Ontogeny of the digestive tract in stinging catfish, *Heteropneustes fossilis* (Bloch) larvae

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Abstract

Heteropneustes fossilis (Bloch) is an important candidate species for diversification of freshwater aquaculture in India. However, high mortality rate during larval rearing is the most serious bottleneck in commercial production of this species. A proper understanding of the ontogenic development of digestive system provide the basis to understand the nutritional physiology of larvae and develop appropriate feeding strategies. In the present study, the ontogenical development of the digestive tract in *H. fossilis* larvae was studied from hatching until 30 day post hatching (dph) at 29 ºC. At hatching (2.8 ± 0.2 mm standard length, SL), the digestive tract was undifferentiated and attached dorsally to the yolk sac. At 1 dph (2.9 ± 0.2 mm SL), the mouth opened and oral valves were visible. At 2 dph (3.0 ± 0.3 mm SL), goblet cells were observed in the buccoparyngaeal cavity. At this age, exogenous feeding started and the intestine was differentiated into the anterior and posterior regions, and the rudimentary liver and pancreas were also seen. Small supranuclear vacuoles were observed in the enterocytes of the posterior intestine at 2 dph. Zymogen granules were observed in acinar cells of pancreas by 3 dph and islets of Langerhans were be visible at 4 dph (3.5± 0.1 mm SL). At the same age, most of the yolk sac reserves were consumed, whereas they were completely exhausted by 5 dph (3.9 ± 0.5 mm SL). Between 4 and 6 dph, the liver elongated in size and started to accumulate lipids in the hepatocytes. Gastric glands were detected at 4 dph, and the pyloric sphincter was completely differentiated at 9 dph (6.1 ± 0.4 mm SL) as an epithelial fold that separated stomach from the anterior intestine. By 13 dph (8.6 ± 0.2 mm SL), profuse gastric glands were visible inside longitudinal mucosal folds of the stomach. The formation of gastric glands and their development was noticed as the last events in the development of the digestive tract in *H. fossilis*. This indicated the end of the larval period and the commencement of the juvenile stage. Considering these observations, it is suggested that *H. fossilis* larvae have a morphologically complete digestive tract by 13 dph. The findings of the study on the development of the digestive system in *H. fossilis* may help in synchronizing the larval stage of development and feeding strategies, and would be helpful in improving larval rearing techniques for catfish species.

Keywords: Ontogeny; *Heteropneustes fossilis;* Digestive tract; Histology; Larvae

1. Introduction

Heteropneustes fossilis (Bloch) commonly known as stinging catfish, belongs to the family *Heteropneustidae* and order siluriformes. This species is a commercially important and popular indigenous catfish in Asian aquaculture, and particularly in countries like India, Thailand, Bangladesh, Pakistan, Nepal, Sri Lanka, Myanmar, Indonesia, and Cambodia (Burgess 1989; Hossain *et al*. 2015; Akand *et al*. 1991). This Fish is mostly preferred for its tender flesh, delicious taste and low fat content, whereas it is also appreciated in traditional medicine. Interestingly, *H. fossilis* is rich in iron content (226 mg per 100g) compared to many other freshwater fishes (Chakraborty and Nur 2012) and due to its high nutritive value, has been recommended as a food for the anaemic patients (Alok *et al*. 1993; Kohli and Goswami 1989). All these characteristics are responsible for a high market demand and economic value (US$ 6-8/kg) of this catfish species. Thus, *H. fossilis* is considered as a highly promising candidate species for diversification of freshwater aquaculture in India (Kutty 2001), due to its adaptability to survive in adverse ecological conditions such as oxygen depleted waters, tolerance to crowding stress and acceptability to pelleted feeds (Tharakan and Joy 1996). However, in India, the culture of this catfish is still not increasing due to non-availability of sufficient quantity of seed because of high mortality rates during larval rearing and a lack of knowledge of their feeding strategies and rearing techniques. Moreover, the seed collection from the wild is negligible due to lack of sufficient rains in the monsoon seasons and habitat alteration (Vijayakumar *et al*. 1998). Therefore, effective and reliable larval rearing methods must be developed for the successful culture of this catfish species in order to ensure a consistent supply of seed.

For effective larval growth and survival, fish must have well developed structurally and functionally digestive system to capture, ingest, digest and absorb food items (Kjorsvik *et al*. 2004). Although fish larvae may be morphologically capable of capturing food items (e.g. zooplankton and inert microdiets) at first feeding (Segner *et al*. 1994), their digestive system needs a series of developmental changes before being fully functional (Govoni *et al*. 1986). The basic mechanisms of organ development do not differ among all the teleosts, but differences takes place in the relative timing of ontogenic events (Treviño *et al*. 2011). Therefore, species-specific studies are required to better understand the nutritional physiology of fish larvae and their developmental features, information that will be of value for synchronizing the stage of larval development with the rearing techniques. There are several studies available on the ontogenesis of the digestive tract of marine fish species (Pena *et al.* 2017; Teles *et al*. 2015; Onal *et al*. 2008), whereas very few studies have been done on freshwater fish species (Verreth *et al*. 1992; Kozaric *et al*. 2008; de Amorim *et al*. 2009; Yang *et al*. 2010; Saelee *et al*. 2011; Pradhan *et al*. 2012; Gisbert *et al*. 2014; Faccioli *et al*. 2016). Despite the potential of *H. fossilis* for inclusion in culture system, there is no study on the ontogenetic development of the digestive tract during larval development. The

histomorphological method is an important tool to study the ontogeny of the digestive tract of fish larvae. Therefore, in the present study, through histology, we report the ontogeny of digestive system of *H. fossilis* during larval development i.e. from hatching to 30 dph. This knowledge may help in developing optimum feeding strategies for the effective larval rearing technique.

2. Material and Methods

2.1. Eggs and larval rearing

Larvae were produced by induced spawning of sexually mature fish of *H. fossilis* in Fish Genetic Resource Center, ICAR-NBFGR, Lucknow, India. Female *H. fossilis* (142–149 g; n = 5) were injected intramuscularly with synthetic hormone Ovarim® (Congruent Pharmachem Pvt. Ltd. Mumbai, India) (dose: 1.0 ml kg^{-1} body weight, BW) and males (84–96 g; n = 5) with the same hormone at the dose of 0.5 ml kg^{-1} BW. Stripping of females, sperm collection and fertilization was done following the protocol described by Puvaneswari *et al*. (2009). Eggs were subsequently incubated in Fibre Reinforced Plastics (FRP) tray incubators at 29.0-30 ˚C with continuous water flow. After hatching (20-22 h post-fertilization), larvae were stocked in three circular FRP tanks at the density of 5 larvae l^{-1} containing 100 litres of water. Tanks were provided with aeration to maintain dissolved oxygen at saturation and also to ensure uniform mixing of live feed. From 2 dph to 10 dph, larvae were fed *ad libitum* three times per day with mixed zooplankton (copepod, cyclops and cladocerans) collected from nearby ponds. After 10 dph, larvae were fed with egg custard and a dry micro diet (Micro Elite 50, Lucky Star®, Taiwan). Excess feed and faeces were daily removed from each tank before feeding. During the experiment, water temperature, dissolved oxygen and pH were recorded daily, and values were as 29.0 -30 ^{0}C, 6.5–7.8 mg l^{-1} and 7.2–7.5, respectively.

