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Ontogenetic development of the digestive system in *Alburnus chalcoides* larvae and juveniles

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Abstract

The growth of the gastrointestinal tract of the larvae and juvenile of *Alburnus chalcoides* was studied after histological analysis using light microscopy, haematoxylin-eosin staining and the mouth development was scanned via electron microscopy. This study focuses on the morphology and histology of the mouth growth and digestive tract of *A. chalcoides* larvae to test the best weaning time for providing practical diet for fry based on the grade of their morphological aspects. It was observed that on the fifth day after hatching, the larvae mouth was opened. On the eighth day, the yolk sac was absorbed by two-thirds. On the same day, food is fed manually. Yolk sac was completely absorbed in 10th day. The histological base of the esophagus was formed by day 3. At day 3, the formation of enterocytes started. Also, the larvae hepatopancreas was formed on 5th day. According to the results, it was observed that after 8 days onwards, larvae of these fish can have a proper diet. At this day, the size of the mouth was 84 µm. when the lips were formed within 20 days and for the mouth angles 45ºand 90º, the food size for mouth, was 168 and 307 µm, respectively.

Keywords: *Alburnus chalcoides*, Ontogenetic, Digestive system, Larvae, Juvenile

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Introduction

The Danube bleak or Caspian Shemaya, *Alburnus chalcoides*, is one of the bony fish species in the Caspian Sea. Its old name was *Chalcalburnus chalcoides* (Mosavi-Sabet, *et al*., 2015). Concerning the economical, biological and ecological aspects, the *A. chalcoides* is one of the most valuable fish (Rahbar *et al*., 2013). It is also very popular for sport fishing. It has commercial value in the southern parts of the Caspian Sea (Mohadasi *et al*., 2013). *A. calcoides* feeds on zooplankton. The *A. calcoides* lives in the rivers adjacent to the Caspian Sea and Aral Sea (Mohaddasi *et al*., 2013). The fish living in the lakes undertake a migration to the upstream part for laying eggs from the start of May to the end of July (Bagherian and Rahmani, 2009).

In recent two decades, the digestion system of the fish larvae has been studied (Zambonino Insante and Cahu 2001) Also, the fish larvae digestion system and their evolutionary route has been greatly studied during the past 20 years (Zambonino Insante *et al*., 2008). It is imperative to possess knowledge regarding the evolutionary and growth rate of the fish digestion system and optimal nutrient absorption during the larval growth stage, in order to be able to study the nutritional physiology of the fish (Kato *et al*., 2004). The studies indicate that for better growth of the fish larvae, the consumption and digestion rate of ingested nutrients and the amount absorbed to the body are of great importance during the larval period (Sarasquete *et al*., 1993). In order to property produce aquatic species, it is important to find the optimal composition of nutrients and the method for maintenance sufficient ingestion of food (Sahlmann *et al*., 2015). After hatching, using live food is required for a short period in order to ensure the survival of fish larvae. Moreover, somatic growth is affected by the morphological constraints applied to the fish behavior (Ramezani-Fard *et al*., 2011). The maximum size of the prey the consumable by the larva is greatly constrained by the mouth size, and it's especially true for the initial stages in which food intake is vital for the survival of larvae.

Growth of digestive system during larval and juvenile stages and designation of weaning time have been studied for several teleost species (Kato *et al*., 2004; Zambonino Infante *et al*., 2008; Ramezani-Fard *et al*., 2011; Sahlmann *et al*., 2015). This research describes the ontogenetic development of digestive system in *A. chalcoides* larvae and juveniles during the first 2 months of the life. The significance of this study is finding the age when larvae are likely to be able to ingest and potentially digest the formulated diets expeditiously.

Materials and methods

Fish collection

A. chalcoides larvae were prepared from Shahid Ansari Center, Rasht, Iran. Random sampling of artificially fertilized eggs was observed on days 1, 2, 3, 4, 5, 7 and 8. The eggs were hatched after 3 days. Five to seven days after hatching the larvae started

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swimming vertically to the surface of the water. Larvae had the ability to use external food items from day 8. Therefore, the Rotifer and Daphnia were used as food for the larvae. Also sampling was carried out from day 10 to 60.

Histological study

Exact 20 larvae were collected at room temperature for 24 hours and then kept in Bouin's solution every day (Ramezani-Fard *et al*., 2011; Khoshnood *et al*., 2015). Then the solution was replaced with 70% ethanol. After 72 hours of sample preparation, 6 micron sections and Haematoxylin-Eosin staining was performed (Khoshnood *et al*., 2014; Zakeri Nasab *et al.*, 2018). The slides were used for light microscopy and photographed by digital camera (Canon A1400). In this study, the oral cavity – pharynx, esophagus, intestine and accessory glands of *A. chalcoides* were studied.

Study of scanning electron microscopy

It was observed that on the fifth day after hatching, the larvae mouth was opened. Also, digestive system was completed in 5-8 days. Also, 5 larvae were prepared to check the state of the mouth, including the upper and lower jaw, according to the shape measure. The larvae were fixed in 4% glutaraldehyde solution at 4Cº (Ramezani-Fard *et al*., 2011). The samples were washed with 0.1 M sodium cacodylate and then stabilized in 1% buffered osmium tetroxide buffer to remove the fixative after 24 h (Ramezani-Fard *et al*., 2011). Then, samples were freeze dried (Freeze Drier, Germany, Model: Christ Alpha1.4 plus). Dried samples were coated with gold and examined by a scanning electron microscopy. The gap of the jaw was estimated by assuming that the mouth opens at a 90º angle during food capture (Ramezani-Fard *et al*., 2011). As this value is the upper limit of food particle size for the fish, a predicted prey size (prey width) was also estimated by assuming a mouth opening of 45º as the most frequent opening angle for feeding larvae of cyprinids species (Ramezani-Fard *et al*., 2011). The projection diagram shows how the linear distance of the oral gap was measured (Fig.1).

