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Original Article

Ontogenetic development of the digestive tract and ultrastructure of the anterior intestinal epithelia in tiger grouper *Epinephelus fuscoguttatus* (Forsskål, 1775) larvae

Kornrawee Aiemsomboon,[a, 1] Wanpen Khammee,[b, 1] Paiboon Bunlipatanon,[c, 1] Uthairat Na-Nakorn[d, *]

[a] Graduate Program in Aquaculture, Graduate School, Kasetsart University, Bangkok, 10900, Thailand
[b] Coastal Aquaculture Research and Development Regional Center (Krabi), Krabi, 81000, Thailand
[c] Phuket Provincial Fisheries Office, Department of Fisheries, Phuket, 83000, Thailand
[d] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, 10900, Thailand

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ABSTRACT

The ontogeny of the digestive tract and ultrastructure of the anterior intestine in *Epinephelus fuscoguttatus* (Forsskål, 1775) larvae were examined using light microscopy and transmission electron microscopy from hatching to 42 d after hatching (DAH). The first developmental stage started at hatching when the digestive tract was a simple tube. The second stage (2–3 DAH), the endo-exotrophic stage, was the time when the mouth of the tiger grouper larvae developed. The third stage (3–24 DAH) started after the depletion of the yolk-sac (3 DAH). The remarkable changes included the appearance of gastric glands at 9 DAH, eosinophilic supranuclear vacuoles appearing in the posterior intestine at 5 DAH and lipid vacuoles found in the interior intestine at 6 DAH which indicated the beginning of protein and lipid absorption. The last stage (after 24 DAH) started when the gastric glands and pyloric caeca were fully developed. The formation of the gastric glands and pyloric caeca indicated a suitable time for weaning. The ultrastructural features of epithelium cells of the anteria intestine showed large lipid droplets at the beginning of the exotrophic stage. From this time onwards, the lipid droplets became smaller, while the endoplasmic reticulum and Golgi complex were well-developed. Upon metamorphosis, the tiger grouper larvae had eosinophilic granule cells (EGCs) in the intestinal epithelia. This substantially increased immunity capability at this time. This study showed that during a critical period of larval survival, the ontogeny coincided with exogenous feeding, while the ultrastructure showed lipid metabolism, hence highlighting the importance of fatty acid in the development of the larvae.

Introduction

The tiger grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775) also known as the brown marbled grouper, is widely distributed in Indo-Pacific tropical and subtropical waters (Randall and Heemstra, 1991). It is popular as a medium-to-high priced live-food fish in the Asian market. Although market demand for this species is increasing which has led to the expansion of aquaculture in many Southeast Asian and Pacific countries (Yamamoto, 2006; Afero et al., 2009), the production of this species together with other grouper species has not been high being 28,456 t in 2015 (FAOSTAT, 2015). This is partly because of a high mortality rate during larval development of these species under intensive culture conditions (Qu et al., 2012).

The larval stage involves morphological and functional changes in the internal organs especially the digestive system (Govoni et al., 1986), which corresponding influence feed digestibility and utilization. The transitional period from endogenous to exogenous feeding is often linked to high mortality of larval fish (Ýufera and Darian, 2007). Moreover, the development of the anterior intestine enterocytes of the larvae plays key roles in digestive physiology as has been shown in orange-spotted grouper (*Epinephelus coioides*) (Primavera-Tirol et al., 2014). The development of the enterocyte ultrastructure with respect to the digestive ontogeny in

* Corresponding author.
E-mail address: ffisurn@ku.ac.th (U. Na-Nakorn).
[1] Equal contribution.

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marine fish larvae (Elbal et al., 2004; Yang et al., 2010; Qu et al., 2012) during start-feeding and starvation (Kjorsvik et al., 1991) and when fed artificial diet (Senger et al., 1993) and under different feeding regimes (Primavera-Tirol et al., 2014) have been previously reported.

Previous studies characterizing the digestive capacity of grouper larvae have been conducted, including the development of the gastrointestinal tract (Quinitio et al., 2004a; b; Aripin, 2010; Qu et al., 2012). The ontogenetic development of coral trout, *Plectropomus leopardus* larvae was divided into four major stages (Qu et al., 2012). Stage I (0–2 DAH) spans the time from hatching to the mouth opening whereby the fish depend exclusively on an endogenous nutritional source. Stage II (2–3 d after hatching; DAH) is the endo-exotrophic period, which starts from the mouth opening and ends at the depletion of the yolk sac. Stages III and IV are exotrophic stages. The start of exogenous feeding marks the beginning of Stage III (3–19 DAH). Stage IV (after 19 DAH) begins when the gastric glands and pyloric appendages appear. Although the ontogeny of digestive enzymes has been reported (Eusebio et al., 2004; McBride, 2004; Fujii et al., 2007; Aripin, 2010; Martínez-Lagos et al., 2014), more detailed information could be obtained from the development and organization of the enterocyte ultrastructure with respect to the digestive ontogeny (Elbal et al., 2004). Despite its importance, limited studies have been done on the ultrastructure of the intestinal epithelia of grouper (e.g. Qu et al., 2012; Primavera-Tirol et al., 2014), and to the authors' knowledge, there are no studies on *E. fuscoguttatus*.