2.2. Larval sampling, growth measurements and histological analysis procedure

Fish larvae were randomly collected daily from hatching to 15 dph and every third day from 18 to 30 dph from each of the three larval rearing tanks. Larvae were anaesthesized using tricaine methanosulphonate (MS-222, Sigma) for measurement of standard length (TL) and BW. Total length was measured for 10 randomly collected larvae using a scale to the nearest of 1 mm, and same larvae were weighed to the nearest 0.001 g with an analytical microbalance. In addition, 10 larvae were fixed in 10% neutral-buffered formalin for further histological analyses. Preserved samples were dehydrated in a graded series of ethanol, cleaned in chloroform and embedded in paraffin blocks. Serial sagittal sections (4-5 µm thick) were cut from each paraffin block using a rotary microtome (Leica RM 2135, Germany), mounted on glass slides and air dried. After that, tissue sections were deparaffined with graded series of xylene and stained with haematoxylin and eosin (H&E) for general

129 histomorphological observations. Periodic acid-Schiff (PAS) staining was used to detect neutral glycoconjugates
130 in mucous cells (Pearse, 1985). All stained tissue sections were permanently mounted (Entellan, Merck Millipore)
131 and observed under an Olympus (BX 53, Japan) light microscope.
132
133 **3. Results**
134 3.1. Larval growth
135 During the experimental period, *H. fossilis* larvae showed an exponential growth in TL and BW from
136 hatching to 30 dph. Average TL of newly hatched larvae was 2.8 ± 0.2 mm (mean ± std dev) and increased up to
137 30.2 ± 0.5 mm at 30 dph (Fig. 1). Similary, larval BW increased from 1.1 ± 0.3 mg at hatching up to 109.8 ± 2.5
138 mg at 30 dph (Fig. 2).
139
140 3.2. Histological development of the digestive system
141 3.2.1. Yolk sac
142 At hatching, the larvae had a large yolk sac filled with acidophilic yolk platelets, which occupied most
143 of the abdominal cavity. Yolk platelets absorption started from 1 dph and between 2-3 dph, most of the yolk sac
144 reserves were consumed. At 4 dph, remnants of yolk were present and yolk was completely absorbed by 5 dph
145 (3.9 ± 0.46 mm TL). No yolk platelets were visible in subsequent histological slides.
146
147 3.2.2. Buccopharynx
148 At hatching, the buccopharyngeal cavity was not visible, while at 1 dph, the mouth opened (Fig. 3a) and
149 two oral valves and oral cavity were visible. The buccopharyngeal cavity was lined with simple squamous
150 epithelium. Exogenous feeding started at 2 dph (3.0 ± 0.3 mm TL) and by this age, goblet cells appeared in the
151 buccopharyngeal cavity (Fig. 3b). At 3dph, taste buds appeared (Fig. 3c) and the number of goblet cells in the
152 folds of the buccopharyngeal epithelium increased as larval development progressed (Fig. 3d).
153
154 3.2.3. Oesophagus
155 At hatching, the oesophagus was not visible in the *H. fossilis* larvae, while at 1 dph, it appeared as a
156 simple and narrow tube, which was lined with a simple cuboidal epithelium (Fig. 4a). At 2 dph, the oesophageal
157 lumen increased in length and connected the buccopharyngeal cavity and the anterior intestine (Fig. 4b).
158 Oesophageal mucous producing cells (goblet cells) were detected at 3 dph along the oesophageal epithelium and

their number increased with the development (Fig. 4c). Between 4 and 6 dph, the oesophagus length increased and it became surrounded by two layers of circular and longitudinal muscular fibres (Fig. 4d). After 6 dph, no histological changes were observed in the oesophagus except an increase in the number and size of goblet cells in the posterior portion of the oesophagus.

3.2.4. Stomach

At 3 dph, the stomach started to develop at the posterior end of the oesophagus with prominent mucosal folds lined with a simple cuboidal epithelium (Fig. 5a). Gastric glands were firstly visible by 4 dph as a cluster of cubic cells on the ventral side of the gastric mucosa of the stomach (Fig 5b). At 6 dph, the size of the stomach increased and clearly differentiated as two distinct regions, the cardiac and fundic regions where gastric glands appeared as clusters of cubic cells (Fig.5c), and the pyloric region where gastric glands were absent. In the glandular stomach, the mucosa was composed of a layer of a simple columnar epithelium with mucous cells (Fig 5d), while gastric glands formed in the submucosa. At 9 dph (6.1 ± 0.4 mm TL), the pyloric sphincter was developed and separated the stomach from the anterior intestine. At 10 dph (6.7 ± 0.1 mm TL), the stomach further increased in size and the mucosal folds became numerous, and the mucosa was covered by an external circular layer of musculature. Gastric glands were mostly developed by 13 dph (8.6 ± 0.2 mm TL), and were composed of a single type of secretory cells and covered by a thin layer of connective tissue (Fig. 5e). The stomach wall in the glandular region consisted of mucosa, a thin *lamina propria*, submucosa, a thin *muscularis* and serosa layers. There were no significant histological modifications observed in the stomach from 13 dph onwards, with the exception of an increase in gastric glands and an increment in size and numbers of mucosal folds. At 21 dph, a morphologically distinct non-glandular stomach was detected (Fig. 5f).

3.2.5. Intestine

Newly hatched larvae had an undifferentiated intestine, whereas at 1dph, it appeared as a short straight tube dorsally attached to the yolk sac (Fig. 6a). The epithelium lining the intestinal lumen was composed of a simple layer of columnar epithelial cells with basal nuclei and prominent microvilli. At 2dph, the posterior part of intestine bent at 90° and the intestine separated into anterior and posterior regions due to the formation of the intestinal valve (Fig. 6b). Small eosinophilic supranuclear vacuoles were firstly observed in the enterocytes of the posterior intestine at 2 dph. At 3 dph, the lumen of the posterior intestine began to expand and at the end, the rectum was formed (Fig. 6c). The increase in length of the intestine was associated to the differentiation of the

mid intestine forming a loop within the abdominal cavity (Fig. 6c). The rectum was lined by a cuboidal epithelium in which goblet cells and mucosal folds were absent. At 4 dph, goblet cells started to differentiate in the anterior and posterior intestinal regions and their number increased with larval development. At 9 dph pyloric sphincter separated the anterior intestine from stomach (Fig.6d). Between 11 and 13 dph (7.3 ± 0.2 - 8.6 ± 0.2 mm TL), supranuclear vacuoles became abundant in the posterior intestine (Fig. 6e). Between 14 and 18 dph (9.2 ± 0.18 - 12.3 ± 0.3 mm TL), supranuclear vacuoles decreased in number in the posterior intestine, whereas they gradually disappeared. At 24 dph, posterior intestine became large with compact longitudinal folds (Fig. 6f). After this, no major histological changes were observed in the intestine until the end of the study with the exception of an increase in the intestine length and the size of mucosal folds.