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Figure 1: Larval mouth: Two black lines on the image indicate the larval lower and upper jaws and disk center shows the point from which angle was measured to be 45º and 90º (Ramezani-Fard *et al*., 2011).

Results

Mouth morphological growth

The mouth is closed from day 1 to 4. According to photos taken at the 5th day of oral clefts and epithelial cells visible. On day 7 and 8 oral cavity is orbicular. On the eighth day, the mouth opens84 µm. For the roundel mouth larvae this occurs on day 10 (Table 1). On day 20, the lips are formed. The length of the already formed lips of the jaws was measured on 20th day (Table 2). Over time, the morphology and size of the mouth, changed during the growth.

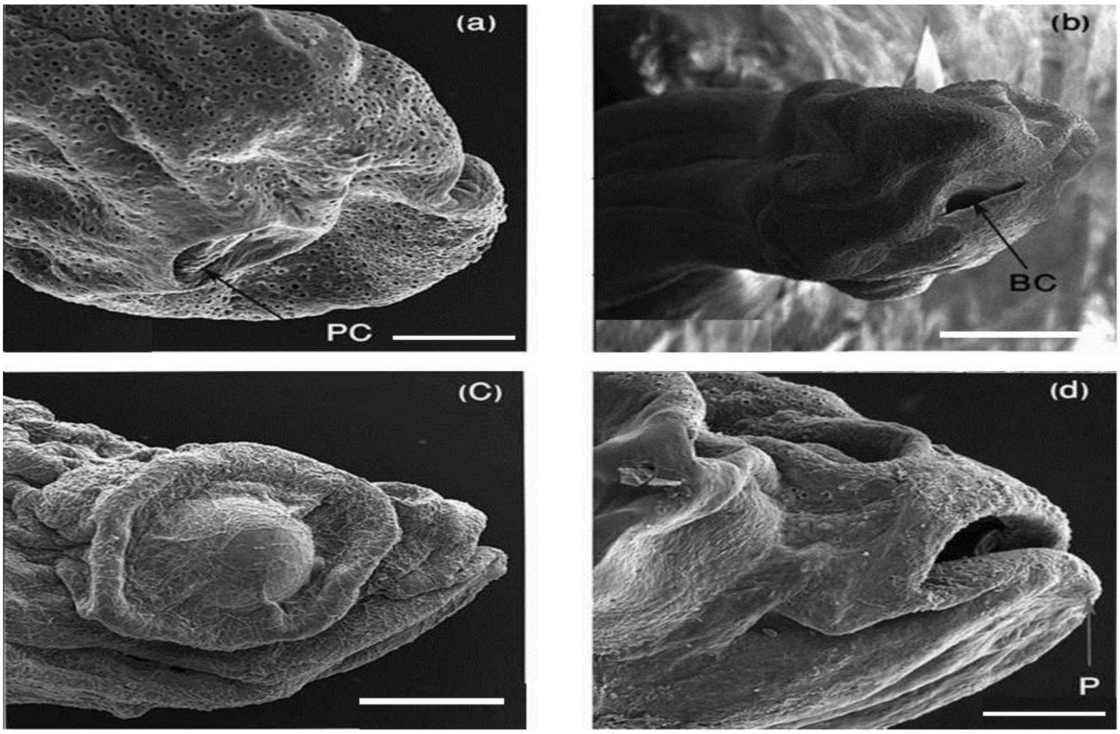

Figure 2: The results of electron microscopy micrograph scan of the mouth. (a) *Alburnus chalcoides* larvae's mouth was opened on 5th day. Pavement cells epithelium were seen in 5th day. Scale bar = 100 µm. (b) Crescent-shaped mouth of larvae at day 10. The buccal cavity is determined on the photo. Scale bar = 200 µm. (c) lips: At day 20, the lips were formed. Scale bar = 200 µm. (d) Papilla. Scale bar = 100 µm.
Pc= pavement cavity epithelium; Bc= buccal cavity; P= papilla

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Table 1: Development of morphometrics in larval *Alburnus chalcoides* mouth cavity size from day 7.

Larval age	Total length (µm)	mouth cavity size
7 Day	5.2 ± 6.8	72 ± 9
8 Day	5.5 ± 4.4	84 ± 7
10 Day	5.8 ± 3.8	97 ± 2
15 Day	6.3 ± 8.2	136 ± 2

Table 2: Development of morphometrics in larval *Alburnus chalcoides* from day 20 to 60. Lower jaw length, upper jaw length and mouth gap (45º and 90º opening) from day 20 (When it was formed lips) to 60 after hatching.

		Length of jaw (µm)		Mouth gap (µm Ø)	
Larval age	Total length (mm)	Lower jaw	Upper jaw	45º	95º
20 Day	7.4 ± 1.4	233.2 ± 14.2	200.0 ± 8.1	168.4 ± 24.1	307.0 ± 13.8
25 Day	9.2 ± 1.8	272.9 ± 31.5	278.3 ± 9.4	205.2 ± 18.5	379.0 ± 11.4
30 Day	12.8 ± 0.6	283.6 ± 9.1	291.5 ± 18.4	219.6 ± 13.3	405.9 ± 30.4
40 Day	17.4 ± 0.2	336.3 ± 21.2	354.5 ± 9.2	264.6 ± 8.2	488.0 ± 23.4
50 Day	26.1 ± 1.2	392.2 ± 6.2	408.2 ± 14.1	306.4 ± 18.1	565.7 ± 9.4
60 Day	33.4 ± 0.6	460.8 ± 13.6	500.0 ± 6.4	369.2 ± 9.8	679.4 ± 41.2

Hatching digestive tract

On day 1, part of the intestine is visible. Enterocyte cells of the intestine and liver hepatocytes can be detected from day 3. The yolk sac on day 1 is still large and larvae use it as a food source. The yolk sac is completely absorbed by the day 10 (Fig. 3).