The aim of the present study was to observe the structure of the digestive tract and the ultrastructure of enterocytes during ontogeny of *E. fuscoguttatus* from hatching to 42 DAH. It is hoped that this information can provide fundamental knowledge of benefit to larval rearing of this species.

Materials and methods

Larvae and rearing conditions

Tiger grouper larvae were obtained from spontaneous spawns of captive broodstock between March and April 2013 at the Coastal Aquaculture Research and Development Regional Center (Krabi), Thailand. Fertilized eggs were collected and incubated in 500 L circular fiberglass tanks with mildly aerated seawater at ambient salinity (approximately 30 psμ) and temperature (28–30 °C). Hatching occurred 19–20 h after fertilization. Then, the larvae were stocked in 182 cm × 250 cm × 110 cm cement tanks at a density of 5 fish/L at an ambient temperature of 29–30 °C. Water replacement was done daily at 10% per day. During the rearing period, the water quality was: salinity 30–33 psμ, dissolved oxygen 7–9 mg/L and pH 7.90–8.50. Fish were held under natural photoperiod conditions throughout the trial.

The feeding regime of the larvae was: 2–6 DAH, ss-type rotifer (*Brachionus* sp., size 80–90 μm), 5–15 individuals/mL; 3–5 DAH, copepod nauplii (*Pseudodiaptomus aurivilli* Cleve), 3–4 individuals/mL; 6–24 DAH, s-type rotifer (size 120–150 μm), 10–15 individuals/mL, plus *Chlorella* sp. as rotifer feed, at a final density of 2–3 × 10^3 cells/mL and *Artemia* nauplii (Salt Creek Inc.; Salt Lake City, UT, USA), enriched with 50 mg/L 'EASY DHA Selco' for 6 h, 5 individuals/mL; 24–34 DAH, thawed, frozen adult *Artemia* purchased from a farm, 5 individuals/mL, five times per day (0800 h, 1100 h, 1300 h, 1600 h and 1800 h); 35–42 DAH, thawed, frozen adult *Artemia* (2–3 individuals/mL) plus artificial diet (NRD G12, INVE Aquaculture Inc.; Phichit, Thailand; protein 55%, lipid 9%, ash 14.5%, particle size 1200–2000 μm), fed to satiation twice (0900 h and 1300 h) per day. The feeding schedule and feed types relating to the growth of the larvae are shown in Fig. 1.

Fish sampling and growth measurements

The fish larvae were randomly collected every day during 0 and 9 DAH, and at 12, 15, 18, 24, 30, 36 and 42 DAH. No food was added to the rearing tank at night prior to sampling which was done in the morning to minimize the effects of exogenous enzymes from live food in the fish guts (Kolkovski, 2001). The total length (L_T) of 10 specimens was individually measured to the nearest 0.1 mm using an ocular micrometer with the aid of dissected microscopy, and a vernier caliper when the larvae were ≥10 mm. Larval growth was determined as the absolute growth rate (AGR, in millimeters per day) and specific growth rate (SGR, % total length/day) according to Hopkins (1992). The AGR was calculated as: $AGR = (TL_f - TL_i)/\Delta t$, and the SGR was determined as: $SGR = 100\ (LnTL_f - LnTL_i)/\Delta t$,

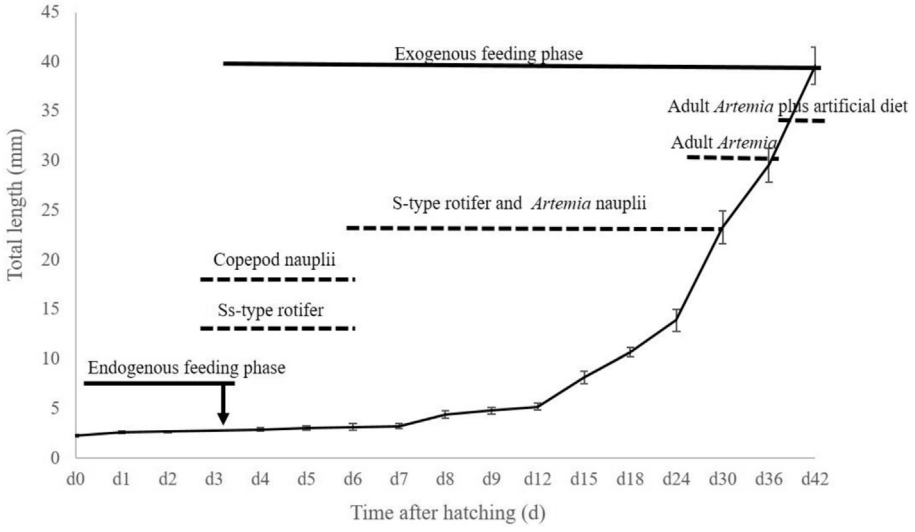

Fig. 1. Total length (±SD, n = 10) of *Epinephelus fuscoguttatus* larvae 0–42 d after hatching. Main feed types and associated feeding schedule are marked by dashed lines; the arrow indicates the onset of exogenous feeding.