3.2.6. Liver and Pancreas

Between 1 and 2 dph, the incipient liver appeared as clusters of spherical cells with basophilic nuclei anteriorly to the digestive tract (Fig. 7a). At 3 dph, the liver was seen as a compact tissue with polyhedral hepatocytes containing a central nucleus and slightly eosinophilic cytoplasm, which were loosely arranged around a central vein and hepatic sinusoids (Fig. 7b). By 3 dph, hepatocytes in *H. fossilis* larvae increased significantly in number and were arranged loosely along the hepatic parenchyma without dividing into distinct lobules. Hepatocytes had a eosinophilic cytoplasm and a central basophilic nucleus with distinct nucleoli. Between 4 and 6 dph, the liver elongated occupying most of the anterior part of the abdominal region, and cytoplasmatic vacuolization started to occur due to synthesis and accumulation of lipid deposits within hepatocyes (Fig. 7c). The storage of lipid in the cytoplasm of hepatocytes increased between 10 and 12 dph (6.7 ± 0.1 - 7.8 ± 0.2 mm TL) and after this age, the level of lipid storage decreased (Fig. 7d). As the larval growth progressed, hepatocytes became more compact, and increased in size.

At hatching, the pancreas was not visible, whereas between 1 and 2 dph, it appeared as a compact basophilic tissue ventrally to the intestine and close to the liver. At 3 dph, the pancreas was clearly distinct and acini of the exocrine pancreas were observed and composed of polyhedral basophilic pancreatic cells with eosinophilic cytoplasm (zymogen granules) (Fig. 7b). During this period, the pancreatic acinar cells were arranged around a central canal and a pancreatic duct was clearly visible. Thereafter, exocrine polyhedral acinar cells and acidohilic zymogen granules gradually increased from 3 dph to 30 dph (3.2 ± 0.14 - 30.2 ± 0.46 mm TL). At 4 dph, the endocrine pancreas appeared inside the exocrine pancreas (islets of Langerhans) (Fig. 7e). After 4 dph,

no histological changes were observed in the pancreas except for an increased in size and number of acinar cells in the exocrine pancreas and an increase in number of islets of Langerhans in the endocrine pancreas (Fig. 7f).

4. Discussion

Regardless of the existence of extensive literature on the ontogenic development of the digestive system in different fish species, this kind of studies are still of value since they are essential for understanding the digestive physiology of larvae, as well as achieving a proper knowledge on the development of the species of interest that allows synchronizing zootechnical procedures with the larval stage of development and environmental conditions. As different fish species showed different developmental patterns which are linked to their phylogenetic position and reproductive guilds, we decided to compare present data on *H. fossilis* with those from other catfish species. In *H. fossilis* larvae, the digestive system development was analysed histologically from hatching to juvenile stage. At hatching, the digestive tract of this species was undifferentiated and its developmental pattern was similar to that described in other catfish species like the neotropical freshwater catfish *Hemisorubim platyrhynchos* (Faccioli *et al*. 2016); the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Gisbert *et al*. 2014); the butter catfish from the Indian subcontinent *Ompok bimaculatus*, pabda (Pradhan *et al*. 2012); the slender walking catfish *Clarias nieuhofii* (Saelee *et al*. 2011); the yellow catfish *Pelteobagrus fulvidraco* (Yang *et al*. 2010); the silver catfish *Rhamdia quelen* (de Amorim *et al*. 2009) and the African catish *Clarias gariepinus* (Verreth *et al*. 1992), even though different catfish species from different geographical areas differ in their timing of organ differentiation and development (Table1). For comparative purposes among different studies dealing with the histological development of the digestive tract in catfish species, we decided to use accumulated degree days (ADD) as all the consulted studies did not included larval size in their descriptions (Gisbert *et al*. 2018). The variability in timing of development of different digestive organs may be due to difference in reproductive guilds, as well as environmental rearing conditions (i.e. tropical and subtropical), especially water temperature that resulted in a much faster organogenesis of the digestive system. In this study, most of the histological changes of the digestive system occurred during the yolk sac absorption period (4 dph, 3.5 ± 0.1 mm TL). During this period, the mouth opened and gut differentiated into four histologically distinct segments (i.e. buccopharynx, oesophagus, incipient stomach and intestine). At the same time, the liver and the pancreas, started to differentiate and achieve their final histological organization.

The beginning of exogenous feeding is a species-specific trait and it is mainly affected by environmental conditions (Yufera and Darias 2007; Qu *et al*. 2012), egg size (Gisbert *et al*. 2000) and reproductive guilds

(Treviño *et al*. 2011). In *H. fossilis* reared at 29.0 – 30.0 ºC, the onset of exogenous feeding was detected at 2 dph when larvae measured 3.0 ± 0.3 mm TL. First feeding in *H. fossilis* was accompanied by a period of mixed nutrition when nutrients were partially obtained from remnants of endogenous reserves contained in the yolk sac and ingested food items. A mixed feeding period is considered to improve the growth and survival of larvae by providing any deficiency of nutrients prior to the complete yolk absorption, as well as serving as a temporary nutrient reserve for the larvae to withstand short periods of food deprivation during the time of transition to exclusively exogenous feeding (Treviño *et al*. 2011). The transition to exogenous food in the presence of yolk reserves generally implies that the alimentary canal is functional, although structural and functional development still continues from the larval to the juvenile and adult forms (Jaeroszewska and Dabrowski 2011). In this sense, to ensure that young larvae can digest, absorb and assimilate nutrients from exogenous food during this period of mixed nutrition, a minimum set of digestive structures and functions must be guaranteed (Segner *et al*. 1993). In the present study, yolk sac reserves were completely exhausted in *H. fossilis* at 145 ADD (3.9 ± 0.46 mm TL); thus, the period of mixed nutrition lasted for 3 days. The onset of exogenous feeding in *H. fossilis* took place at 58 ADD similarly to *O. bimaculatus* (54 ADD; Pradhan *et al*. 2012) and *C. gariepinus* (55 ADD; Verreth *et al*. 1992). These three species were the ones where first feeding occurred earlier, whereas in *R. quelen* it took place quite late (120 ADD; Silveira *et al*. 2013). During the mixed nutritional period, the most relevant histological changes in the digestive systems of *H. fossilis* were the differentiation of intestine, appearance of liver, pancreas, zymogen granules in the pancreas, stomach and gastric glands. In *H. fossilis*, the intestine was differentiated into anterior and posterior regions at 58 ADD (2 dph), which was similar to *O. bimaculatus* (54 ADD, Pradhan *et al*. 2012). Incipient liver and pancreas were observed at 58 ADD in *H. fossilis*, similar results were reported in other catfish *P. fulvidraco* (48 ADD, Yang *et al*. 2010) and *C. nieuhofii* (40-53 ADD, Salee *et al*. 2011). Zymogen granules (precursors of pancreatic digestive enzymes) were detected in the exocrine pancreas of *H. fossilis* at 87 ADD after the onset of exogenous feeding. Similarly, zymogen granules were also detected after the onset of exogenous feeding in *S. glanis* (Kozarić *et al*. 2008) and *C. gariepinus* (Verreth *et al*. 1992) at 69-115 ADD and 55 ADD, respectively. In the present study, the stomach started differentiating at 87 ADD and gastric glands first appeared at 116 ADD. Similar results have been observed in *C. nieuhofii* (106 ADD, Salee *et al*. 2011) and *C. gariepinus* (110-138 ADD, Verreth *et al*. 1992).

