Ontogenic development of digestive accessory glands in larval and juvenile Vimba bream, *Vimba vimba* (Pallas, 1814)

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Abstract

The current study investigated histological development and histochemistry of digestive accessory glands of Vimba bream *Vimba vimba* from hatching through 60th day after hatching (DAH). The samples were randomly taken from day 1 (hatching) until the full absorption of the yolk sac at day 7 and the day after that, then with 2, 5, and 10 days intervals until 60 DAH. The development of the digestive accessory glands was followed histologically using light microscopy after Haematoxylin-Eosin and Periodic-Acid Schiff (PAS) staining. A primordial liver was observed at 1 DAH in the ventral region of the yolk sac, pancreas at 2 DAH, gall bladder and bile duct were developed at 4-5 DAH. At the same time when larvae were growing, digestive accessory glands continued differentiation and, at 5 DAH, hepatocytes of liver with eosinophilic cytoplasm and basophilic granules showed regular shape around sinusoids. Coinciding with the liver development, sinusoids developed as well and hepatic cells increased in size. Also, at this stage, eosinophilic zymogen granules of exocrine portion of pancreas were observed. Secretory activity of digestive accessory glands began with the emergence of PAS positive cells followed by secretion of neutral mucopolysaccharides and glycogen compounds in liver cells of larvae at 4 DAH and pancreatic cells of larvae at 4-7 DAH. The results of this study can be effective in identifying growth limiting factors in breeding larvae, reducing casualties during exogenous feeding, and even developing a proper diet for its digestive system.

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Introduction

Vimba bream, *Vimba vimba* is one of the most valuable migratory fish species of Caspian Sea which migrates to upstream areas of Caspian Sea for spawning. It is introduced as an endangered species whose populations in Caspian Sea have decreased dramatically in recent years (Abdoli and Naderi, 2009).

In fish hatchery, after yolk sac absorption, larvae feeding is very important because the highest fish casualties occurs during this period, i.e. in transition from endogenous to exogenous feeding (Moshayedi, 2016). Thus, among different stages of fish growth, larvae or early stages of growth is a critical phase because of adaptation of embryonic period (feeding from yolk sac) with post-embryonic period (Bisbal and Bengston, 1995). Therefore, the highest casualties are observed at this transition stage.

In recent decades, histological techniques and microscopic studies have been very common in order to understand physiological status of different fish organs. Investigating the developmental changes of digestive organs, especially their secretory glands, which is related to ingestion and digestion in the larval stage, seems necessary. These studies are effective in determining the mechanism of nutrition and the effects of primary dietary regimens on developmental system of these organs (Gisbert *et al*., 1999).

Accessory glands are playing a significant role in both digestive enzymes secretion and nutrients metabolism mechanisms. The digestive tract and accessory glands of many marine fish larvae undergoes numerous morphological and physiological changes during ontogeny that can substantially influence larval survival under culture conditions. Although, larval fish may be morphologically capable of capturing food items at first feeding however, the digestive system needs a series of changes before being fully functional (Okan Kamaci *et al*., 2009).

During the last decade, several researches have been performed on histological development of accessory glands of fish larvae in order to know about the nutritional capabilities of young larvae and established feeding protocols for optimizing larval rearing, including: Ontogenic development of digestive tract as well as digestive accessory glands in Malaysian mahseer, *Tor tambroides* (Ramezani-Fard *et al*., 2011), *Schizothorax zarudnyi* (Shahriari Moghadam *et al*., 2014), *Chitala chitala* (Mitra *et al*., 2015), *Clarias gariepinus* (Ikpegbu *et al*., 2012), Atlantic Salmon, *Salmo salar* (Sahlmann *et al.,* 2015), Common carp, *Cyprinus carpio* (Moshayedi, 2016), Sterlet, *Acipenser ruthenus* (Wegner *et al*., 2009), *Solea selegalensis* (Ribeiro *et al*., 1999), and *Amphiprion percula* (Gordon and Hecth, 2002).

Despite case studies on digestive system of *V. vimba* and its morphometric structure, there is lack of information on ontogenic development of accessory glands and their secretory activities. The role of Cyprinids in protein supply and their economic importance is significant. In addition, lack of adequate studies on different histological fields of some species necessitates further studies to analyze their microscopic structures at different stages of their growth and development. Moreover, understanding the

initial stages of fish development is a basic requirement for breeding a species since with better knowledge of the initial development process of a species; it is possible to provide larviculture protocols in its breeding management plans.