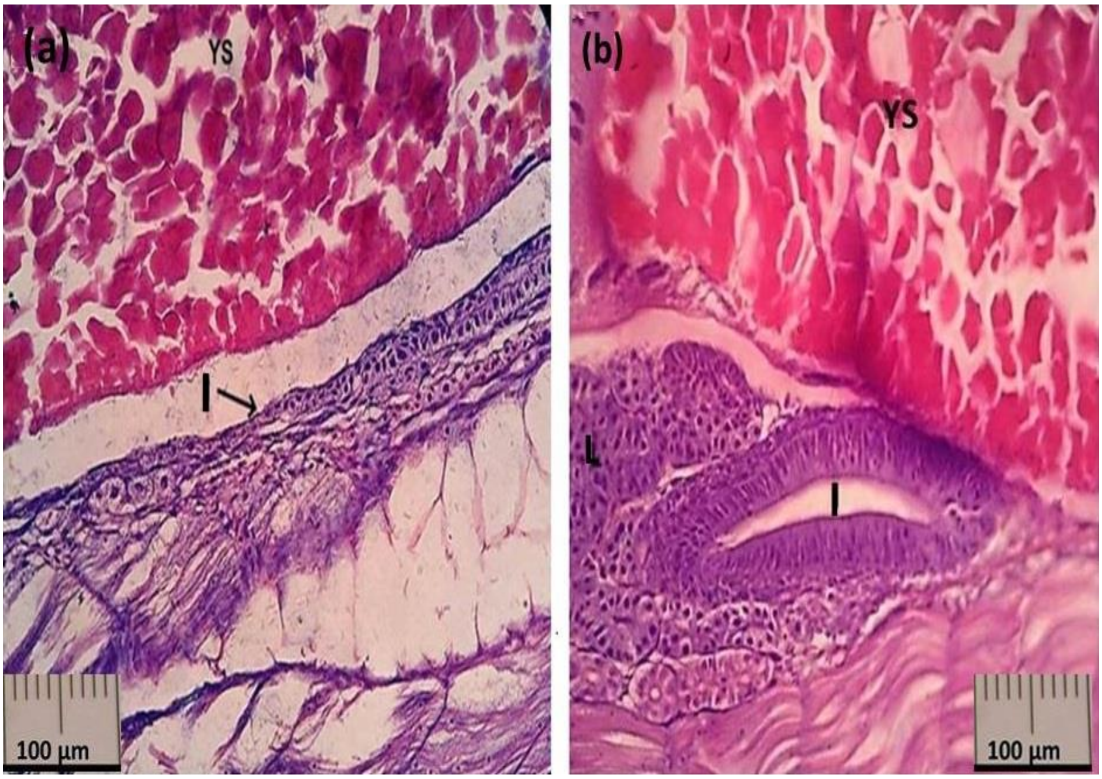

Figure 3: Histology of the early digestive tract: (a) Longitudinal-section: part of intestine on day 1. (b) Longitudinal-section: Enterocyte cell I= intestine; YS= yolk sac; L= liver

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The mouth and oral cavity - pharynx

For the one-day larvae, the mouth was closed and did not open until the fourth day. The oral cavity was covered with a layer of pavement cells epithelium, and cartilage was identified early in the form of small pieces on day 5 (Fig. 4a). The pharynx was very short and frail at the end of the gill filaments. During the growth of the oral cavity, simple epithelial cells transformed to the stratified type (Fig. 4b). Simultaneous with the opening of the mouth, pharynx can be observed clearly. Pharynx consists of cubic cells. In addition, goblet cells can also be observed in this area (Fig. 4c), and with growth development, their number increases. From day 7 onwards, taste buds can be observed in the pharynx (Fig. 4d). Taste buds were seen in the mouth highlighted from day 20 and on this day pharyngeal teeth redundancies also was observed (Fig. 4e).