In the present study, mucous cells were detected by 58 ADD (2 dph), coinciding with the onset of exogenous feeding. These observations were similar to those reported in *P. fulvidraco* (Yang *et el.* 2010). In contrast to these results, mucous cells were observed before the mouth opening and taste buds appeared at 3 dph

(4.8 ± 0.13 mm SL) in the neotropical carnivorous freshwater catfish, *Hemisorubim platyrhynchos* (Faccioli *et al.* 2016) or just coinciding with mouth opening in *P. punctifer* (Gisbert *et al*. 2014). The earlier development of mucous cells in the buccopharyngeal compared to other regions of the digestive tract, and the apparition of taste buds might be attributed to their importance in foraging behaviour, prey capture and ingestion (Rønnestad *et al.* 2013). The production of mucosubstances from buccopharyngeal and oesophageal goblet cells plays an important role in lubricating the digestive mucosa and preventing its abrasion by food items (Hirji and Courtney 1983; Scocco *et al*. 1998). In addition, the neutral glycoconjugates produced from oesophageal globets cells could also cooperate in the digestion of food and its transformation into the chyme, as well as participating in the absorption of easily digested substances (Sarasquete *et al*. 2001). In *H. fossilis* larvae, goblet cells appeared in the anterior and posterior region of the oesophagus at 58 ADD. Similarly, in *O. bimaculatus* and *P. punctifer*, these cells were detected at 54 – 81 ADD (2-3 dph) and 42 ADD (2dph), respectively.

The first histological signs of stomach development in *H. fossilis* were observed as a dilatation of the gut at the end of posterior oesophagus at 3 dph (87 ADD) when larvae were 3.2 ± 0.1 mm SL. Gastric glands started to differentiate one day later at 4 dph (116 ADD), marking the beginning of stomach development. The stomach was completely developed with prominent folds, a pyloric sphincter and gastric glands were abundant in the cardiac region between 10 (6.7 ± 0.1 mm SL) and 13 dph (8.6 ± 0.2 mm SL). In this sense, gastric glands appeared at 116 ADD in *H. fossilis*, whereas in other catfish species gastric glands appeared at (49 ADD) in *R. quelen* followed by *P. fulvidraco* (72 ADD); *C. nieuhofti* (106 ADD), *C. gariepinus* (110-138 ADD); between 115 to 161 ADD in *S. glanis* (Kozariac *et al*. 2008); *P. punctifer* (196 ADD) (Gisbert *et al*. 2014) and *O. bimaculatus* (216 ADD) (Pradhan *et al*. 2012). The appearance of gastric glands and the presence of mucous cells in the gastric mucosa generally indicate the formation of a functional stomach (Stroband and Kroon 1981), which is also a histological criterion to differentiate larvae from juveniles (Tanaka 1971; Sarasquete *et al*. 1995; Chen *et al*. 2006). However, recent data from Solovyev *et al*. (2016) reported that in some cases, it may exist a mismatch between gastric gland formation and pepsin detection, indicating that special care needs to be taken when extracting conclusions regarding stomach functionality and acid digestion, especially when dealing with weaning strategies. Regardless of the recommendations provided by the former authors and the lack of studies evaluating the digestive capacities in *H. fossilis*, we recommend that this species may be weaned when larvae are 377 ADD (13 dph; 8.6 ± 0.2 mm TL).

In the present study, supranuclear vacuole were first observed by 58 ADD (2 dph) and reduced in number after 406 ADD (14 dph; 9.2 ± 0.2 mm TL) when gastric glands were fully developed. These results have also been reported in other catfish species like *H. platyrhynchos* (Faccioli *et al*. 2016), as well as in different marine and freshwater species (Gisbert *et al*. 2004; Qu *et al*. 2012; Yúfera *et al*. 2014; Peña *et al*. 2017) among others. According to Govoni *et al*. (1986), the presence of acidophilic supranuclear inclusions in enterocytes reflects pinocytotic protein absorption and intracellular digestion, which is considered as an adaptation to compensate for the absence of functional stomach. The emergence of gastric glands and disappearance of supranuclear vacuoles in the posterior intestine indicated complete development of the digestive capacities of fish larvae (Govoni *et al*. 1986; Chen *et al*. 2006).

In the present study, first acidophilic zymogen granules were observed after the onset of exogenous feeding at 87 ADD (3 dph). Similarly, zymogen granules have been observed after the onset of exogenous feeding in *S. glanis* at 69-115 ADD (Kozaric *et al*. 2008). In contrast, the zymogen granules were present before the onset of exogenous feeding in *O. bimaculatus* (27 ADD, Pradhan *et al*. 2012) and *P. punctifer* (42 ADD, Gisbert *et al*. 2014). These zymogen granules are considered to be the precursors of trypsin and chymotrypsin enzymes, which play an important role in extracellular proteolytic digestion (Walford and Lam 1993; Zambonino Infante and Cahu 2001). In the present study, synthesis and accumulation of lipids in the hepatic parenchyma started between 116 ADD (3.5 ± 0.1 mm TL) and 174 ADD (4.4 ± 0.4 mm TL). The storage of lipid in the cytoplasm of hepatocytes increased between 290-348 ADD (6.7 – 7.8 mm TL), and after 377 ADD (8.6 ± 0.2 mm TL), the level of lipid storage started decreasing. Similar results were observed in *S. glanis* (Kozaric *et al*. 2008); *O. bimaculatus* (Pradhan *et al*. 2012) and *P. punctifer* (Gisbert *et al*. 2014). These changes may be attributed to different digestive capacities during early ontogeny, but also to different lipid requirements along larval development (Rønnestad *et al*. 2013) .

In conclusion, the ontological analysis of digestive tract obtained from this study suggested that *H. fossilis* larvae can be fed with artificial diet from 13 dph, as they have a morphologically complete digestive system. The pattern of digestive system development was found similar to most of the catfish and other teleost species, except for the timing of organ differentiation, which directly affects the functionality of the digestive system, as well as larval rearing procedures. However, further research must be conducted to assess the digestive enzyme secretion to confirm the functionality of the digestive tract and evaluate the feeding strategies to develop better larval rearing practices for this species.

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Figure Captions

Fig.1. Growth in length (mm) of *H. fossilis* larvae from hatching to 30 days after hatching.

Fig. 2. Growth in weight (mg) of *H. fossilis* larvae from hatching to 30 days post hatching.

Fig. 3. Longitudinal histological sections of the buccopharynx in *H. fossilis*. (a, b) General view of histological section of larva at 1 and 2 dph, respectively showing oral valve, mouth and buccal cavity. (c, d) Larva at 3 dph showing taste buds in the buccopharyngeal cavity and goblet cells. *Abbreviations*: *BC*, Buccal cavity; *GC*, Goblet cells; *OV*, Oral valve; *TB*, Taste buds; Staining: haematoxylin-eosin.