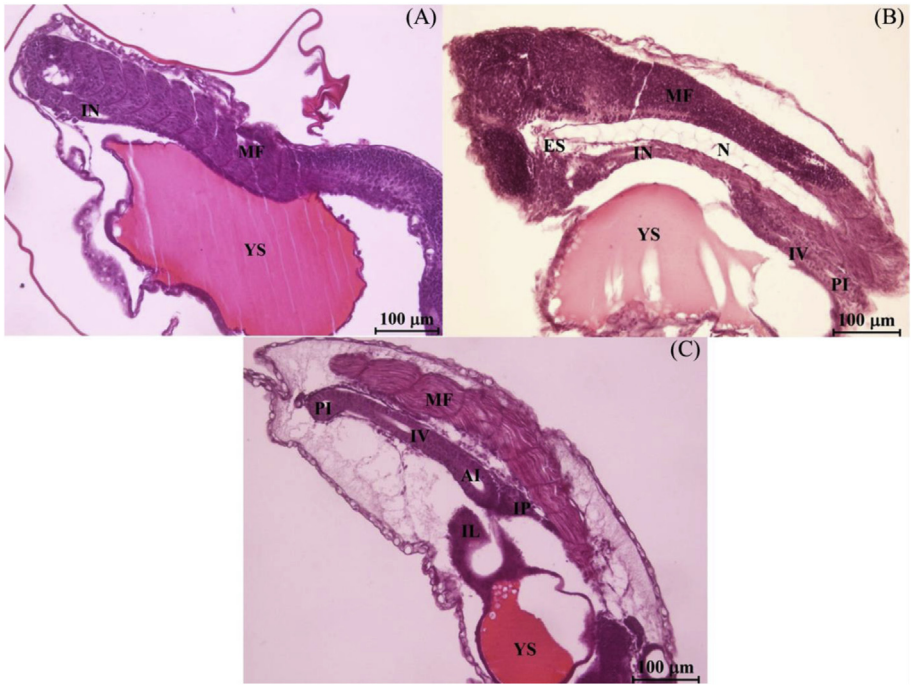

Fig. 2. Sagittal section of *E. fuscoguttatus* larva: (A) at 0 d after hatching (DAH), note the nearly straight gut lying dorsally to the yolk sac; (B) 1 DAH, the intestinal valve appears and separates the intestine; (C) 2 DAH, note the incipient liver lying between the digestive tract and the yolk sac, where AI = anterior intestine; ES = esophagus; IL = incipient liver, IN = incipient intestine, IP = incipient pancreas, IV = intestine valve, MF = muscular fiber, N = notochord, PI = posterior intestine, YS = yolk sac and scale bar = 100 µm.

where TL_i and TL_f are the initial and final total length in millimeters, respectively, and Δt is the time interval in days between sampling dates. Ten specimens were used to describe the morphology of a digestive tract.

For histological observation, specimens (n = 10) were fixed with Bouin's fixative, while the samples for ultrastructure observation (n = 5) were fixed with 2.5% glutaraldehyde adjusted to pH 7.2 with 0.1 M phosphate buffer at 4 °C. The 0–11 DAH specimens were processed as whole bodies, while the 12–42 DAH specimens were dissected, but only the body part including the digestive tract was collected.

Histological study

Each fixed sample set (n = 10) was dehydrated in a graded series of ethanol, embedded in paraffin blocks and sectioned into serial sagittal and transverse sections (5 µm thick) using a microtome MRS3500 (Histo-Line Laboratories; Milan, Italy). Then, the tissues were stained with hematoxylin and eosin (H&E) stain for general histological observation. The sections were examined and photographed under a microscope (CX41; Olympus Corporation, Tokyo, Japan) equipped with a digital camera (U-TVO.5XC-3; Olympus Corporation, Tokyo, Japan).

Ultrastructural study

The pre-fixed samples were post-fixed in 2% osmium tetroxide (OsO_4) at pH 7.2 with 0.1 M phosphate buffer for 2 h and then dehydrated in a graded series of acetone, infiltrated and embedded in Spurr's resin (Spurr, 1969), respectively. Ultrathin sections (70 nm) obtained with an ultramicrotome (EM UC7; Leica; Wetzlar, Germany) were stained with uranyl acetate and subsequently with lead citrate (Hoppert and Holzenberg, 1998) and examined using a transmission electron microscope (HT7700; Hitachi; Tokyo, Japan) at 80 KV.

