Ontogeny of digestive and immune system organs of larval and juvenile kelp grouper *Epinephelus bruneus* reared in the laboratory

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**ABSTRACT:** A histological examination was made of the ontogenetic development of the digestive and immune systems of the larval and juvenile kelp grouper *Epinephelus bruneus* reared in the laboratory. The liver, gall bladder, pancreas and the demarcating region between the intestines and rectum were formed within 3 days post-hatch (dph). During the preflexion phase (within 16 dph), revolution of the intestine concluded, and pharyngeal teeth and the mucous cells of the esophagus were differentiated. In the transitional period to the juvenile stage (25 dph), the blind sac of the stomach, gastric glands and pyloric caeca began to form. From the viewpoint of the differentiation phase of the adult-type digestive system, the kelp grouper is similar to Heterosomata, hitherto reported. The primordial thymus, kidney and spleen were present at 12, 1 and 6 dph, and the small lymphocytes in these lymphoid organs appeared at 21, 30 and 33 dph, respectively. The developmental sequence of the lymphoid organs and the appearance ages of the lymphoid organs and small lymphocytes in the lymphoid organs in the kelp grouper are similar to those of other marine fish previously reported, except for the Pacific bluefin tuna *Thunnus orientalis*.

**KEY WORDS:** digestive system, *Epinephelus bruneus*, immune system, juvenile, kelp grouper, larvae.

**INTRODUCTION**

Groupers are in demand as a new aquaculture species in East and South-East Asia† ‡§ because of their high prices, and the artificial larval rearing of groupers has been practised. The kelp grouper *Epinephelus bruneus* is one of the most economically important species for aquaculture in Japan. However, there is high mortality during the early life stages of artificially reared larvae, and more detailed information on their early growth and development is necessary to improve larval rearing techniques.

Knowledge of the digestive system developmental changes associated with food assimilation processes is essential for understanding the nutritional physiology of larval fish. In addition, the age at which immune competence is attained is an important factor in determining at which developmental stage the animal is most vulnerable, and which treatment can be most effectively administered, if necessary. This knowledge might help identify the limiting factors during larval rearing, reducing bottlenecks in the weaning process and synchronizing the stage of development with rearing technology and feeding practices. Thus, following a previous study on the growth and morphological development, in the present study, the development of the digestive and immune systems during the larval and juvenile stages in kelp grouper reared in the laboratory has been investigated.

**MATERIALS AND METHODS**

The egg collection and rearing conditions of the sample larvae and juveniles have been described previously. The rearing water temperature ranged from 23.4 to 25.8°C (25.0°C average). The feeding scheme was as follows: rotifer *Brachionus* sp. from 3 to 31 days post-hatch (dph); *Artemia* nauplii (*Artemia salina*) from 20 dph; eggs and larvae of *Pagrus major* from 32 dph; and formulated feed from 18 dph until the end of rearing (45 dph). Twenty fish were sampled daily for 45 dph from the
same tank used in a previous study. These fish were fixed in Bouin’s fluid (ScyTek Laboratories, West Logan, UT, USA) and preserved in 80% ethanol. For light microscopy, the fish were embedded in paraffin and serial sections (3–7 μm) were stained with hematoxylin–eosin and/or with periodic acid Schiff (PAS) reaction. To confirm the accumulation of lipid in the juvenile stage, the Nile blue sulfate method was used.

RESULTS

Digestive system

Mouth, pharynx and esophagus

Longitudinal sections of larvae at different numbers of days post-hatch are shown in Figure 1. At 2 dph (total length [TL], 2.93 mm), the mouth had opened and oral valves had appeared. The rudimentary gill arches were visible under the epithelium of the posterior pharynx and the external gill slit had formed. By 3 dph (TL, 2.94 mm; Fig. 1e), the digestive tract had opened completely from the mouth to the anus. The esophagus, composed of single layer of cuboidal cells, was observed and the border region to the rudimentary stomach was distinct. At 7 dph (TL, 3.50 mm; Fig. 1c), the mucous cells of the esophagus were observed and longitudinal folds had developed in the border region between the esophagus and the rudimentary stomach. By 12 dph (TL, 5.01 mm; Fig. 2), mucous cells had appeared in the pharyngeal cavity and the pharyngeal teeth had formed.