Fig.4. Longitudinal histological sections of the oesophagus in *H. fossilis*. (a) Detail of the abdominal cavity of a 1 dph larva, note the short oesophagus, the rudimentary intestine and the large yolk sac. (b) Detail of the abdominal cavity of a 2 dph larva showing an elongated oesophagus and large yolk sac. (c) Detail of the posterior buccopharyngeal cavity with goblet cells along the oesophageal epithelium at 3 dph. (d) Oesophagus showing longitudinal folding in a larva aged 6 dph; *Abbreviations*: *CM*, Circular muscles; *GC*, Goblet cells; *I*, Intestine; *OE*, Oesophagus; *LM*, Longitudinal musculature; Staining: haematoxylin-eosin.

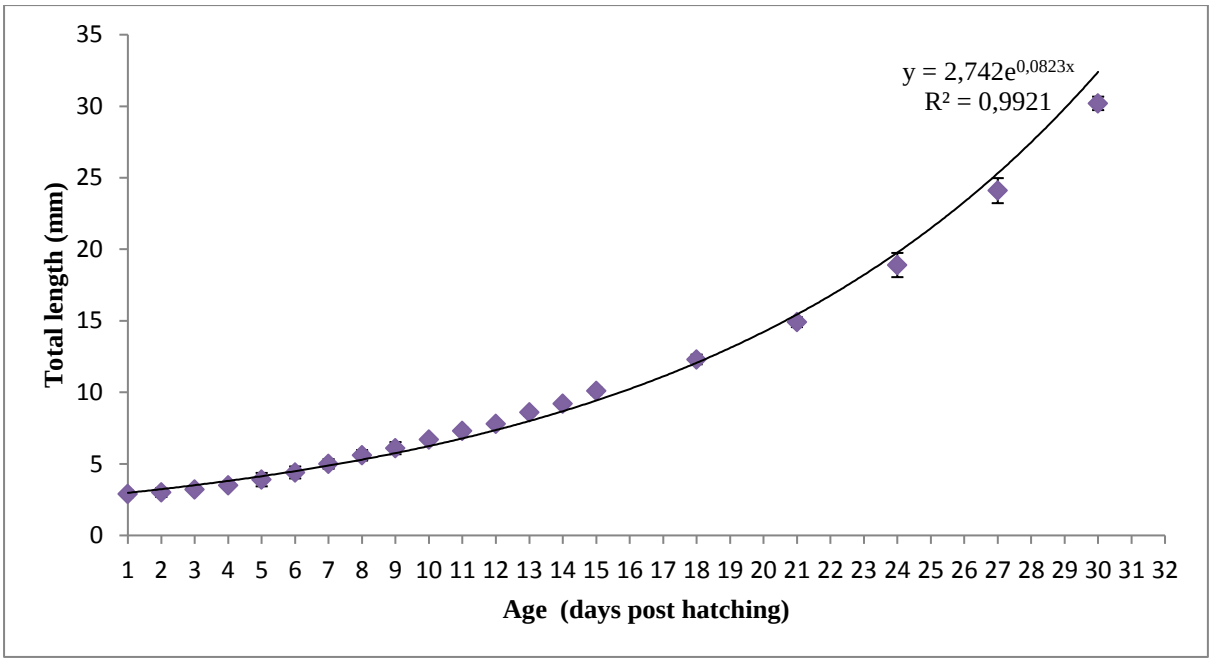
Fig. 5. Longitudinal histological sections of stomach in *H. fossilis*. (a) Detail of the abdominal cavity in a 3 dph larva showing the development of the intestinal swelling into the non-glandular stomach. (b) Image showing gastric glands differentiation at 4 dph. (c) Increase in gastric glands number at 6 dph. (d) Image of the stomach at 7 dph stained with Periodic acid Schiff (PAS) showing the gastric mucosa lined with neutral mucous. (e) Changes in gastric glands development and number at 13 dph. (f) General view of non-glandular stomach at 21 dph;. *Abbreviations*: *GG*, Gastric glands; *GME*, Gastric mucosal epithelium; *I*, Intestine; *NGS*, Non-glandular stomach; *OE*, Oesophagus; *S*, Stomach; Staining: haematoxylin-eosin, PAS.

Fig. 6. Longitudinal histological sections of the intestine in *H. fossilis*. (a) An undifferentiated intestine in a larva aged 1 dph. (b) Intestine divided into the anterior and posterior regions in a larva aged 2 dph. (c) Intestine showing anterior intestine, posterior intestines, middle intestine and rectum at 3 dph. (d) General view of the abdominal cavity showing the formation of the pyloric sphincter that separated the anterior intestine from the stomach at 9 dph. (e) Detail of the intestine with the presence of supranuclear vacuoles within enterocytes in a 11 dph larva. (f) Detail of the posterior intestine with large and compact longitudinal folds in a specimen aged 24 dph. *Abbreviations*: *AI*, Anterior intestine; *BC*, Buccal cavity; *GG*, Gastric glands; *I*, Intestine; *L*, Liver; *MI*, Middle intestine; *OE*, Oesophagus; *PI*, Posterior intestine; *PS*, Pyloric sphincter; *R*, Rectum; *S*, Stomach; *SNV*, Supranuclear vacuole; *YS*, Yolk sac; Staining: haematoxylin-eosin.

Fig. 7. Longitudinal histological sections of the accessory digestive glands, liver and pancreas, in *H. fossilis*. (a) Detail of the abdominal cavity at the level of the connection of the oesophagus with the anterior intestine showing the incipient development of the liver dorsally to the yolk sac in a larva aged 2 dph. (b) Detail of the developing liver and exocrine pancreas at 3 dph. (c) Detail of the hepatic parenchyma showing an increase in fat accumulation within hepatocytes at 6 dph, as well as the exocrine and endocrine pancreas with islets of Langerhans; (d) Liver showing a moderate inclusion of lipid deposits in a larva aged 15 dph. (e) Exocrine pancreas with zymogen granules within pancreocytes and onset of formation of islets of Langerhans (endocrine pancreatic tissue) at 4

dph. (f) Detail of the exocrine pancreas with zymogen granules and endocrine pancreatic tissue (islets of Langerhans) in a larva aged 10 dph. *Abbreviations*: *OE*, Oesophagus; *I*, Intestine; *IL*, islets of Langerhans (endocrine pancreas); *L*, Liver; *P*, Exocrine pancreas; *YS*, Yolk sac; *YP*, Yolk platelets. Staining: haematoxylin-eosin.

Fig. 1.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519 Fig. 2.