The present study aims to describe the main histological and histochemical changes of digestive accessory glands in order to provide fundamental knowledge on the hatchery management for commercial aquaculture.

Materials and methods

In this study, broodstocks of *V. vimba* were taken during their reproductive migration to southern rivers of Caspian Sea in May 2016 and then they were transferred to Shahid Ansari hatchery, rearing and bony fish stock recruitment center of Rasht for artificial propagation. After fertilization, the eggs were transferred to incubation section. Then, they were transferred to glass incubators to spend the rest of their incubation period in these incubators. Water temperature during the incubation period was (19±1.65) °C. After that, the larvae were transferred into rectangular traps and then to a cultivating pool. The samples were randomly taken on a completely random basis, in the morning of first, second, third, fourth, fifth, sixth, seventh and eighth DAH, then with 2, 5, and 10 days interval, until 60th DAH (being released into the sea). Almost 10 samples were taken from each phase. Larvae's' feeding was carried out by live food (zooplanktons such as Rotifer) and formulated fish meal (SFC-0, 2-3 times per day, Mazandaran Animal and Aquatic Feed, Iran).

The larvae samples were randomly selected and because of their small size, the whole fish fixed in Bouin solution for subsequent histological studies. The samples dehydrated in an ascending series of ethanol, cleared in xylene, and impregnated with paraffin (Bio-Optica, Italy). Paraffin blocks were sectioned using a microtome (Leica RM 2145) and 5μm-thick sections were mounted on a glass side. The Haematoxylin- Eosin and Periodic-Acid Schiff stains were used to describe the development of the digestive accessory glands under a light microscope (Nikon Eclipse E600, Japan) for histological and histochmical observations (Pearse, 1985; Khoshnood *et al*., 2011).

Results

At first DAH, the liver cells were composed of small basophilic polygonal cells with small cytoplasm (Fig. 1-A). At 3 DAH, liver (L) and pancreas (P) were separately distinguishable and a thin layer of connective tissue was observed between them (Fig. 1-B). Until third DAH, hepatic cells showed irregular and scattered shape and, at the same day, significant signs of liver formation appeared as a specific glandular structure in the anterior part of digestive tract. Liver tissue showed sinusoids which contained red blood cells (Fig. 1-B). At fifth DAH, hepatocytes with almost central nucleus and foamy cytoplasm containing light lipid vacuoles were observed (Fig. 1-C). Sinusoids with red blood cells (Fig. 1-C) were observed too. At fifth DAH, when vacuolization of hepatocytes began, sinusoids increased (Fig. 1-C). Between 7-8th DAH, the liver increased in size and, lipid vesicles were observed, as well. Moreover, sinusoids

became more expanded and basophilic hepatocytes became paler (Fig. 1-D).

During larval growth, liver structure continued differentiation and, at 8th DAH, more vacuoles were observed to store glycogen and lipids, the number of which increased on the following days (Fig. 1-D). At 20th DAH, with increasing of hepatocytes, larvae showed the largest liver size, when compared with its previous days (Fig. 1-E).

At 30th DAH, the liver grew notably in size and central veins (Fig.1-F), lipid vacuoles (Fig. 1-F), and sinusoids were observed (Fig. 1-F).