Stomach and pyloric caeca

At 2 dph, the rudimentary stomach, composed of a single layer of cuboidal cells, had formed (Fig. 1a). The esophagus and stomach were linearly located but the stomach had started to enlarge at 4 dph. By 6 dph, the stomach had enlarged and become an ellipse. In the pyloric region, the wall of the stomach had become thicker and the epithelium had changed to a columnar shape at 7 dph, and longitudinal folds had developed in the rudimentary stomach at 9 dph (TL, 3.90 mm; Fig. 1b). The blind sac had formed, gastric glands had begun to appear in the stomach and the pyloric caeca had

Fig. 1 Longitudinal sections of larvae at (a) 2 dph, (b) 9 dph, (c) 7 dph, (d) 1 dph, (e) 3 days post-hatch (dph) and (f) 10 dph. ab, air bladder; es, esophagus; gb, gall bladder; gc, goblet cell; it, intestine; li, liver; no, notochord; mc, mucous cell; og, oil globule; pa, pancreas; ph, pharynx; re, rectum; rst, rudimentary stomach; yo, yolk sac.
appeared in the pyloric portion by 25 dph (TL, 12.31 mm; Fig. 3a). By the end of the study, at 45 dph, the size and number of the gastric glands and pyloric caeca had increased and a complex internal structure was observed in the caeca (TL, 29.98 mm; Fig. 3c).

**Intestine**

The intestine, composed of a single layer of columnar and cuboidal cells, had formed at 1 dph (TL, 2.90 mm; Fig. 1d). The intestine and rectum were clearly differentiated with the development of a sphincter between mid-gut and hind-gut. The fish had begun feeding and a little intestinal digestion of rotifers was observed at 3 dph. The intestine had commenced coiling, and many acidophilic globules were observed in the rectal epithelia at 7 dph. Goblet cells (PAS-positive cells) were found in the region close to the rectum in the intestinal epithelium at 10 dph (TL, 4.19 mm; Fig. 1f). The intestines had finished coiling by 12 dph. By the end of the study, at 45 dph, many...
acidophilic microglobules were observed in the rectal epithelia.

Accessory organs (liver, pancreas and gall bladder)

By commencement of feeding at 3 dph, the liver, pancreas and gall bladder had formed. The pancreas had appeared above the border region between the rudimentary stomach and gut, and pancreatic islet and acidophilic zymogen granules were observed. The liver and gall bladder were located under the rudimentary stomach between the gut and oil globule. By 8 dph, the pancreas had extended posteriorly along the intestine. At 10 dph, marked glycogen deposition was observed in the liver. In the juvenile stage (33, 35 and 45 dph; Fig 3b,c,d), numerous vacuoles were observed in the pancreas of the juveniles, and these vacuoles were positive for Nile blue sulfate stain. However, few Nile blue-positive vacuoles were observed in the livers of juveniles.

The relationship between the age differentiation of the characteristics of the digestive system and the developmental stages of larval and juvenile kelp groupers.

Immune system

Thymus

At 12 dph there was a distinct thymus under the pharyngeal epithelium above the gill arch (Fig. 6a). At this stage the thymus consisted of basophilic-staining blast-like cells (3–4 mm in diameter) with large pale nuclei. These cells increased in number and many reticular cells (4–6 mm) with large pale nuclei and long cytoplasmic processes were observed at 15 dph, and the cytoplasmic processes formed a reticulum within the thymic parenchyma. These cells became deeply stained with larval growth, and the early stages of thymic zonation were apparent at 20 dph. The smaller cells (3.0–3.5 mm) with deeply staining nuclei were observed at 21 dph (Fig. 6b). The smaller cells were small lymphocytes, as identified by their small dense nucleus with a high nuclear to cytoplasmic ratio. By 30 dph distinct zonation was marked (Fig. 6c).
Kidney

The excretory kidney was present as tubules situated between the yolk sac and the notochord by 1 dph (Fig. 7a). By 3 dph, obvious tubules were found with large blast-like cells (6–7 mm) between the tubules. As development continued, the blast-like cells increased in number and became smaller and darker staining (4.0–5.5 mm, at 7 dph). By 15 dph, there was a distinct increase in hematopoietic tissue and a variety of cell types was evident. Small lymphocytes (3.0–3.5 mm) with deeply staining nuclei and a high nuclear to cytoplasmic ratio were evident by 30 dph (Fig. 7b).

Spleen

The primordial spleen, which consisted of blast-like cells (3–4 mm), had appeared by 6 dph and was located close to the gut (Fig. 4a). Progression was rapid; by 8 dph the capsule was distinct and reticular cells (3–6 mm) with large pale nuclei and long cytoplasmic processes were observed (Fig. 4b). At 10 dph the blood vessel supplying the spleen was distinct. The organ remained mainly erythroid throughout the larval stage. At the early juvenile stage (33 dph) small lymphocytes (3.0–3.5 mm) with deeply staining nuclei and a high nuclear to cytoplasmic ratio were observed (Fig. 4c).

