

The Ontogeny of the Digestive Tract and Associated Organs of Humpback Grouper (*Cromileptes altivelis*) Larvae

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Abstract

The ontogeny of the digestive tract and associated organs in humpback grouper (*Cromileptes altivelis*) larvae was observed until 40 days after hatching (DAH). At 1 DAH (2.27 ± 0.07 mm), the digestive tract existed as a simple tube with the mouth and anus still closed. The mouth started to open on 2 DAH (2.41 ± 0.12 mm) but was not fully functioning. At the same time, the epithelium of the intestine and rectum started to fold while the liver and pancreas started to appear. The yolk sac was fully absorbed at 3 DAH (2.50 ± 0.07 mm), and mucous cells appeared at the esophagus. On 4 DAH (2.58 ± 0.08 mm), numerous acidophilic supranuclear vacuoles appeared and the epithelium cell on esophagus started to increase rapidly. On 6 DAH (2.61 ± 0.14 mm), the intestine differentiated into midgut and hindgut. Goblet cells, pharyngeal teeth, taste buds and the tongue appeared on the buccopharynx at 8 DAH (3.24 ± 0.38 mm). The stomach was divided into cardiac and fundic regions on 16 DAH (5.03 ± 0.18 mm). Gastric glands which were distributed between the fundic stomach and the midgut, formed on 25 DAH (10.29 ± 2.20 mm). The formation of the fundic stomach signalled the starting point of weaning and as the most important stage in ontogeny.

Introduction

Recently large-scale hatchery production of humpback grouper (*Cromileptes altivelis*) fry has developed, but its survival rate is still very low. The most critical phase occurs at the larval to adult stage or during 40 days after hatching (Kohno et al. 1993). The high mortality at this stage was mostly caused by dietary factors and disease.

Grouper larvae are extremely sensitive even to a minimal mechanical disturbance particularly during metamorphosis. In the hatchery phase, grouper larvae are small and fragile with small reserves of endogenous nutrition and low initial feeding rates (Ordonio-Aguilar et al. 1995). These factors are considered to be a fundamental cause of the high mortalities and delayed development of fish larvae (Kohno et al. 1997). Therefore, the study of larval fish nutritional physiology of humpback grouper should provide information to solve some of these problems (Govoniet et al. 1986; Segneret et al. 1993). Furthermore, the histological development

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of the larval fish digestive system should be studied because most physiological functions are based on the development of this organ (Hamlin et al. 2000; Govoni, 2004).

Successful development of the digestive system is crucial for the survival and growth in fish larvae because an efficient digestive system enables fish to capture, ingest, digest and absorb food (Kjorsvik et al. 2004). Although larval fish may be morphologically capable of capturing food items at first feeding (Segner et al. 1994; Bisbal and Bengtson, 1995), the digestive system needs a series of developmental changes before being fully functional to digest food (Govoniet et al. 1986; Canino and Bailey, 1995). Knowledge on the structural development of the digestive system is essential to understand the digestive physiology and determine the appropriate timing to wean fish larvae (Watanabe and Kiron, 1994; Baglione et al. 1997; Cahu and Zambonino Infante, 2001). The objective of this study was therefore to examine the structural changes during the ontogeny of the digestive system of hatchery-reared humpback grouper from hatching until metamorphosis, aiming to understand the sequence of organ development and provide fundamental knowledge on hatchery management for humpback grouper larvae.

Materials and Methods

Eggs of humpback grouper were collected from the spawning tank through the outlet with a fine net (400 μm). The eggs were transferred into 0.5 to 1.0 m^3 transparent circular tanks for incubation and unfertilized eggs were removed by siphoning. About 100,000 pieces of fertilized eggs were spread evenly in two different tanks and the tanks were covered with plastic to reduce fluctuation of temperature. The larvae were reared in yellow 5-ton tank and were provided with mild aeration. The green water was introduced to the rearing tank on 2 DAH until 30 DAH. In addition, rotifers were introduced into the rearing tank at day 2 and increased gradually over time. The waste material was siphoned out daily from the bottom of the rearing tank after 12 days. From day 15 onward, small-size commercially formulated diets with a particle size of 200-400 μm were used and finally from day 17 onward, newly hatched *Artemia* were introduced to the rearing tank. The water in the rearing tank was changed daily once the larvae started to feed on the artificial diet and *Artemia*. At day 30, a flow-through system was used in the rearing tank to avoid water quality problems. The larvae were reared in the rearing tanks for 40 days, a period which corresponded to the end of larval development.