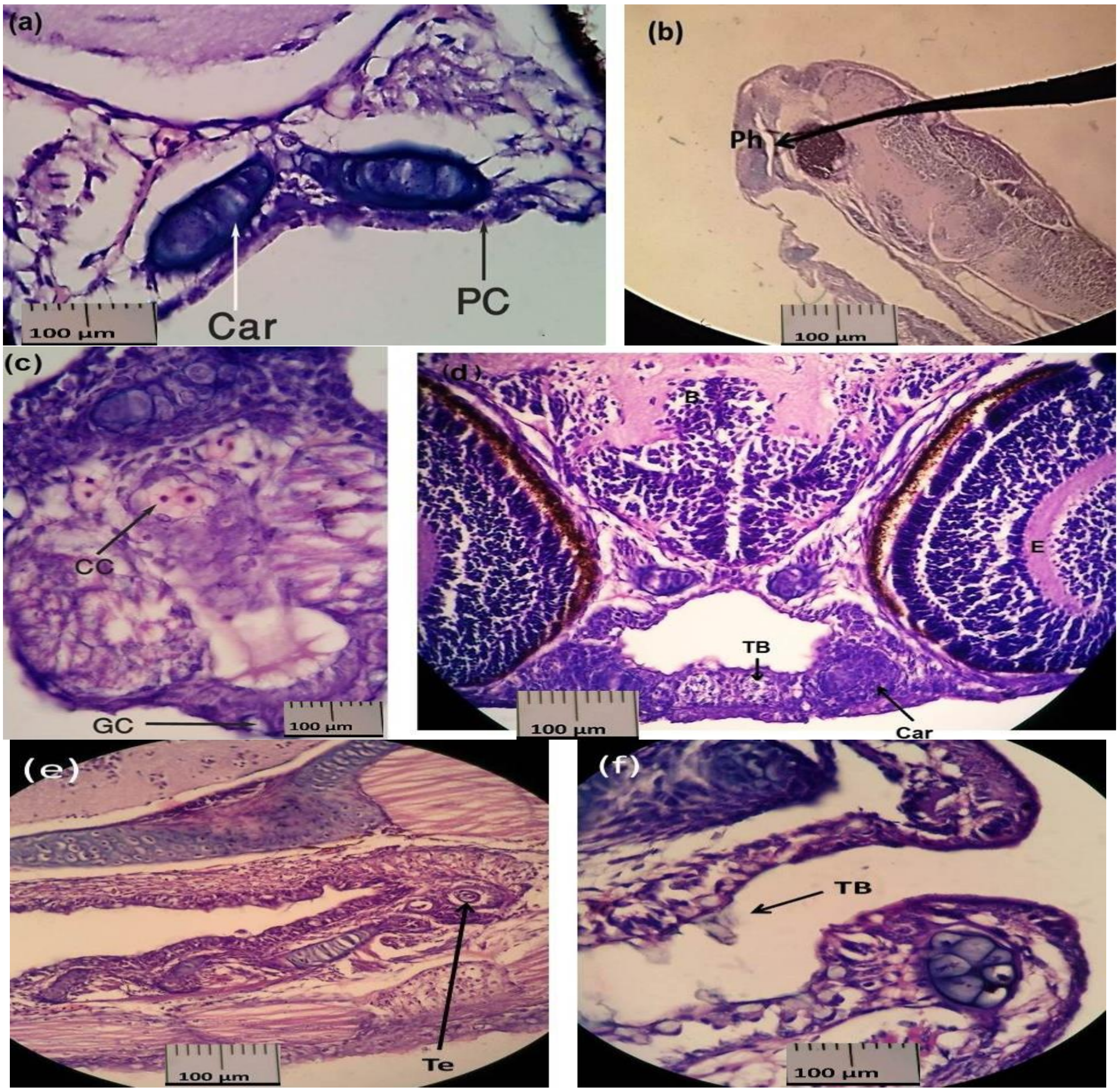

Figure 4: Histology of larval oral cavity – pharyngeal: (a) Cross-section: oral cavity on day 5. (b) Longitudinal-section: Pharynx on day 5 is concordant with mouth's opening. (c) Cross-section: Pharynx consists of cubic cells. In addition, goblet cells can also be observed in this area (d) Cross-section: Taste buds can be seen from day 7. (e) Longitudinal-section: Pharyngeal teeth on day 20 (f) Longitudinal-section: Taste buds on day 20.

Car= cartilage; PC= Pavement cell; CC= cubic cell; GC= goblet cell; E= eye; TB= taste bud; Te= Teeth

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Esophagus

Esophagus is formed after 3-4 days. The muscular layer can be observed around the esophagus. Goblet cells increase in number on day 5 (Fig. 5a), and the taste buds can be seen in abundance on the 7th day (Fig. 5b). On day 4, esophagus has a narrow curvature, and is connected to the intestine and this connection gets stronger in 15 days (flash) (Fig. 5c). Esophagus horny cells can be observed from day 8 and their number increases from day 20 onwards (Fig. 5d). The number of taste buds is increased by day 20 and protuberant can be observed with the secretion of mucus cells being very high.