Thus, in the final stages of growth, liver structure allocated considerable amount of abdominal cavity to itself, considering that hepatic cells increase and proliferation was the most important developmental characteristics of liver.
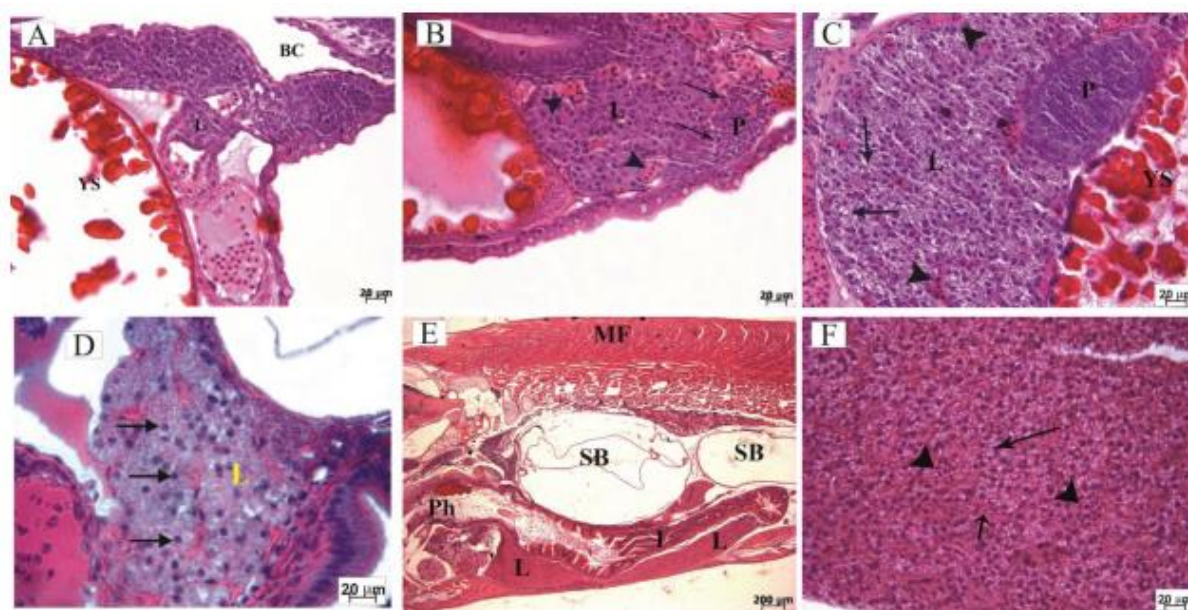

Figure 1: Histology of larval hepatopancreas (H&E).
(A) Appearance of Liver at 1th DAH; (B) Differentiated liver and pancreas with sinusoids (Arrowheads) and a thin layer of connective tissue between liver and pancreas at 3th DAH (Arrows); (C) Hepatopancreas at 5th DAH including Eosinophilic cytoplasm with light lipid vacuoles (Arrows) and sinusoids (Arrowheads); (D) Sinusoids with more expanse at 8th DAH m(Arrows show hepatocytes); (E) Expanded liver in the abdominal cavity at 20th DAH; (F) Hepatic tissue with central veins (Arrowheads) ; lipid vacuoles (Big Arrow) and sinusoids (Small Arrow) at 30 [th] DAH; BC, Buccal Cavity; I, Intestine; L, Liver; MF, Muscular Fibers; N, Notochord; P, Pancreas; Ph, Phaynx; SB, Swimming Bladder; YS, Yolk Sac.

After histochemical analysis of digestive accessory glands (PAS staining), the following results were obtained:

At 4th DAH, the liver showed PAS-positive hepatocytes (Fig. 2-A) and fat vacuoles (Fig. 2-A).
Between 5-7th DAH, larval hepatic tissue showed PAS-positive hepatocytes and fat vacuoles (Fig. 2-B).

At 7th DAH, hepatocytes were stained by PAS staining (Fig. 2-C) which is indicative of available polysaccharides, and consequently glycogen.

With the onset of exogenous feeding at 8th DAH, bolder PAS-positive granules and fat vacuoles were observed (Fig. 2-D); Epithelium brush border of Intestine also showed positive reaction to PAS staining

(Fig. 2-D). Intestinal lumen is marked with (*).

The same histochemical pattern was observed at 10th and 15th DAH; more stained glycogens were observed during PAS staining at 15th DAH (Fig. 2- E).