To determine the digestive system, 20 fish were randomly sampled and fixed in Bouin's solution for 4-6 hr. The samples were transferred to 70% alcohol solution before tissue processing. Samples were dehydrated by immersing in graded ethanol and xylene to ensure the penetration of wax in the tissues. After the dehydration process had been completed, the samples were placed in cassettes individually and embedded in paraffin wax. After that, the cold block was cut in series of sections of 5 μm thick, mounted in glass slides, and air dried overnight before staining with haematoxylin and eosine (HE). All of these protocols followed the histological techniques described by Drury and Wallington (1967), and Kiernan (1990).

Results

The developments of the digestive system are shown in Fig. 1. On the first day after hatching (1 DAH) the mouth, buccal cavity and pharynx were undifferentiated and still closed (Fig. 1a). The anterior parts of larvae consisted of yolk sac and oil globule. Yolk sac was still visible at this phase and was slowly absorbed until 3 DAH. On 3 DAH, the mouth was open and the oil globule was still visible and disappeared at 4 DAH. Taste bud firstly appeared on 5 DAH and on 13 DAH, goblet cells were visible in the stratified squamous epithelium of the pharyngeal cavity.

On 2 DAH, the esophagus which appeared as a simple tube similar to the incipient gut, and was lined by a single layer of squamous cell started to develop, and the simple layer of cuboidal epithelium formed (Fig.1b). Longitudinal folds of epithelium appeared on the pharyngeal cavity and became complex and intensified on 16 DAH. Two days after exogenous feeding on 5 DAH, longitudinal folds formed along the esophagus, and became abundant on 9 DAH. On 9 DAH, the lining of esophagus completed the transformation from a simple layer of cuboidal epithelium to one of columnar epithelium. The goblet cells firstly appeared on stratified epithelium esophagus and the micro villi of esophagus also formed (Fig. 1c). Goblet cells on epithelium folds became abundant by 31 DAH until 40 DAH.

On 1 DAH, the primordial stomach appeared as a bulge at the end of the esophagus on 2 DAH and stomach started to open and enlarged by 3 DAH (Fig. 1d). On 7 DAH, stomach started to fold and was pushed ventrally by the developing swimming bladder. Accessory organs such as swimming bladder, gall bladder, and kidney were visible at this age. Stomach became more elongated and folds were abundant to create the pyloric stomach on 10 DAH. By 16 DAH, the pyloric sphincter separated the stomach from the midgut and the stomach was composed of the cardiac, fundic and pyloric regions (Fig. 1e). Proliferations of gastric glands occurred at 34 DAH and continued to grow onward. The fundic stomach became wider and elongated and was distributed around the stomach wall. Pyloric caeca appeared as a single protrusion of the midgut just below the pyloric region of the stomach. The fundic region was elongated as the larvae grew and formed the largest portion of the stomach on adult stage. The fundic stomach evolved into the largest part of the stomach and was the main storage for food (Fig. 1f).

The incipient intestine of a newly-hatched larva was lined by a single layer of columnar cells (Fig. 1g). At this stage, intestine and rectum were still closed. On 2 DAH, the epithelium cells of intestine developed and increased in number. The intestinal tract began to open on 3 DAH simultaneously with the onset of exogenous feeding. The intestinal valve divided the intestine into midgut and hindgut at 6 DAH (Fig. 1h). On 7 DAH intestine became coiled, and lipid vacuola and acidophilic supranuclear vacuoles appeared on midgut, hindgut and rectum. The appearance of lipid vacuola and acidophilic supranuclear vacuoles at this stage was an indicator of the initial protein digestion and absorption in the gut. On 22 DAH the number of goblet cells appeared and increased with the ontogeny differentiation of the intestinal mucosa. On 40 DAH, goblet cells increased by number, and numerous lipid vacuola and acidophilic supranuclearvacuola developed on intestinal folds (Fig. 1i).