DISCUSSION

In many species, the major differences in the digestive system between larvae and adults are the absence of a functional stomach and pyloric caeca in the early stages of life. In Heterosomata,9–13 gastric glands and/or a pyloric caecum are
formed during the metamorphosis phase (transitional phase to the juvenile stage). Tanaka reported that in spite of age-wise variations, the gastric glands are always formed at approximately the same phase, namely at approximately two-thirds of the postlarval stage, in four species: red sea bream *Pagrus major*, black sea bream *Acanthopagrus schlegeli*, scorpion fish *Sebastiscus marmoratus* and ayu *Plecoglossus altivelis*. In addition, gastric glands are formed during the latter half of the postlarval stage in larvae such as yellowtail *Seriola quinqueradiata*, Japanese whiting *Sillago japonica*, milkfish *Chanos chanos* and freshwater goby *Chaenogobius annularis*. Tanaka also reported that the differentiation time of the pyloric caecum corresponds to the respective transitional period from larvae to juvenile in the four species mentioned above. In contrast, in scombriform fishes, such as Pacific northern bluefin tuna *Thunnus orientalis*, skipjack tuna *Euthynnus pelamis*, yellowfin tuna *Thunnus albacares*, striped bonito *Sarda orientalis* and Spanish mackerel *Scomberomorus niphonius*, which show precocious development, the gastric glands and the blind sac of the stomach are formed during the earlier half of the postlarval stage, and the pyloric caecum is formed by the middle of the postlarval stage. In the present study, these three characteristics were formed at 25 dph (transitional phase to the juvenile stage) in the kelp grouper. Thus, from the viewpoint of the differentiation phase of the adult-type digestive system, the kelp grouper is similar to Heterosomatia.

In a similar way to most marine finfish species, kelp grouper hatchlings possess an undifferentiated, rudimentary digestive system. The first major morphological changes in the digestive system of the larvae happen during the first 2 dph, when larvae are exclusively dependent on endogenous reserves; the liver, gall bladder, pancreas and the demarcating region between the intestine and rectum form within 3 dph, and acidophilic zymogen granules are observed in the pancreas. Although enzymatic data are not available for kelp grouper larvae, several studies have described the presence of enzymatic activity at first feeding in the larvae of other fish species. Kelp grouper first-feeding larvae have a mouth size that is in the smallest size group. The small mouth size has been suggested as one of the causes of heavy mortalities in grouper during the early developmental stages. Thus, the rotifer size at the time of feeding commencement is an important factor for the achievement of a high survival rate.

The second major morphological changes in the digestive system of kelp grouper larvae are the differentiation of the gastric gland and the formation of the pyloric caecum at 25 dph. In California halibut larvae, Gisbert *et al.* reported that the presence of goblet cells in the fundic stomach, secreting a large amount of neutral mucous substances might indicate that gastric glands are already functional, although enzymatic data (pep-
sin secretion) are not available. In the present study, goblet cells and a large amount of neutral mucosubstances were observed in the stomach by 31 dph. Subsequently, the early juveniles commenced feeding on fish eggs and larvae at 32 dph and an accumulation of lipids was observed in the pancreas. These remarkable changes, including food habit, protein digestion and energy accumulation during the transitional phase to the juvenile stage might indicate the importance of quality, amount and variety of food in this period. Future work must focus on the ontogeny of enzymatic secretions to provide precise information about the functionality of the digestive tract and to evaluate the effect of different feeding strategies on digestive tract maturation.

Knowledge of lymphoid organs in teleosts is based on anatomic and histological observation. The thymus and kidney are considered to be the major primary lymphoid organs; the latter is the equivalent of bone marrow in adult mammals, the source of B lymphocytes. In general, the available data from studies on the effect of thymectomy on the maturation of the fish immune system support correlations among the histological maturation of the teleost thymus, the appearance of lymphocytes in the peripheral lymphoid organs and the development of cell-mediated immune responses. The presence of small lymphocytes in both the thymus and kidney by 30 dph might be important in the immune system development of kelp grouper. The histogenesis of the lymphoid organs and small lymphocytes in the lymphoid organs in five marine fishes are shown in Table 1. The developmental sequence of the lymphoid organs and the appearance ages of the lymphoid organs and small lymphocytes in the lymphoid organs in kelp grouper are similar to those of red sea bream, yellowtail and Japanese flounder Paralichthys olivaceus.

A problem in the larviculture of groupers is the intermittent occurrence of viral nervous necrosis (VNN), which has caused mass mortalities in the hatchery and has probably contributed to the low survival rates seen in these species. The disease apparently affects most species of groupers and is transmitted from the brood fish or through the rearing water (reviewed in the study by Marte). The occurrence of VNN with high mortalities is also observed in larval and juvenile kelp grouper. In other fish species, including sevenband grouper, vaccinations against VNN have been attempted and preventive effects have been observed experimentally. For vaccination against an infection during the larval and juvenile stages, knowledge of the age fish are able to mount an adaptive immune response is required in order to prevent the effect of immune tolerance. Further studies are needed to determine the functional maturity of the immune system in this species.

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