In general, hepatic cells reaction to PAS staining was positive due to compressed glycogen deposits between hepatocytes and it became more intense, with increased glycogen deposits, during the final stages of development (Fig. 2–F).
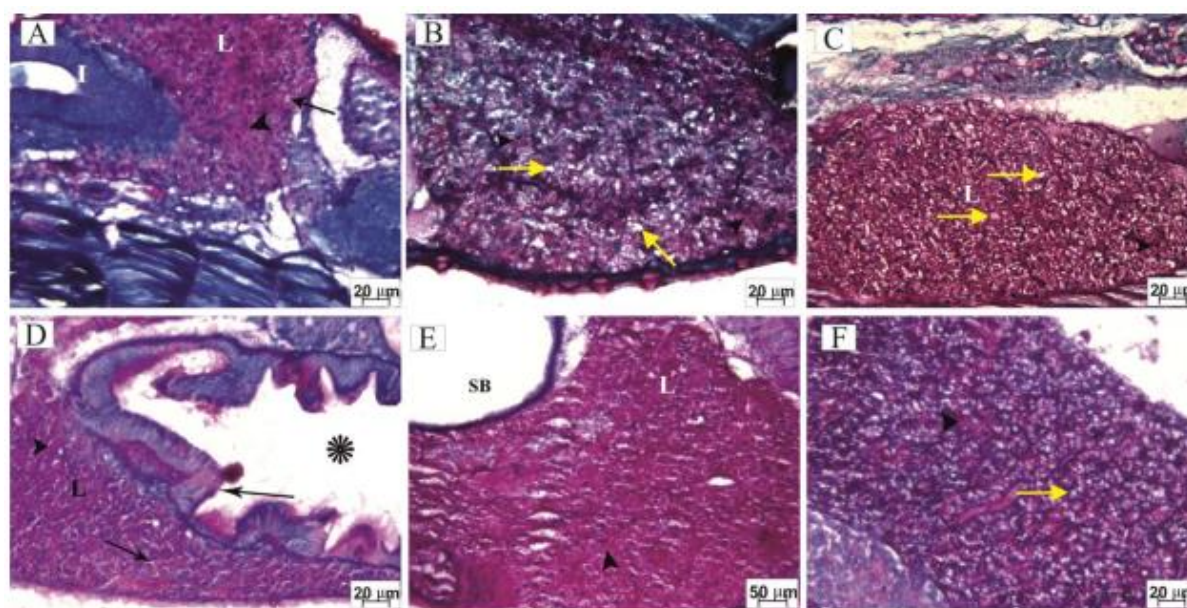

Figure 2: Histochemistry of larval hepatopancreas (PAS Staining).
(A) Longitudinal section of *V. vimba* larval liver at 4th DAH (PAS-Positive),Hepatocyte(Arrowhead), Fat vacuole(Arrow); (B) Hepatic cells positive for PAS at 5th DAH, Hepatocyte (Arrowheads), Fat vacuole(Arrows); (C) Accumulation of glycogen–containing vacuoles in hepatocytes at 7th DAH (Arrows and Arrowheads); (D) Hepatocytes (PAS positive) with vacuoles at 8th DAH (Arrow and Arrowhead); Big Arrow showed PAS positive brush border of Intestine; * showed lumen; (E)–(F) PAS Positive hepatic cells at 15th DAH, 30th DAH (Arrowheads), (Arrow shows fat vacuole). I, Intestine; L, Liver; SB, Swimming Bladder.

The pancreas was distinguishable from the liver at 3-4th DAH, by strongly basophilic cells (Fig. 3-A). At 7th DAH, pancreatic tissue showed exocrine cells (Fig. 3-B) and traces of developing endocrine portion of pancreas (Langerhans islands) (Fig. 3-B). At this stage, acinar pancreatic cells were easily detectable because of available light eozinophilic zymogen granules in cytoplasm. Also, a few number of PAS-positive zymogen granules were observed on the same day (Fig. 3-C).

As the intestinal length was increasing and folding began, pancreas was observed adjacent to the anterior intestine. So that, between 8-10th DAH, basophilic pancreatic cells and eosinophilic zymogen granules observed (Fig. 3-D) at apical section of acinar cells. At 15th DAH, exocrine portion of pancreas has increased in size and showed larger and bolder PAS positive granules (Fig. 3-E).

During the growth process, vacuoles grew in size and constituted a major part of the cytoplasm. At 20th DAH, fat cells (Fig. 3-F) were observed around and between pancreatic tissues.