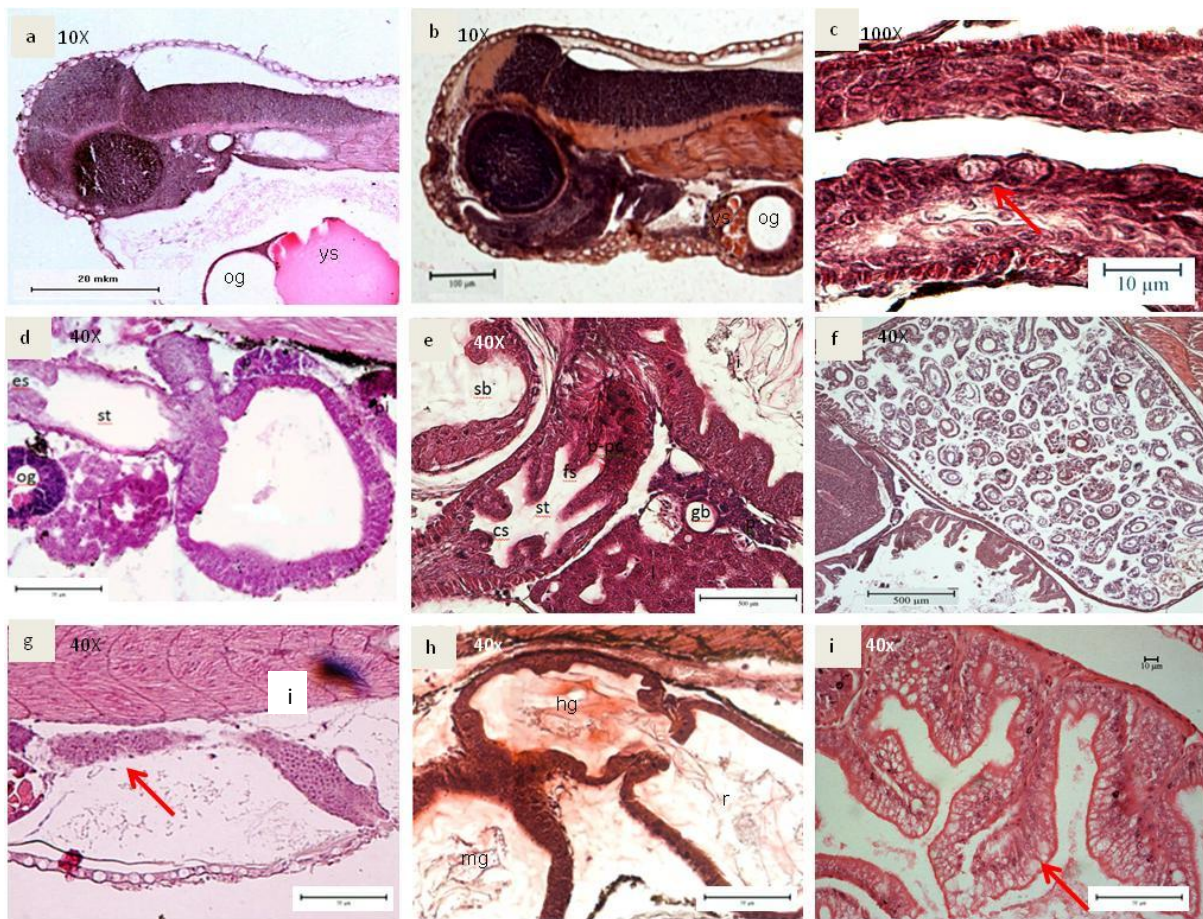


Fig. 1. Sagittal section of the humpback grouper larvae during the development; a. Newly hatch larvae; b. Larvae on 2 DAH; c. Esophagus on 9 DAH; d. Stomach on 4 DAH; (e) Stomach on 16 DAH; (f) Stomach on 40 DAH; (g) Intestine on 2 DAH; (h). Intestine on 6 DAH; (i) Intestine on 40 DAH; Abbreviations: es: esophagus; rst: rudimentary stomach; y: yolk sac; og: oil globule; l: liver; i: intestine.

The liver and pancreas were not differentiated at hatching. A cluster of basophilic pancreatic cells appeared on 2 DAH adjacent to the incipient intestine behind the liver. Between 2 and 3 DAH, the liver and pancreas started to develop from a cluster of undifferentiated round eosinophilic cells and extended in the posterior region of the abdominal cavity, between the yolk sac and the intestine (Fig. 2a). By 4 DAH, preliminary gall bladder appeared, and on 6 DAH, the hepatocyte cells were more defined in a spherical shape and became more contiguous on 9 DAH. Also on 6 DAH, acidophilic zymogen granules were also apparent in the centre of acini (Fig. 2b). On 16 DAH, the liver was enlarged and hepatocytes number increased rapidly and showed many vacuoles. On 19 DAH, lipid vacuoles in the liver became abundant and the zymogene in the pancreas were also developed. By 25 DAH, lipid vacuoles on hepatocyte cytoplasm increased rapidly. The hepatocyte cytoplasm also increased rapidly and was full of lipid vacuoles by 37 DAH (Fig. 2c).