Results

Growth and larval development

The total length of larval fish averaged 2.27 ± 0.17 mm at hatching (0 DAH), and increased to 39.61 ± 1.86 mm at 42 DAH (Fig. 1). The absolute growth rate and specific growth rate were 0.89 mm/d and 6.81% TL/d, respectively.

Histology of digestive tract development

At hatching, the digestive tract of *E. fuscoguttatus* larvae appeared as a straight and undifferentiated tube, not opened to the exterior (undifferentiated buccopharynx), lying dorsally to a large yolk sac (Fig. 2A). The lumen of the intestine appeared at 1 DAH (Fig. 2B). At this time, an intestinal valve appeared and separated the intestine into the interior intestine and posterior intestine. During yolk-sac absorption (1–2 DAH), the rudimentary digestive system differentiated into the buccopharynx, esophagus and intestine. The posterior intestine was bent at 90° in an L shape. At 2 DAH, the mouth opened and the incipient liver and pancreas were visible between the digestive tract and the yolk sac (Fig. 2C). During this period, the incipient stomach appeared as a bulge at the posterior end of the esophagus.

The fish larvae started feeding at 3 DAH, and the brush borders in the apical region of the columnar cells were obvious. At this time, the swim bladder, intestine, liver and pancreas were all developed.

The buccopharyngeal cavity and esophagus were well developed at 9 DAH, and the stomach was visible as an extension of the esophagus (Fig. 3A) and the esophagus goblet cells were first found at 12 DAH (Fig. 3B). At 18 DAH, a longitudinal fold which was composed of loose connective tissue appeared and increased both in height and length with the growth of the larva (Fig. 3C).

As described in the previous section, the stomach was first observed at 9 DAH and gradually increased in size and number of

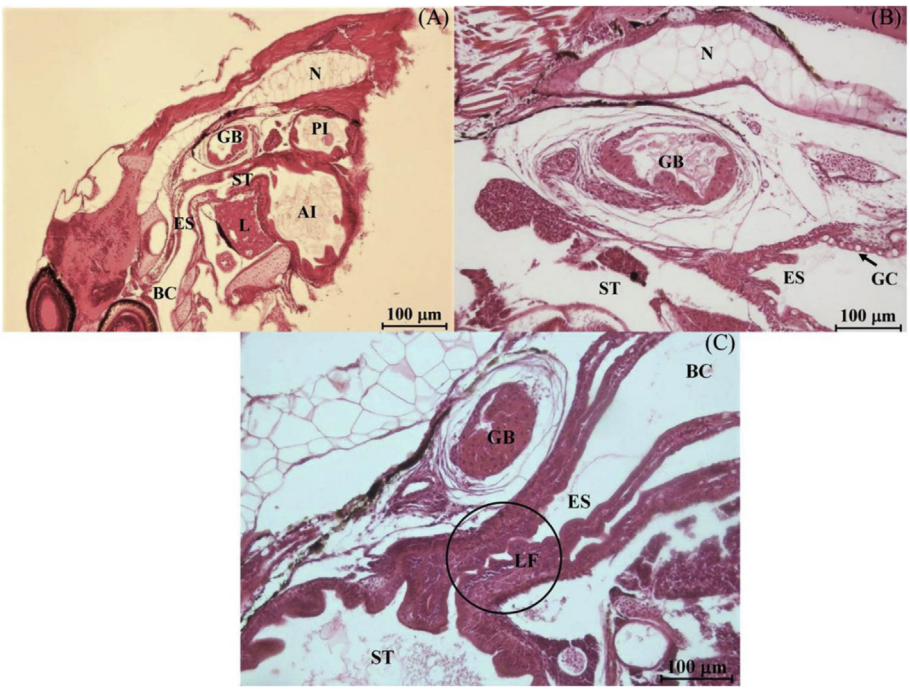

Fig. 3. Sagittal section of *E. fuscoguttatus* larva: (A) at 9 d after hatching (DAH), general view of a larva, note the buccopharyngeal cavity and esophagus (scale bar = 100 µm); (B) at 12 DAH, a detail of the esophagus, note the presence of goblet cells (arrow) in the esophagus (scale bar = 100 µm); (C) at 18 DAH, the longitudinal fold (circled) appeared in the esophagus (scale bar = 100 µm), where AI = anterior intestine, BC = buccopharyngeal cavity, ES = esophagus, GB = gas bladder, GC = goblet cell, L = liver, LF = longitudinal fold, N = notochord, P = pancreas, PI = posterior intestine and ST = stomach.

mucosal folds (Fig. 4A) as the larva grew. The gastric glands were fully differentiated at 24 DAH and increased in number with the growth of the larva (Fig. 4B and C). Goblet cells appeared and increased in number and the liver increased substantially in size at 24 DAH. At 42 DAH, the cardiac, fundic and pyloric portions of the stomach could be distinguished.