Since 20th DAH until the end of the study, pancreatic tissue structure showed no significant changes, except increasing size and number of pancreatic acinies. PAS staining pattern also showed that cells staining tendency increased until the end of the sampling (Fig. 3-G).
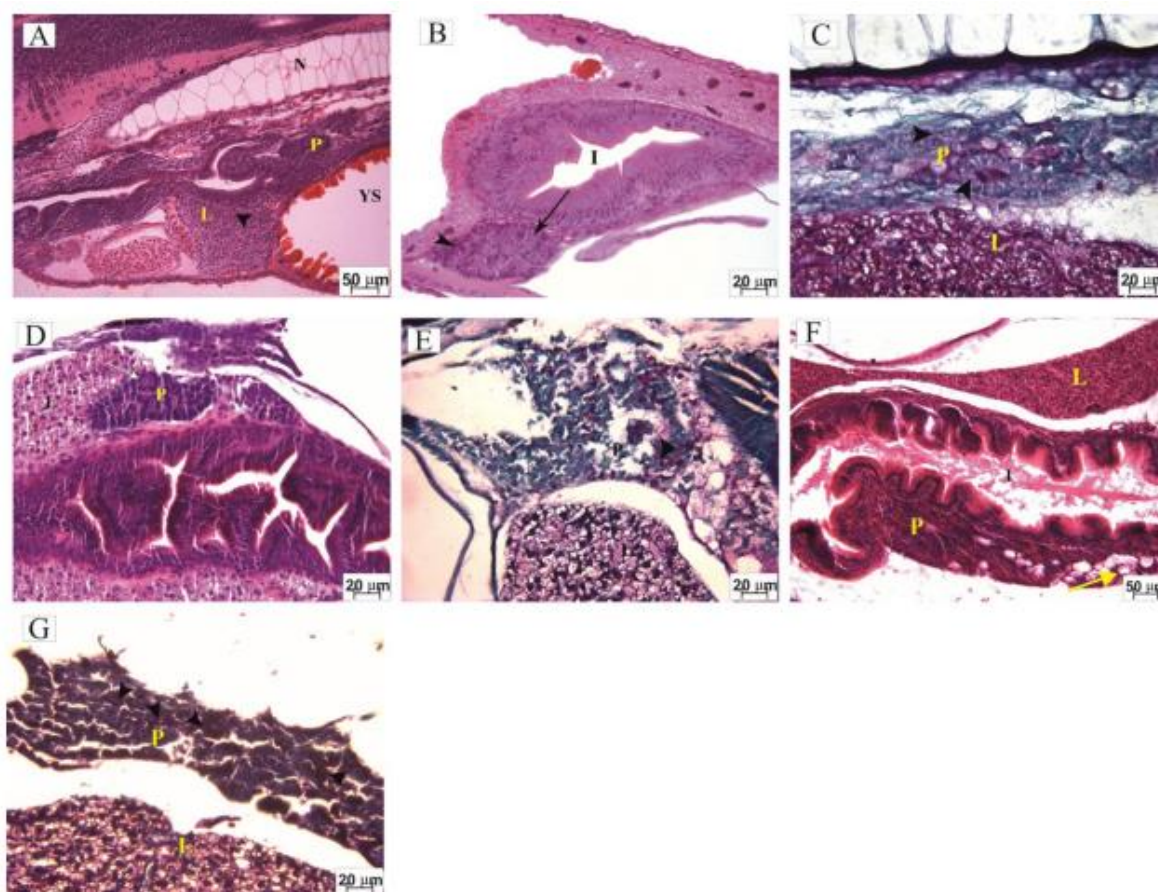

Figure 3: Histology and Histochemistry of larval pancreas.
(A) Pancreas region at 4 DAH (H&E staining); (B)Fully differentiated pancreatic tissue composed of exocrine cells with zymogen granules (Arrowhead), endocrine cells (Arrow) at 7th DAH; (C) PAS–Positive Cells (Arrowheads); (D)Pancreatic tissue with zymogen granules (Arrow) at 10th DAH (H&E staining); (E) PAS positive zymogen granules at 15th DAH(Arrowhead); (F) Fat cells between and around pancreatic tissue at 20th DAH(Arrow) (H&E staining); (G) PAS positive pancreatic cells at 40th DAH (Arrowheads). I, Intestine; L, Liver; N, Notochord; P, Pancreas; YS, Yolk Sac.

At 4th DAH, gall bladder and bile duct tissue formation gradually began. Thus, in microscopic examination of histological sections, at 5th DAH, gall bladder was covered with simple squamous epithelium (Fig. 4-A) which was surrounded by a smooth muscular layer (Fig. 4-A) on the exterior surface and was observed between two liver lobes. Gall bladder was observed with simple columnar epithelium at 6th DAH. Bile duct was observed with simple cuboidal epithelium (Fig. 4-B) the source of which are hepatic primordial cells.

As the fish grew, gall bladder become surrounded by a hepatic tissue and develops a wide lumen.

At 15th DAH, gall bladder and bile duct (Fig. 4-C) which stained positive for PAS could be seen.

During larval growth, gall bladder epithelium got thicker and, except for increased in size and thickness of gall bladder, no other changes were observed until the end of sampling; Fig. 4-D indicates transverse section of gall bladder (GB) and bile duct at 60th DAH.