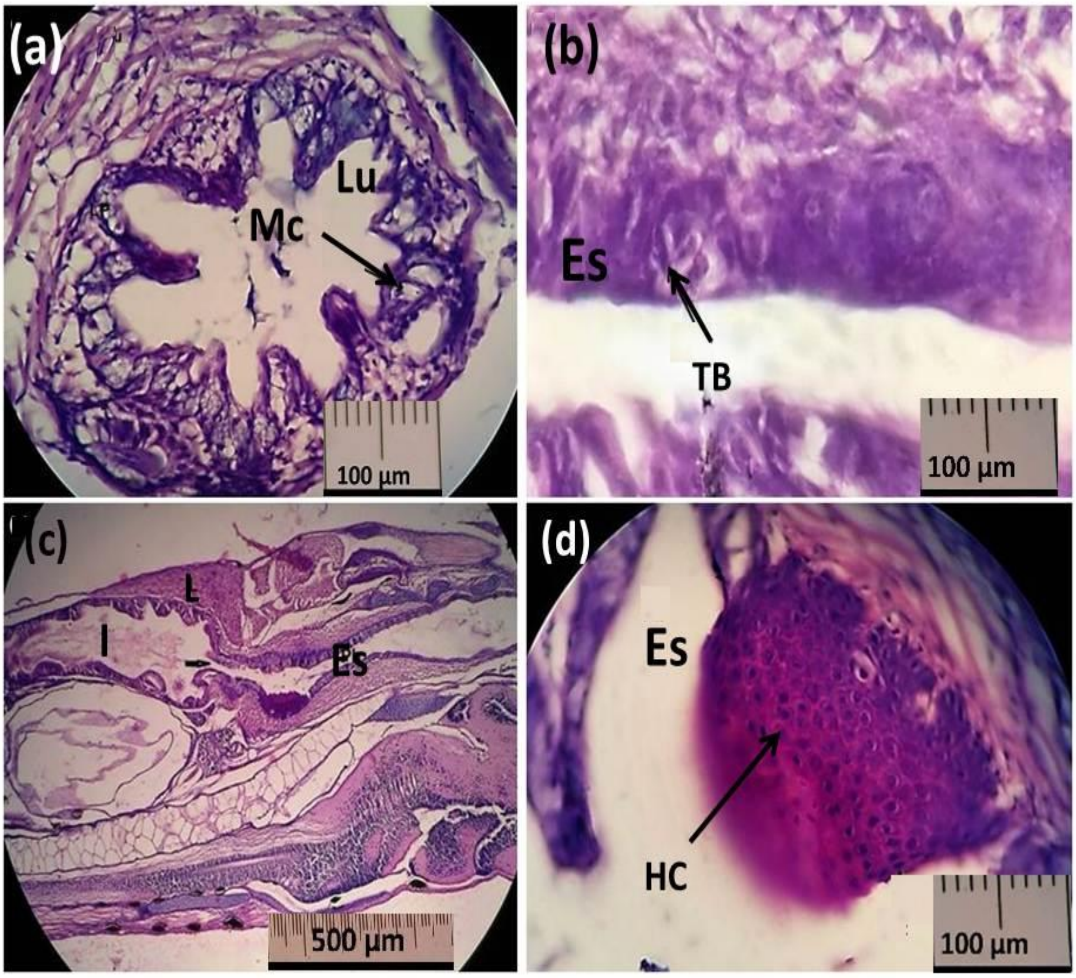

Figure 5: Esophagus in development: (a) Cross-section: Goblet cells on day 5. (b) Longitudinal-section: taste buds on day 7. (c) Longitudinal-section: Esophagus connected to the intestine completely marked on the 15-20th day. (d) Cross-section: Horny cells, from day 20.

I= intestine; L= liver; Es= esophagus; HC= Horney cell; TB= taste bud; Mc= mucus cell; Lu= lumen

Intestine

On the first day, the posterior part of the intestine was formed. On the second day, the bowel was more completed, and on day 4, it was attached to the esophagus. Intestinal epithelium consists of cells formed as a column. On day 5 goblet cells were mingled with the intestine cells, and from day 7 onwards the number of goblet cells

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increased and in the anterior part of the intestine too, most of the posterior segment was observed and the difference between the posterior and anterior parts of the rest was visible. By day 5, there were no wrinkles on the intestine (Fig. 6a), while on day 7, wrinkles could be observed in the anterior part of the intestine. From day 8-10 it folded over more deeply and there were more wrinkles in the anterior part of the intestine as compared to the posterior. Before day 10, there were no intestinal microvilli (Fig. 6b). But, from day 10 onwards, finger-like microvilli could be seen across the depth of the intestine and the discharge of mucus cells was increased.

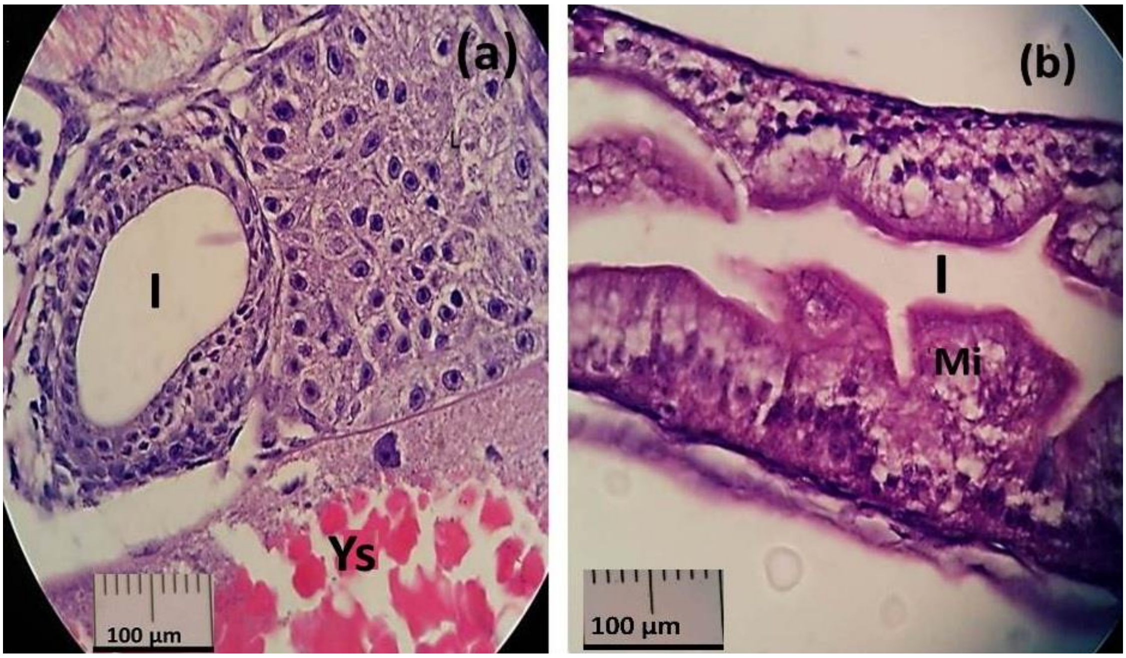

Figure 6: Intestine: (a) Cross-section: Day 5, intestine without wrinkles (b) Longitudinal-section: Day 10, intestines, has wrinkles and finger-like microvilli.
I= intestine;
Ys= yolk sac; Mi= microvilli

Accessory glands

On day 3, the liver was formed. Liver cells were polygonal and had a spherical nucleus. On the 8th day of liver cells formation, the liver ducts and blood vessels (sinusoidal) could be seen. On day 4, pancreatic could be detected. On day 8, the posterior part of the body could be seen in the hepatopancreas (Fig. 7a). It was more complete in 15-20 days, and then was separated from the anterior portion of the pancreas and liver which could be seen separately. Gallbladder was observed from day 4 and after 5-7 days, association with intestinal tract was clear. On day 20, its size was larger than previous days (Fig. 7b).