At 15 DAH, pyloric caeca appeared in the region between the stomach and the beginning of the anterior intestine (Fig. 5A). The

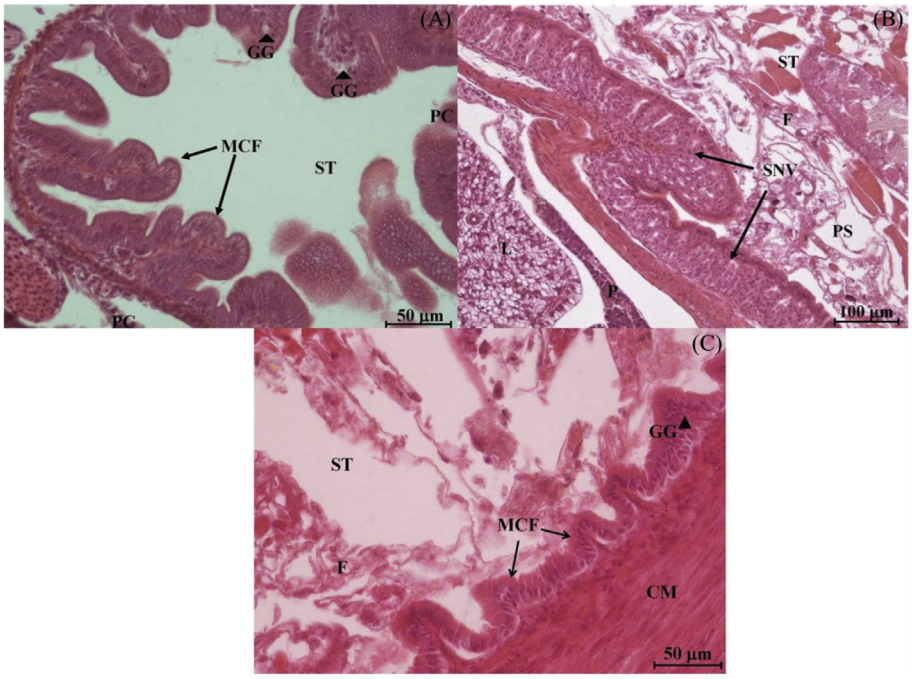

Fig. 4. Sagittal section of *E. fuscoguttatus* larva: (A) the larval stomach at 24 d after hatching (DAH), showing the gastric glands (arrowheads) and mucosal fold (arrows) (scale bar = 50 µm); (B) the stomach at 36 DAH, note the presence of supranuclear vacuoles (arrows) in mucosa of the pyloric stomach (scale bar = 100 µm); (C) the gastric glands at 42 DAH, and mucosal fold (arrows) (scale bar = 50 µm), where CM = circular muscle, F = food content, GG = gastric glands, L = liver, MCF = mucosal fold, P = pancreas, PC = pyloric caeca, PS = pyloric stomach, SNV = supranuclear vacuoles and ST = stomach.