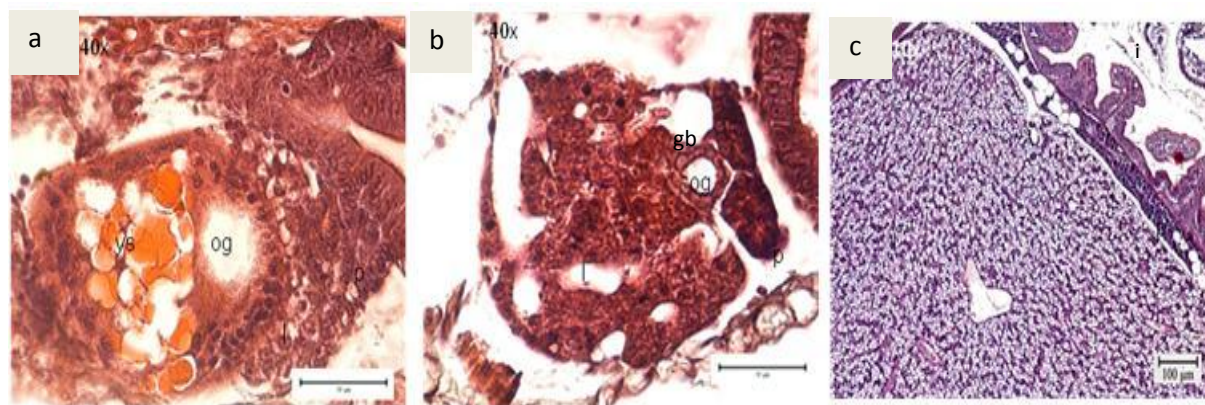


Fig. 2. Sagittal section of the liver and pancreas during the development of humpback grouper larvae (a) 2 DAH; (b) 6 DAH; (c) 37 DAH; Abbreviations: ys: yolk sac; og: oil globule; l: liver; p: pancreas; gb: gall bladder; i: intestine.

Discussion

The digestive tract of humpback grouper larvae was an undifferentiated tube before exogenous feeding, which is similar to the development of tiger grouper (*Epinephelus fuscoguttatus*) (Ariza, 2010), kelp grouper (*Epinephelus bruneis*) (Kato et al. 2004), dusky grouper (*Epinephelus marginatus*) (Glamuzina et al. 1998), leopard grouper (*Mycteroperca rosacea*) (Martinez et al. 2009), and green grouper (*Epinephelus coioides*) (Quinitio et al. 2004). On 5 DAH lipid vacuoles appeared in the enterocytes of the anterior midgut. Structural changes of the larval intestine occurred on 6 DAH at the time when the intestinal valve divided the intestine into midgut and hindgut. According to Watanabe (1984), the appearance of supranuclear vacuoles indicates pinocytosis and intracellular digestion on protein. Furthermore, lipid vacuoles were observed in enterocytes in the anterior midgut, indicating the possible lipid absorption in the fish gut (Deplano et al. 1991; Sarasquete et al. 1995; Pena et al. 2003).

The appearance of gastric glands coincided with a general increase in enzyme activities indicating an increase in the digestive capacity of the larvae preceding metamorphosis. According to Tanaka (1971) and Stroband and Dabrowski (1981), the juvenile stage begins when the gastric glands develop, stomach shows digestive activity, and pyloric caeca appear. The appearance of gastric glands marked the formation of a functional stomach (Stroband and Kroon, 1981). Gastric glands increase digestive efficiency, but the timing of gastric gland development varies greatly among fish species. In particular for grouper species, information on the development of the digestive tract is still very limited. Gastric glands are usually detected at 20–30 DAH (Chen et al. 2006). The formation of the pyloric caeca indicates the last major change of the digestive system in fish larvae (Bisbal and Bengtson, 1995; Hamlin et al. 2000). Development of pyloric caeca is the final morphological change in the digestive tract showing that the fish attain the juvenile stage (Bisbal and Bengtson, 1995).

In the present study, we did not observe goblet cells in the esophagus, stomach and intestine until 40 DAH. Gisbert et al. (2004) found the goblet cells in the fundic region of the stomach in California halibut by 30 days, but in yellowtail kingfish the goblet cells were not

found in the stomach until 36 days (Chen et al. 2006). Hamlin et al. (2000) reported that goblet cells in the pyloric caeca were more than those in the intestine in haddock, indicating that the distribution of goblet cells in the digestive tract varies among species.

At the marine fish hatcheries, the weaning of humpback grouper larvae usually starts on 13 DAH when the cardiac stomach, fundic stomach and pyloric sphincter are formed. In the present study, pyloric sphincter separated the stomach from the midgut then comprising the cardiac and pyloric regions. However, other studies have suggested that weaning should start after the appearance of gastric glands (Segner et al. 1993) which, in the present study was seen at 25 DAH since the digestion enzyme needed for the digestion process is secreted by this gland.

Conclusion

In conclusion, the digestive system ontogeny of humpback grouper larvae follows a pattern similar to those of other marine finfish species, especially that of grouper. The digestive system was ready to process exogenous food by the end of the first developmental phase, but the digestive system became mature when gastric glands and pyloric caeca appeared at the onset of the third phase on 25 DAH. The formation of the fundic stomach is suggested to be the appropriate point to wean humpback grouper onto commercial pellets. A better understanding of the ontogeny of fast-growing fish larvae can thus help to improve its rearing techniques and survival rate.

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