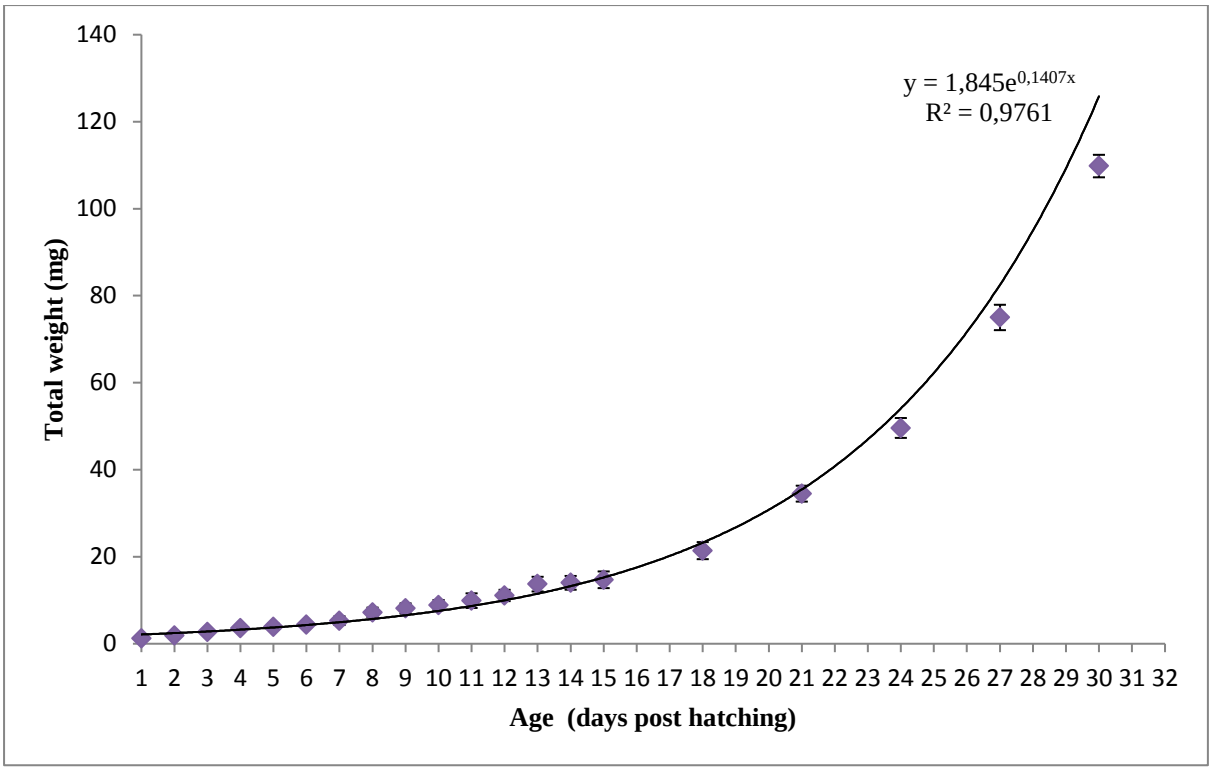

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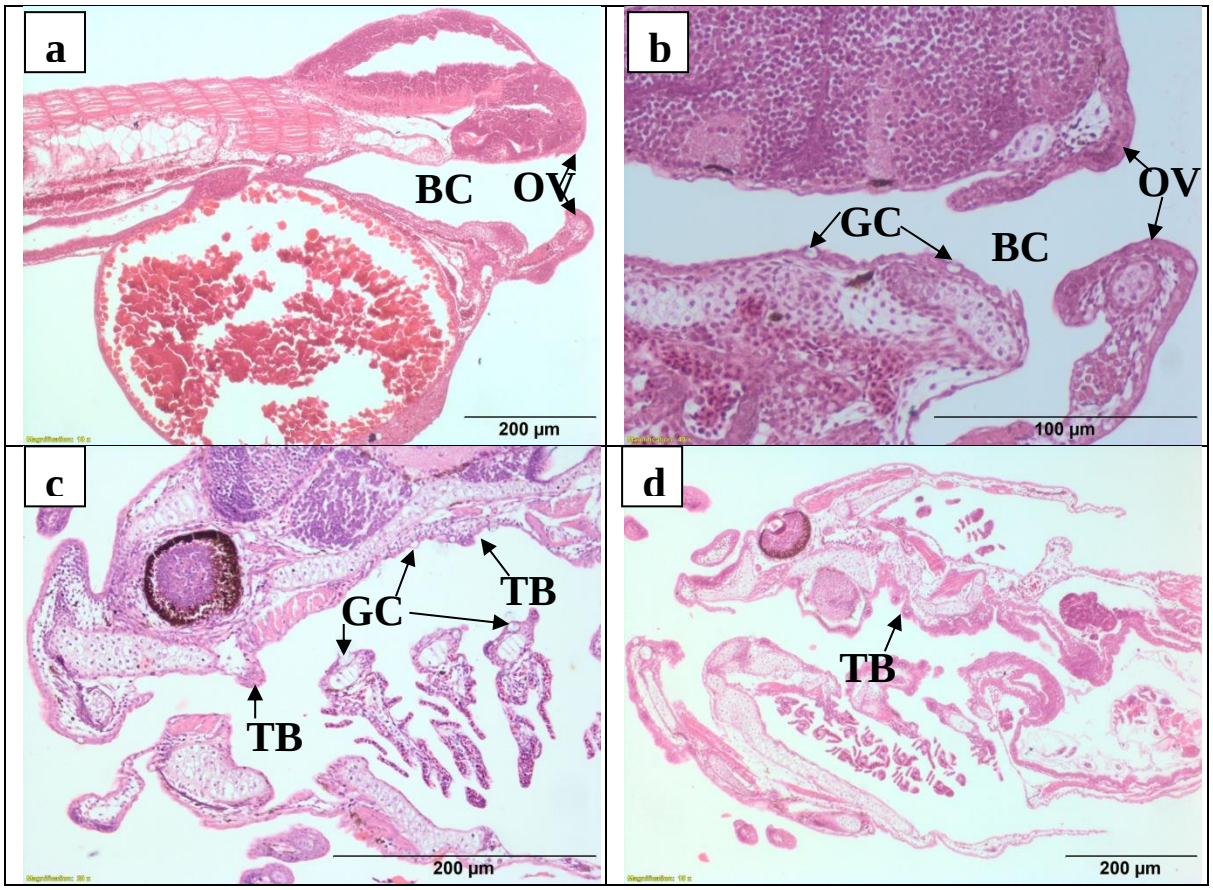
Fig. 3.