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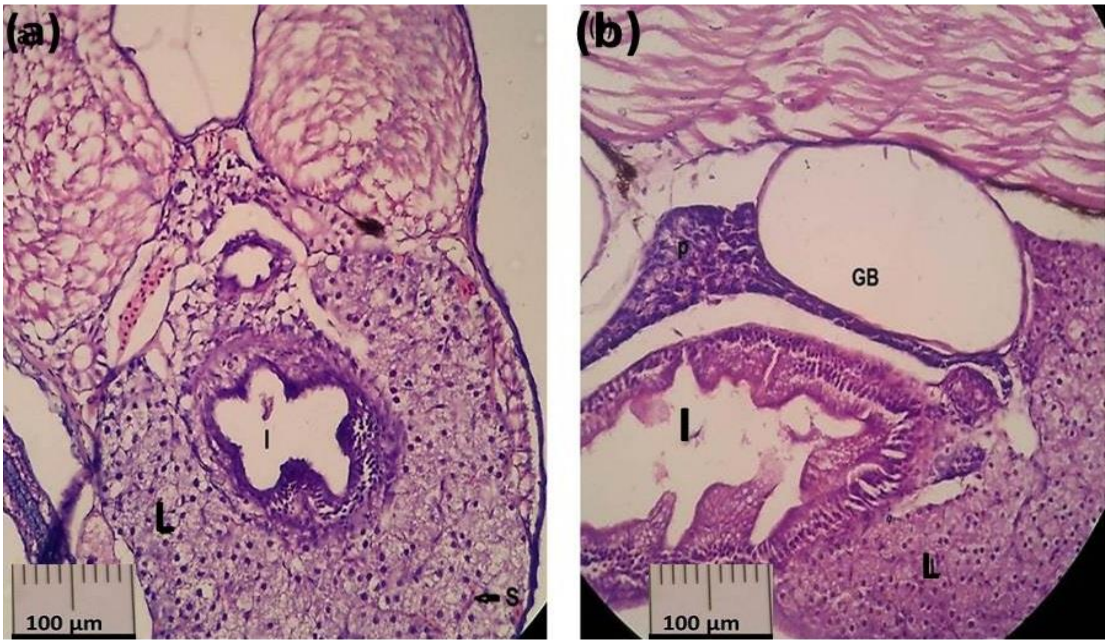

Figure 7: Accessory glands during growth: (a) Cross-section: Hepatocytes and sinusoidal on day 8. (b) Longitudinal-section: Gallbladder enlargement, more specifically in the posterior part of the hepatopancreas on day 20.
I= intestine; S= sinusoid; L= liver; GB= gall bladder; P= pancreas
In digestive system of this fish, significant changes were not observed from the day 25 and only morphological changes were found. In fact, growth of organs was more than previous days (Fig. 8a-e).

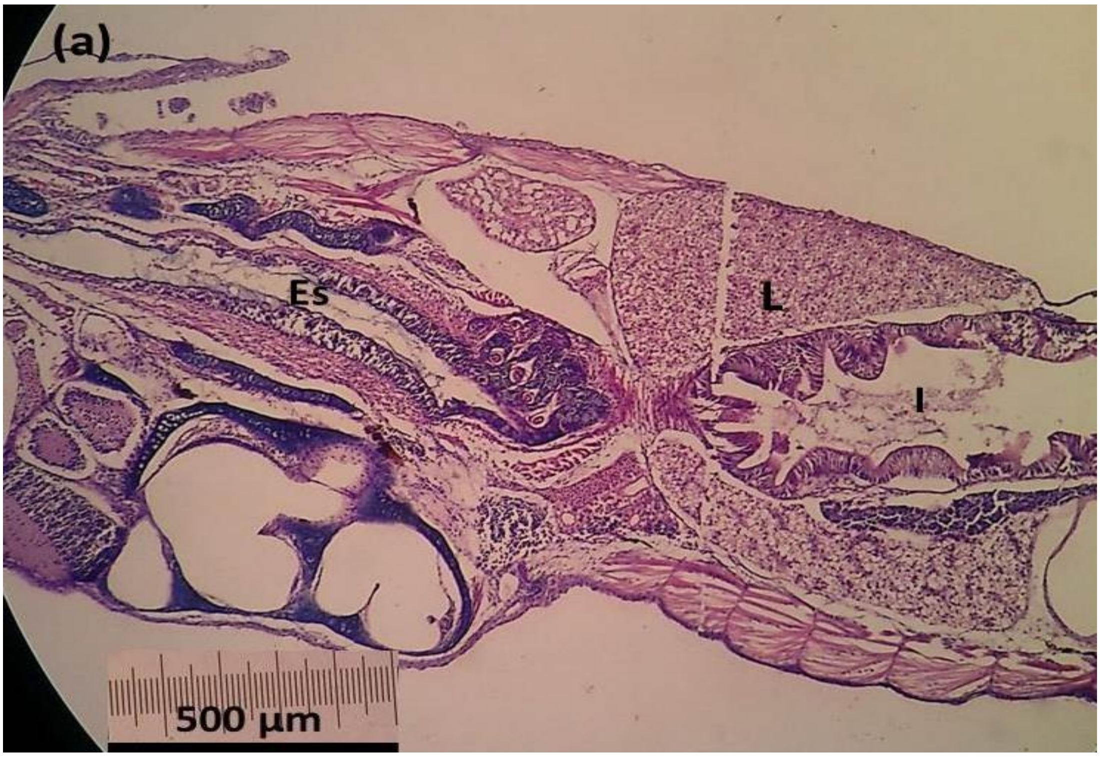

Figure 8: Longitudinal-section: (a) Digestive system after 30 days.