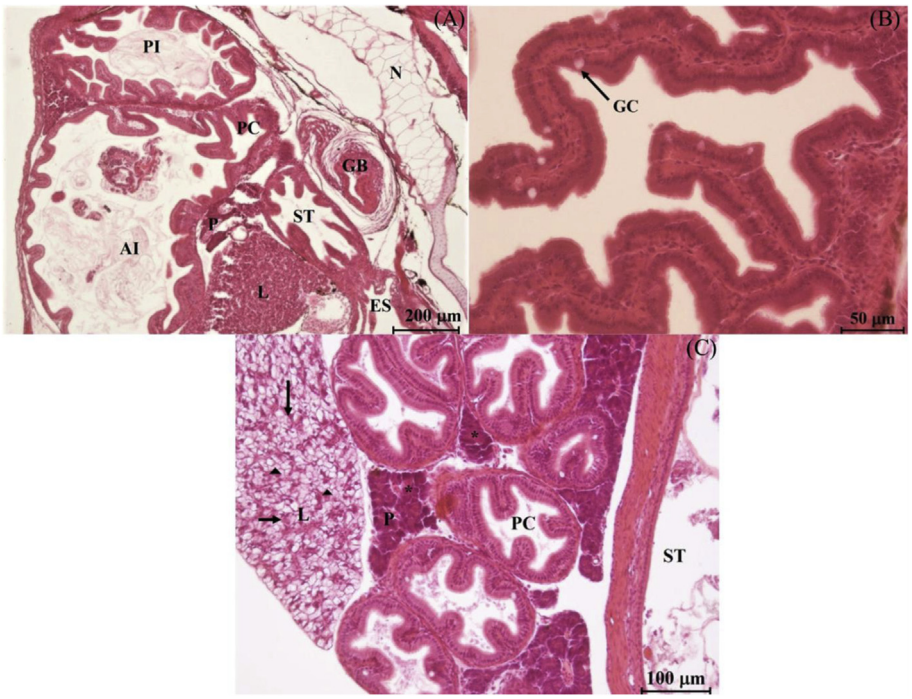

Fig. 5. Sagittal section of *E. fuscoguttatus* larva: (A) the pyloric caeca at 15 d after hatching (DAH), showing the different characteristic areas of the digestive system (esophagus, gas bladder, stomach, lipver, pancreas, anterior intestine, posterior intestine) with scale bar = 200 µm; (B) intestine of a larva at 30 DAH, showing the mucous membrane fold and goblet cells (arrow) with scale bar = 50 µm; (C) liver, pancreas, and pyloric caeca at 36 DAH, showing the hepatocytes (arrows) and vacuoles for lipid storage (arrowheads) in the liver and zymogen granules (asterisks) in the pancreas, with scale bar = 100 µm, where: AI = anterior intestine, ES = esophagus, GB = gas bladder, GC = goblet cell, L = Liver; N = notochord, PI = posterior intestine, P = pancreas, PC = pyloric caeca and ST = stomach.

histology structure of the pyloric caeca was similar to that of the anterior intestine.

At 36 DAH, numerous vacuoles appeared in the liver for glycogen and lipid storage (Fig. 5C), and the hepatocytes cytoplasm was full of lipid vacuoles. From 36 DAH to 42 DAH, the liver and pancreas increased in size without any substantial structural changes.

All of the digestive organs were visible in the histological sections, and became developed between 24 DAH and 36 DAH. During this stage, the most obvious changes in the morphology of the digestive tract were: 1) the formation of the stomach, 2) the appearance of small vacuolar inclusions in the enterocytes of the medium intestine, 3) liver enlargement and 4) embedding of the exocrine pancreas into the medium intestine.

There was also a rapid increase in the number of gastric glands and in the size of the stomach at 24 DAH. At 30 DAH, the intestine loop was formed to accommodate the increasing length of the digestive tract inside the abdominal cavity (Fig. 5A). As the larva grew, the mucosal folds were deeper and more abundant, and the number of goblet cells in the posterior intestine increased. Folds of both anterior and posterior intestinal mucosa occupied most of the intestinal lumen (Fig. 5B). The morphology of the digestive tract resembled that of the adult by day 36 DAH, with an enlarged, fully developed stomach containing a large number of gastric glands. The stomach was connected to the anterior intestine through the pyloric sphincter, which was covered by a thick muscular wall.

Ultrastructure of anterior intestinal epithelia of tiger grouper

At hatching, the intestinal epithelia of *E. fuscoguttatus* larvae had mitochondria and electron-lucent lipoprotein particles in the enterocytes, microvilli appeared in the lumen surface of the anterior intestinal epithelial cells. The nucleus was oval or irregularly contoured and had one or two prominent nucleoli (Fig. 6A). Microvilli started to develop as granular cytoplasmic extensions from the apical membrane of enterocytes and were fully differentiated within 24 h after hatching with evenly structured microvilli in columnar enterocytes.

The mucus granules first appeared in the intestinal epithelium at 3 DAH. The time of mucus granule appearance was associated with the first external feeding. The mucosa epithelium of the anterior intestine consisted of tall, columnar absorptive cells with a nucleus located basally and microvilli at the apical region, interspersed occasionally with goblet cells without microvilli (Fig. 6B). Lipid droplets were first observed at this age.

On day 6 after hatching, large supra- and infranuclear lipid droplets were observed together with the small mitochondria (Fig. 7A). Lateral chylomicrons associated with lamellar structures were observed near the inter-enterocytes space and beside the basal laminar. Lamellar structures were located in the mid-basal portion of the cell, in close association with mitochondria (Fig. 7B). Grouped lipid particles were surrounded by membrane located in the supranuclear cytoplasm.

At 24 DAH, the mitochondria changed into a more elongated shape, while rough endoplasmic reticulum in cytoplasm of absorptive cells of an anterior epithelium were prominent (Fig. 8A). Apoptotic bodies and pyknotic nuclei were also observed (Fig. 8B). At 36 DAH, enterocytes had infranuclear lipid droplets (Fig. 9A).

A centrally placed spherical nucleus, surrounded by abundant endoplasmic reticula was a character of the enterocytes at 42 DAH. Apical mucus granules full of mucus were observed near the terminal web. Thin and irregular microvilli were observed in the enterocytes with very large apical lipid droplets. The number of mucus granules increased as the larvae developed and became very abundant by 42 DAH. The infranuclear lipid droplets appeared surrounding the nucleus. An eosinophilic granule cell (EGC) with dark-stained cytoplasmic granules was observed also (Fig. 10).