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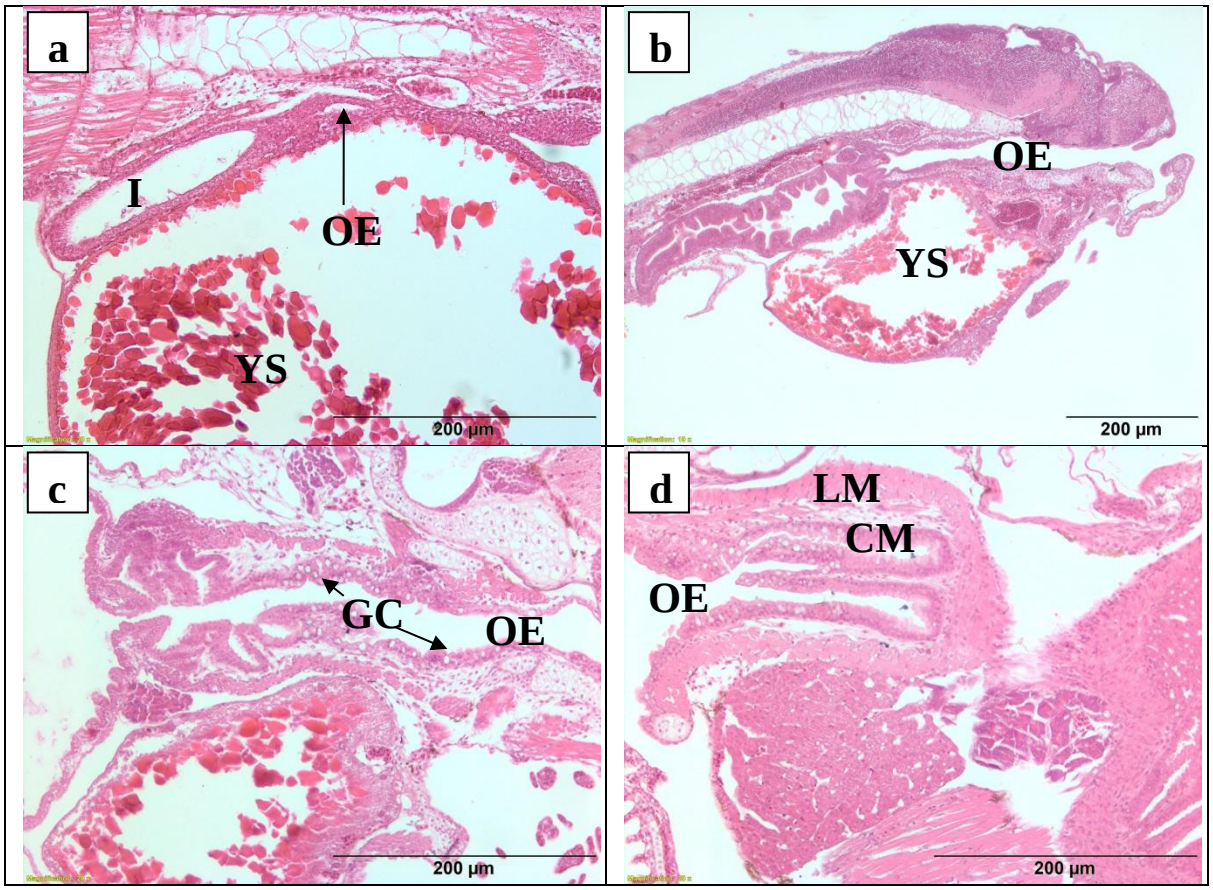

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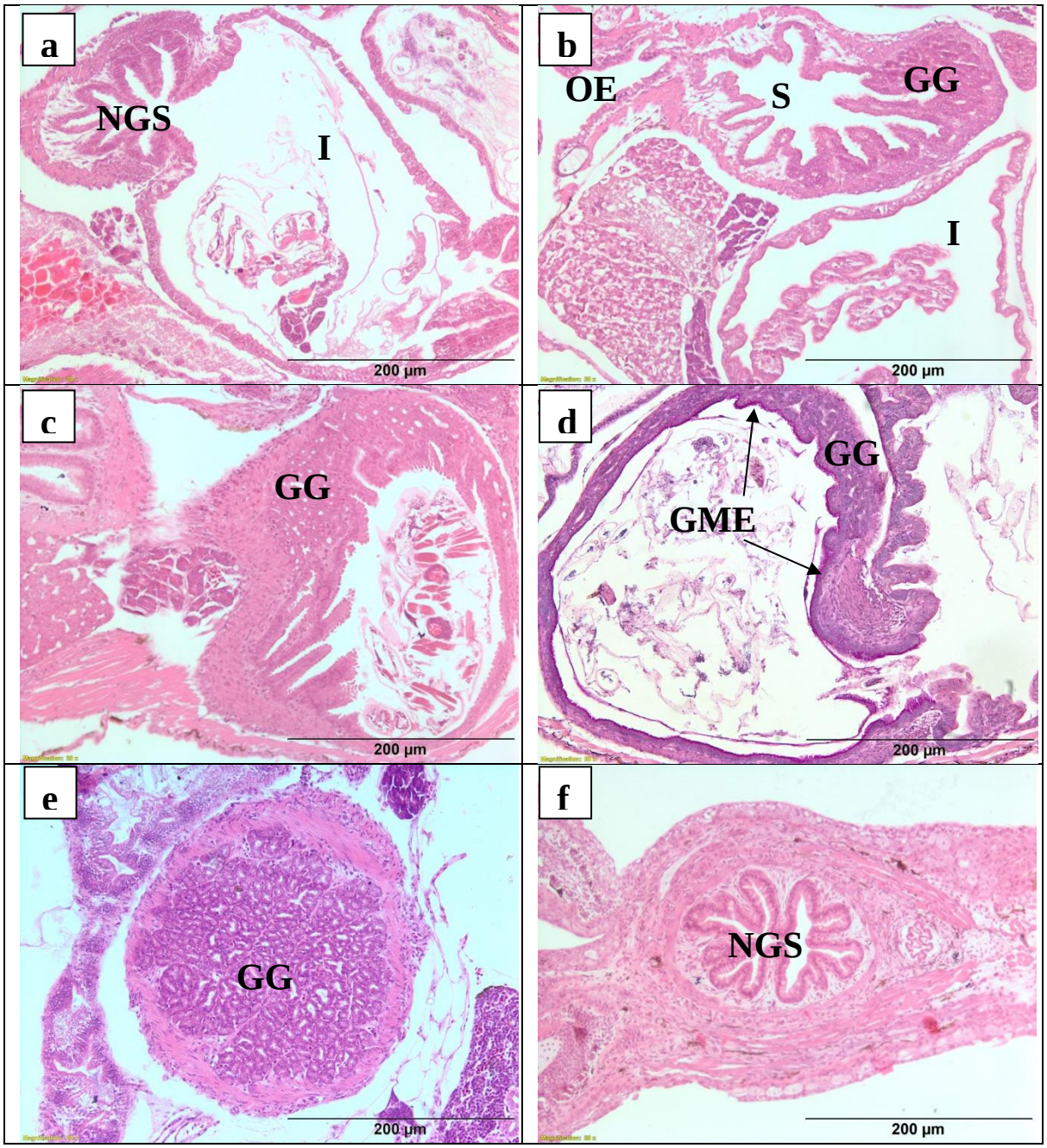
563 Fig. 5.