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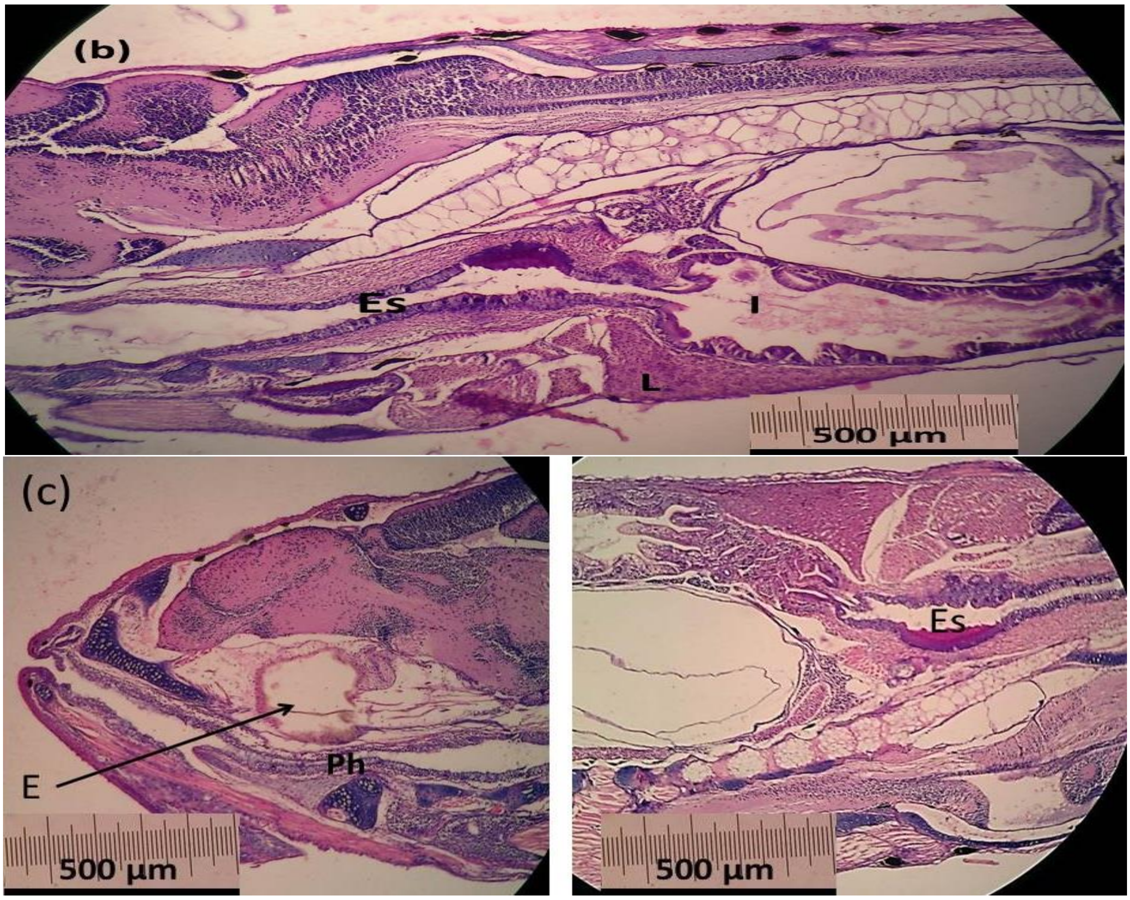

Figure 8: Longitudinal-section: (b,c) Digestive system after 40 days.