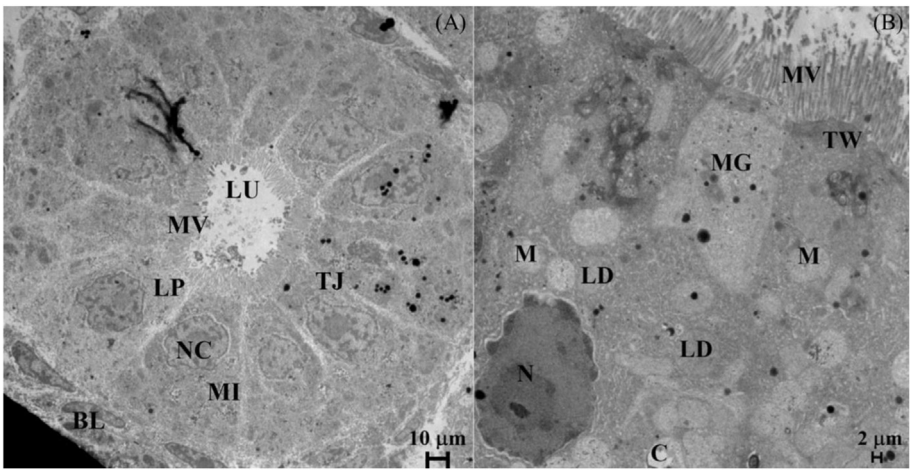

Fig. 6. Transmission electron micrographs of the intestine of *E. fuscoguttatus* larva: (A) intestinal epithelium of a 0 d after hatching (DAH) larva, showing columnar enterocytes delineated by evenly structured, apical microvilli, tight junctions, nucleus and basal lamina developed within 24 h in the undifferentiated gut, and lipoprotein particles, with scale bar = 2 μm and magnification = ×1500; (B) intestinal epithelium of 3 DAH larva, showing enterocytes with well-organized microvilli, mucus granules through the terminal web, nucleus and some mitochondria, note the terminal web and electron-opaque supranuclear lipid droplets, with scale bar = 2 μm and magnification = ×2000, where BL = basal lamina, C = chylomicrons, L = lumen, LD = lipid droplets, LP = lipoprotein particles, M = mitochondria, MG = mucus granules, MV = microvilli, N = nucleus, TJ = tight junctions and TW = terminal web.

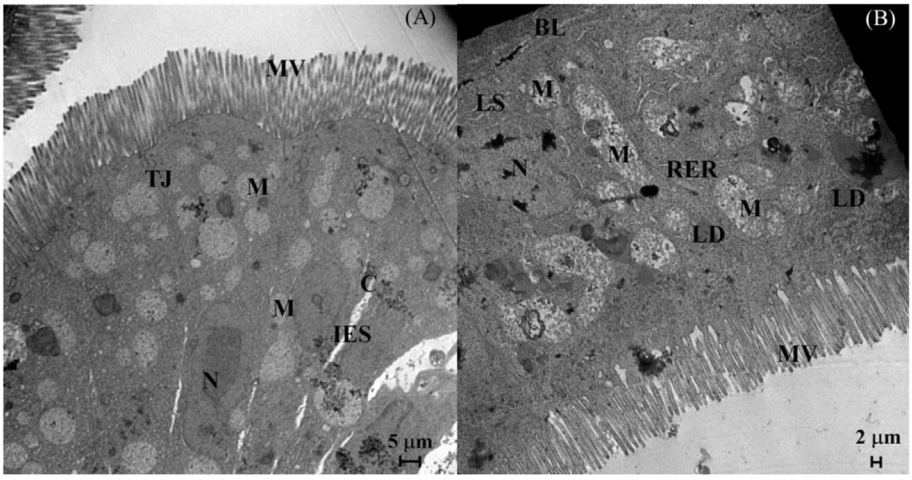

Fig. 7. Transmission electron micrographs of the intestine of *E. fuscoguttatus* at 6 d after hatching (DAH): (A) intestinal epithelium of a larva, showing apical microvilli, tight junctions, chylomicron near the inter-enterocytes space, nucleus and mitochondria, with scale bar = 5 μm and magnification = ×1500; (B) a section showing large supra- and infranuclear lipid droplets, the rough endoplasmic reticulum and the basal mitochondria associated with lamellar structure, with scale bar = 2 μm and magnification = ×2500, where BL = basal lamina, C = chylomicrons, IES = inter-enterocytes space, LD = lipid droplets, LS = lamellar structure, M = mitochondria, MV = microvilli, N = nucleus, RER = rough endoplasmic reticulum and TJ = tight junctions.

Discussion

The development of the digestive tract of *E. fuscoguttatus* based on histological and ultrastructure features was similar to that of coral trout (Qu et al., 2012), and thus, in the present study, it was divided into four stages according to Qu et al. (2012): the endotrophic stage (Stage I), the endo-exotrophic stage (Stage II) and the exotrophic stage (subdivided into Stages III and IV).