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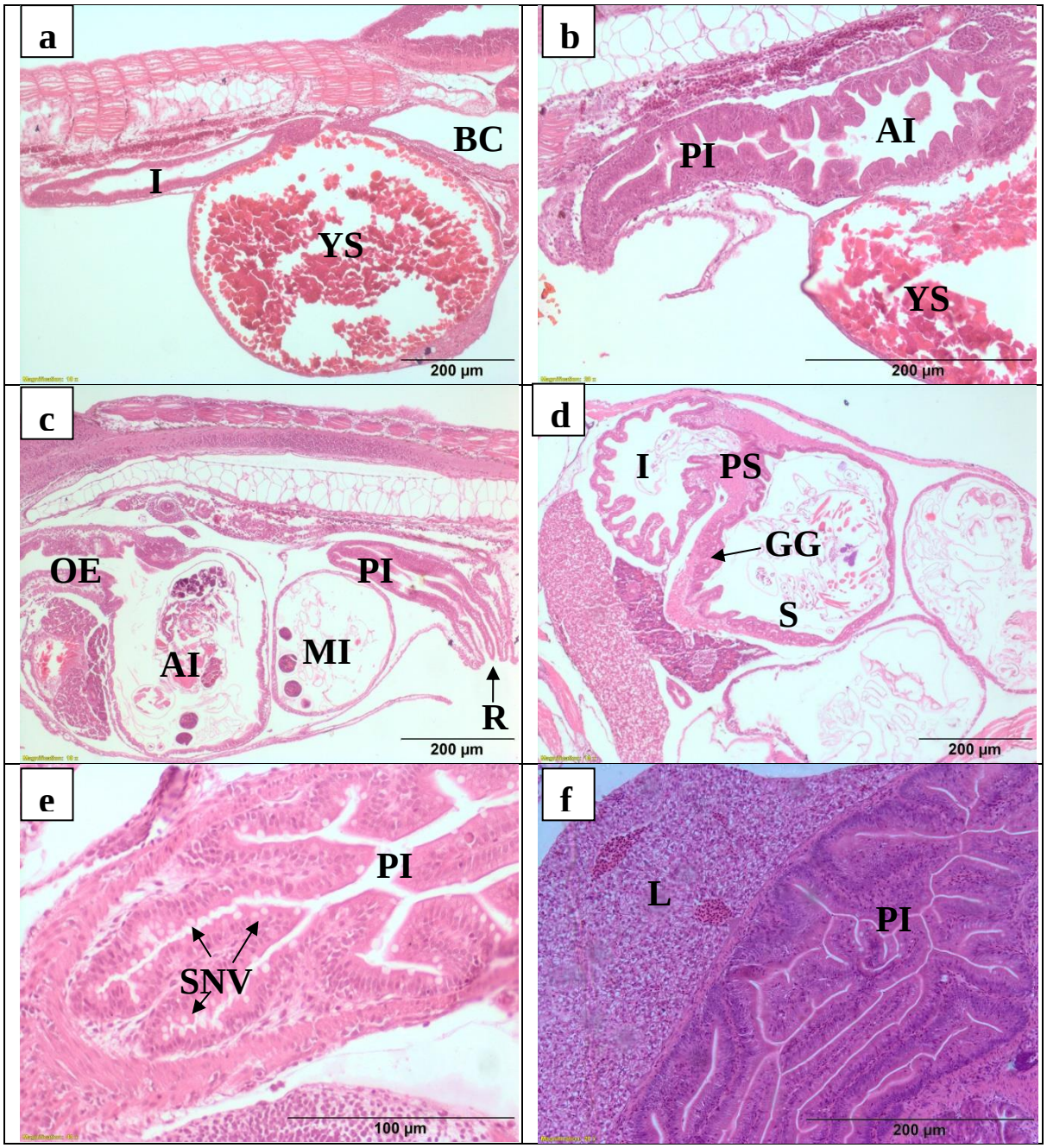
571 Fig. 6.



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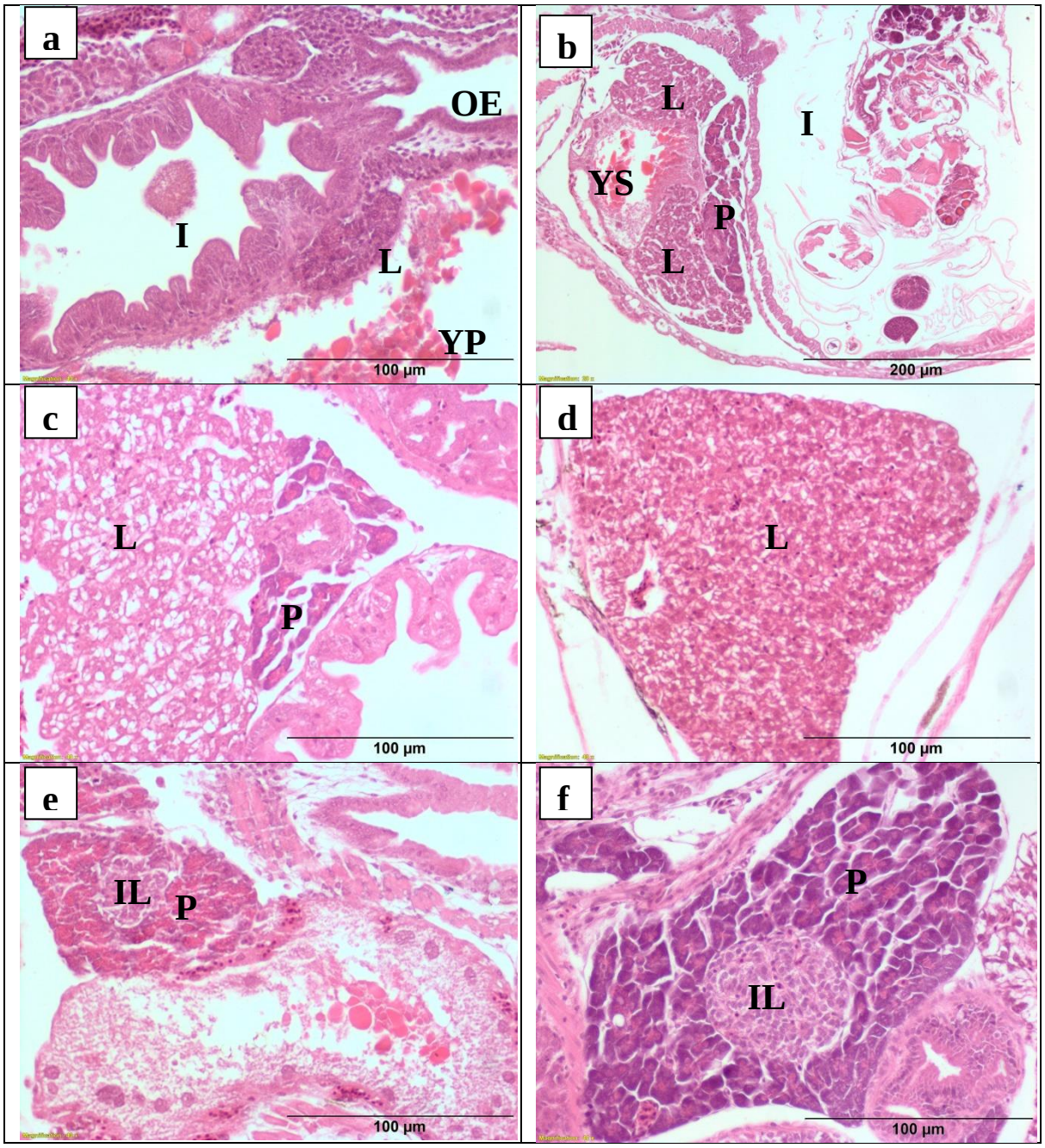
579 Fig. 7.



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