, since some herbivorous fish exhibited short intestines and small intestinal coefficients. This general relationship

above would allow classify the diet of Qinling lenok with 0.61 IC value was mainly carnivorous feeding type.

The pyloric caeca of Qinling lenok are very similar to the intestine in histological level, but an unfolded mucosa and much longer villi. Generally, the pyloric caeca vary considerably in their number, form, size and arrangement, and constitute a characteristic feature of the intestinal tract of many families of fishes (both freshwater and marine fishes), but Faccioli *et al.* (2015) had been discussed the pyloric caeca are related phylogeny rather than a feature associated to diet. It may be possible that fish with the pyloric caeca was to economize the space for the adaptation of the alimentary canal in the smaller space, where by the length of the intestine has become much less, and the pyloric caeca have arisen evidently to compensate for and supplement the functions of the alimentary canal (Rahimullah, 1945). The highly density of microvilli "caecal villi" is evidently meant for the increase of the absorptive surface; and thus supplements the function of the "intestinal villi" in the fishes which possess caeca. The present study showed that numerous goblet cells were found in the intestine and pyloric caeca of Qinling lenok. Nasruddin *et al.* (2014) had been declared that larger quantity of hard solid foods would need extra lubrication, so goblet cells helped to lubricate - feed items. The function of this mucous secretion in the *Senegal sole* intestine (Arellano *et al.*, 2002) is variable (lubrication, absorption and

transport of macromolecules, enzymatic cofactors, against a variety of factors from acid and hypertonic media to pathogenic microorganisms and general mucous secretion) and acts as a physical barrier between the mucosa and environmental agents (Pajak and Danguy, 1993; Scocco *et al.*, 1997). The mucus secreted by mucous cells may also provide complementation for the degradation (Anderson, 1986).

In ultrastructural level, mucous and glandular cells in the stomach were easily found, according to Ostos-Garrido *et al.* (1993) of which two kinds of cells were involved in producing gastric juice. There were a well-developed tubule-vesicular system, Golgi apparatus and mitochondria in the glandular cells, indicated this type participating in formation of zymogen granules and hydrochloric acid, similar results were reported in *Lates niloticus* by Namulawa *et al.* (2015). The presence of large number of glandular cells in the stomach in the present study could explain how Qinling lenok is able to effectively break down whole fish, including the bones and the scales using chemical digestion. The surface of the intestinal border was lined with a dense layer of microvillus. That is typical of epithelia in which active transport is occurring and consequently, probable functional capabilities might be inferred (Bisbal and Bengston, 1995). Also, the pinocytotic channel was observed at the apical cytoplasm of enterocyte close to microvilli. Pinocytotic inclusions in the enterocyte provide evidence that it play a role in digestion of proteins. Besides, the enterocytes in the anterior intestine

of *Oreochromis mykiss* act as a role of osmoregulation apart from absorption nutrients has been discussed by Abaurrea-Equisoaín and Ostos-Garrido (1996). Ultra structurally, unlike intestine epithelium, high density of lipid droplets in the pyloric caeca was observed, which mainly concerned with fat-absorption. The caeca was demonstrated as one of the main sites for lipid absorption in previous studies (Rahimullah, 1945; Carrassón *et al.*, 2006). Lipids are hydrolyzed as fatty acids and mono glycerides, following re-synthesis in the endoplasmic reticulum. Then, they are stored as lipid droplets in the epithelial cells (De Silva *et al.*, 1980). These epithelial cells of pyloric caeca provided a temporary site for depositing fat droplets. In some fishes, the caeca show endocrine role (Beorlegui *et al.*, 1992), but no similar secretory was found in present study.

In conclusion, the digestive tract of Qinling lenok had a-four-layer structure: mucosa, submucosa, muscularis, and serosa. The U-shaped stomach and short intestine was in consistent with its carnivorous feeding type. Striking glandular cells with a great amount of pepsinogen granules in the stomach, enterocytes with well-developed microvillus of the intestine and epithelia of pyloric caeca with high density of lipid droplets, as well as high amount of goblet cells was observed. Further research should be carried out to elucidate the processes of digestion and absorption of nutrients referring to the feeding pattern for artificial culture of this species in the future.

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