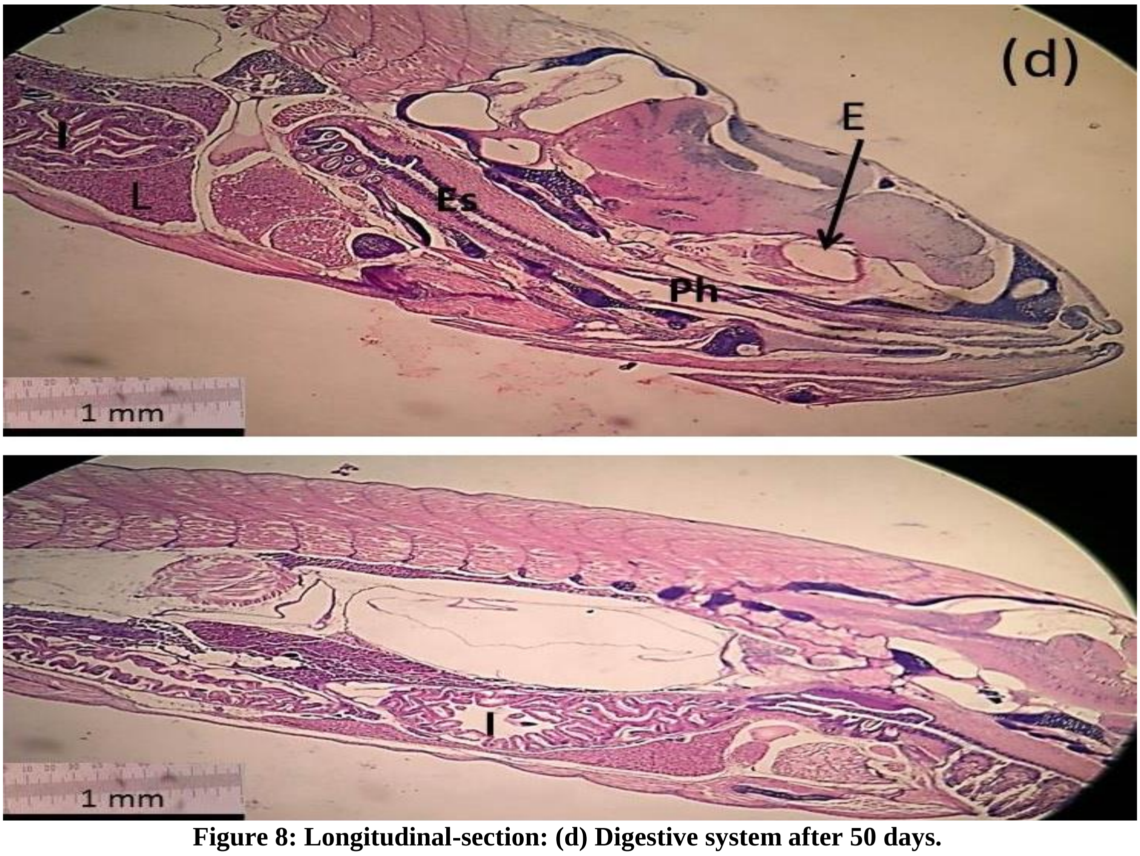

Figure 8: Longitudinal-section: (d) Digestive system after 50 days.

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Figure 8: Longitudinal-section: (e) Digestive system after 60 days.

Discussion

Significant differences were observed between this fish and other species. Mouth was closed for the larvae up to 4 days old and opened at the beginning of day 5. While for Malaysian mahseer, *Tor tambroides*, the mouth was opened from day 1 (Ramezani-Fard *et al*., 2011) and for *Oplegnathus fasciatus* mouth was opened by day 3 (He *et al*., 2012). Newly hatched larvae of *Diplodus puntazzo* absorbed endogenous food reserves (oil globule and yolk sac) during the first two days and then anus and mouth opened at 3 days (Okan Kamaci *et al*., 2010). The digestive system comprises buccal cavity, pharynx and esophagus, anterior and posterior intestine and accessory glands. *A. chalcoides* do not have real stomach. Intestinal is smooth at first and then becomes wrinkled. *A. chalcoides*, deeper wrinkles enable more efficient digestion of food. Glands are included the liver and pancreas. Exocrine pancreatic is hepatopancreas in the posterior part. Gallbladder is formed by day 4. The intestinal tract takes 5-8 days to be completed. The fish taste buds were observed on the 7th day and their number increased from day 10 onwards. The larvae of *T. tambroides* taste buds were formed by epithelial cells on the lips at day 4, and by 5 days, coinciding with the start of exogenous feeding, taste buds, and goblet cells were secreted (Ramezani-Fard *et al*., 2011) and at 8–9 days, the taste buds, tooth, and goblet cells appeared in the buccopharynx of rock bream, *Oplegnathus fasciatus* indicating that the larvae had acquired the ability of palatability evaluation and swallowing reflex (He *et al*., 2012). In the larvae of *O. fasciatus*, at day 8, the taste buds can be appeared (He *et al*., 2012).

In the research conducted by Albrecht *et al*. (2001) it was found that the existence of taste buds on the external surface of the lips might indicate the ability of fish to choose the food consumption. Oral larvae were developed on day 8 (when the mouth

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hole size is 84 µm), but on day 20 the lips and mouth size were 168 and 307 µm, respectively. For the *T. tambroides* larvae, the sizes of 248 and 413 µm have been reported (Ramezani-Fard *et al*., 2011). These measurements help in choosing the optimal feeding for the larvae based on the time (Ramezani-Fard *et al*., 2011). Dabrowski and Bardega (1984) suggested that the size of food or prey ingested by fish larvae is also affected by the density of the linked food. They have observed that prey found in the gut of coregonid larvae (*Coregonus pollan*), collected from Lough Neagh, were 13–26% of the mouth size; however, in an enriched aquarium with a high density of the same zooplankton, the larvae chose larger prey with a size between 40-60% of the mouth size. In *A. chalcoides* goblet cells were seen for the first time in 5 days. The maximum and minimum ranges for the days of their emergence were 5 and 7, respectively. By day 20, they were increased in the number and had a lot of mucus secretion. But the first goblet cell in the digestive tract of *T. tambroides* was appeared at 2 days, while in larvae of *Oplegnathus fasciatus* they appeared in the esophagus at 8 days, and for the larvae of buccopharyx, at 9 days. As larval development proceeds, the degree of stratification of the epithelium, as well as the number of goblet cells and taste buds increases in the buccopharyngeal mucosa. At the end of the larval stage, buccopharyngeal papillae, which are involved in food predigestion and transport processes, appear at the posterior region of pharynx in California halibut and yellowtail flounder (Zambonino Insante *et al*., 2008). The enormous secretion and population of goblet cells in the esophagus have a lubricating role in food transportation as well as a saliva-like function in protecting the mucosa of the alimentary canal against physicochemical damage and bacterial attack (Ramezani-Fard *et al*., 2011). Furthermore, a pregastric digestion role has been suggested for the large quantity of mucus in the post-esophagus of fish. The latter function might have a prominent role in the digestion process of stomachless fish such as *T. tambroides* (Ramezani-Fard *et al*., 2011).

This study showed that *A. chalcoides* larvae's yolk sac endures up to 10 days uses. From day 8, the larvae have an ability to digest their meals. On this day, the digestive system was completed. Nevertheless, more physiological studies are essential in order to check the digestive enzymes activity and to obtain a clear definition regarding the age at which the digestive tract becomes totally functional. Therefore, the precocious digestive system in *A. chalcoides* larvae will enable useful knowledge to better usual larval rearing practices and feeding protocols, and will decrease weaning costs for this species.

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