With regard to chemical pattern, epithelial layer PAS staining tendency also increased as the fish grew (Fig. 4-E).
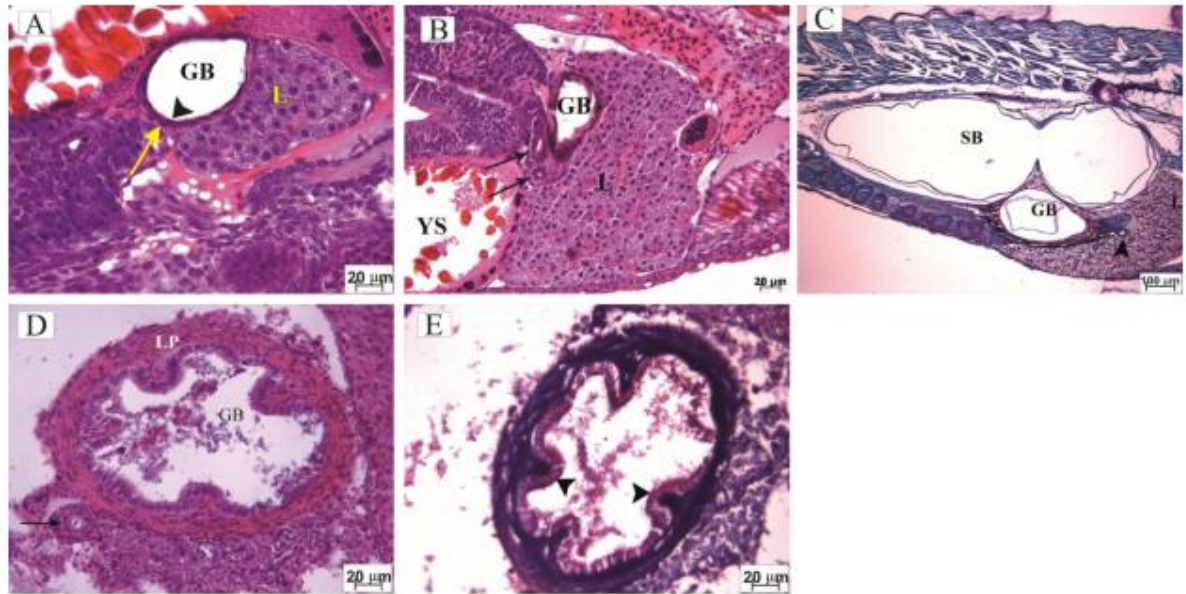

Figure 4: Histology and histochemistry of larval gall bladder.
(A) Longitudinal section of gall bladder with simple squamous epithelium (Arrowhead) at 5th DAH (Arrow shows muscular around GB); (B) Simple cuboidal epithelium of bile duct at 6th DAH (Arrows); (C) Gall bladder and bile duct PAS positive cells(Arrowhead) at 15th DAH (PAS staining); (D) Transverse section of gall bladder with thick layer at 60th DAH (Arrow shows bile duct); (E) PAS positive gall bladder cells at 60th DAH (Arrowheads). GB, Gall Bladder; L, Liver; LP, Lamina Propria; SB, Swimming Bladder; YS, Yolk Sac.

Discussion

Newly hatched *V. vimba* has a primordial liver similar to *Schizothorax zarudnyi* (Shahriari Moghadam *et al*., 2014) of family Cyprinidae, Halibut (Gisbert *et al*., 2004); Haddock (Hamlin *et al*., 2000), and yellow tail kingfish (Chen *et al*., 2006).

Digestive accessory glands appear at 2-3th DAH of some species; the same pattern is identified in Gilthead sea bream (Elbal *et al*., 2004), Common dentex (Santamaria *et al.,* 2004), and Common Pandora (Micale *et al*., 2006) which may be due to vital role of these organs at the time of mixed feeding (intracellular digestion).

Primordial liver development in *V. vimba* larvae was accompanied with intestinal differentiation which indicates the presence of biliary enzymes at the beginning of active feeding and cooperation in digestion of eaten food. This is consistent with observations of Caspian roach*, Rutilus rutilus caspius* (Yaghoubi, 2012) and *C. carpio* (Moshayedi, 2016).

Liver is structured of a mass of compact tissues of polygonal hepatocytes with a central nucleus and small cytoplasm. As larval grew, liver

differentiated and, at 5th DAH, hepatocytes were regularly observed with eosinophilic cytoplasm and basophilic nucleus, as in a common carp, at 8th DAH, hepatocytes were regularly observed around sinusoids (Moshayedi, 2016).

Microscopic studies showed that size of the hepatocytes depends on their physiological activity and the cell size is different in high and low activities (Zambonino Infante *et al*., 2008).

In general, during endogenous feeding, spherical hepatocytes differentiate themselves from central basophilic nucleus and a distinct eosinophilic cytoplasm and appear in a polygonal shape. After being transferred to exogenous feeding stage, hepatocytes increased in size and number and were observed compressed between sinusoids and around the central vein (Hoehne–Riton and Kjorsvik, 2003).

During endogenous feeding, differences between liver glycogen levels of different species may be associated with yolk lipid reserves and energy metabolism in larval period. After beginning of exogenous feeding, vacuolization of hepatocytes can be affected by their reserves. Nucleus position in the cytoplasm of hepatocytes depends on the amount of nutrient accumulation (Zambonino Infante *et al*., 2008).

At the same time of exogenous feeding in *V. vimba*, more vacuoles can be observed in the liver which store glycogen and lipid and number of vacuoles increases in the following days.

Furthermore, during *Petenia splendida* larval hatching, a tubular form of liver was observed which was composed of compact hepatocytes. As larval grew, liver size grew and cytoplasm with lipid content as well as glycogen storage in compressed form was observed among hepatocytes (Trevino *et al*., 2011). Moreover, at 4th DAH, *Acipenser gueldenstaedtii* showed hepatocyte cytoplasm with multiple fat vacuoles and glycogen deposit (Ostaszewska and Dabrowski, 2009).

In *V. vimba* larval pancreas at 3th DAH, pancreatic tissue with basophilic cells were observed which could be distinguished from hepatic cells, with dark basophilic cytoplasm, at 5th DAH. Also, before starting active feeding, eozinophilic zymogen granules of the pancreatic exocrine cells were identified; the same pattern is detected in many other species (Chen *et al*., 2006; Ribeiro *et al.*, 1999; Gisbert *et al*., 2004).

These results indicate the importance of pancreatic secretions during the development of larval's digestive tract (Zambonino Infante and Cahu, 2001).

In a fish, during larval stage yolk sac plays a major role in the synthesis and accumulation of digestive enzymes for digestion at the onset of exogenous feeding (Darias *et al.*, 2006). In addition, before exogenous feeding, presence of zymogen granules (pancreatic enzyme precursors), in pancreatic exocrine cells, approves the significance of pancreatic secretions during development of a larval with no gastric glands (Gisbert *et al*., 2004).

The presence of gallbladder and bile duct stimulates liver function and bile production (Ma *et al.*, 2005).

During the larval period of *V. vimba*, the gall bladder has observed near the anterior intestine, yolk sac, and liver. With larval development, when active feeding starts, the gall bladder is surrounded by the hepatic tissue and contains a large lumen. Bile can enter the intestine through bile ducts.

The presence of the gall bladder and bile duct opening occurs at 5th DAH, while in *S. zarudnyi* larval gall bladder was observed at 1th DAH (Shahriari Moghadam *et al.*, 2014).

Gall bladder observed for the first time, between liver, pancreas and yolk sac, at 3th DAH in Ballan Wrasse larvae (Dunaevskaya, 2010), at 20th DAH in Common dentex, and between two liver lobes in *Pseudosciaena crocea* larvae at 5th DAH (Ma *et al.*, 2005).

Histochemical analysis showed the presence of polysaccharides and subsequently glycogen in the liver. In this study, existence of PAS-positive hepatocytes, between 5-7th DAH, is a proof for this result. In comparison with other cyprinids, glycogen deposit of *Chalcalburnus tarichi*, at 4th DAH, was identified by PAS staining (Unal *et al.*, 2001). Also, *R. rutilus caspius* showed PAS-positive reaction due to having glycogen deposits stored in a compressed form between hepatocytes (Yaghoubi, 2012). In addition, PAS-positive zymogen granules and gall bladder epithelium with polysaccharide secretions were observed in *V. vimba*.

The results of present study indicated that organization and sequence of differentiation of various accessory glands structures in *V. vimba* larvae are similar to other cyprinids and that the larvae at the beginning of exogenous feeding were sufficiently developed to fully utilize food.

Acknowledgment

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