Stage I started at the time of hatching and ended at 2 DAH, when the mouth opened. During this stage, the tiger grouper larvae depended entirely on the endogenous nutrition from the yolk sac and oil globule. Like most marine teleost larvae, the tiger grouper had an undifferentiated straight digestive tract on the dorsal part of the yolk sac at hatching showing a squamous epithelium of undifferentiated cells. Later on, a monostratified layer of columnar cells with basal nuclei and an eosinophilic border developed. The accessory glands (the liver and pancreas) also started to differentiate at this time. The ultrastructural features of the lipoprotein particles in the periblast may have been derived from the yolk sac and oil globule and hydrolyzed by acid phosphatase and nonspecific esterase, respectively, as has been reported in newly hatched *E. coioides* larvae (Quinitio et al., 2004b; Primavera-Tirol et al., 2014).

The tiger grouper had a relatively short endo-exotrophic stage from the mouth opening (2 DAH) to the complete absorption of the yolk sac (3 DAH). During this phase, the intestinal lumen expanded rapidly, brush borders of the intestinal epithelium were really distinguishable, while the liver and pancreas became differentiated, which coincided with a previous report in the same species (Aripin, 2010), and in other groupers, such as *E. coioides* (Quinitio et al., 2004a); *P. leopardus* (Qu et al., 2012); *Mycteroperca rosacea* (Martínez-Lagos et al., 2014). The histology of the digestive tract suggested early functioning of the liver and pancreas when yolk absorption was completed; so that the larvae are able to ingest, and

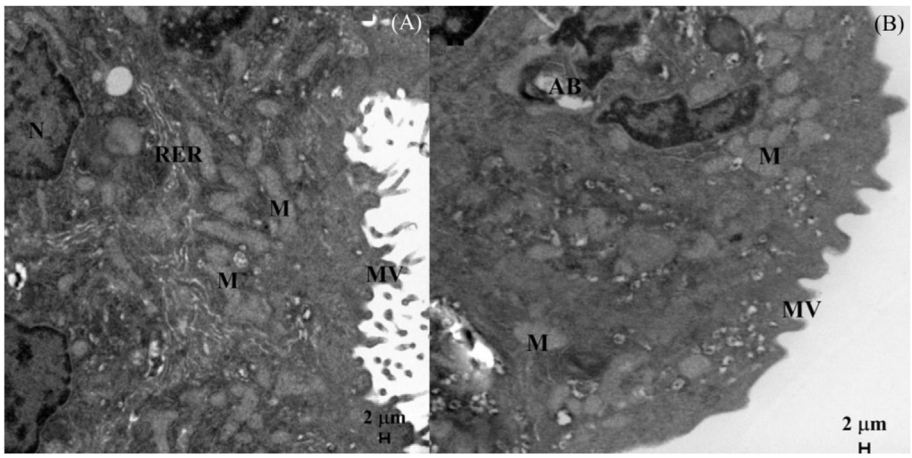

Fig. 8. Transmission electron micrographs of the intestine of the 24 d after hatching (DAH) *E. fuscoguttatus*: (A) intestinal epithelium of a 24 DAH larva, showing a presence of elongate mitochondria, rough endoplasmic reticulum and nucleus, with scale bar = 2 µm and magnification = ×4000; (B) section showing apoptotic bodies and microvilli, with scale bar = 2 µm and magnification = ×4000, where AB = apoptotic bodies, M = mitochondria, MV = microvilli, N = nucleus and RER = rough endoplasmic reticulum.

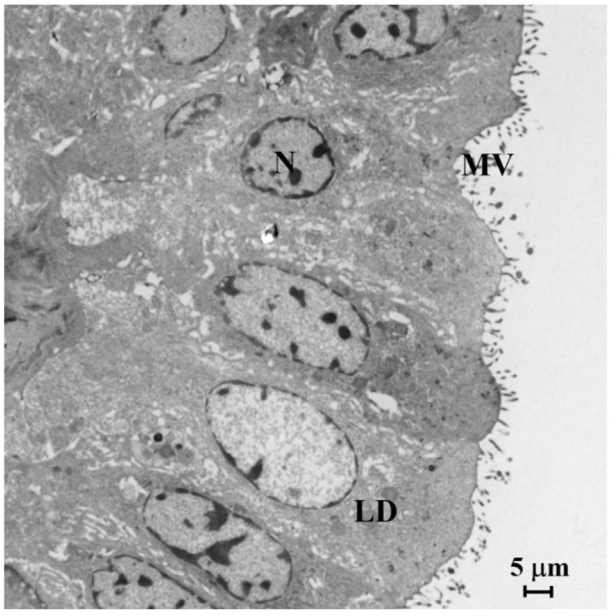

Fig. 9. Transmission electron micrograph of the intestine of the 36 d after hatching *E. fuscoguttatus*, for intestinal epithelium of a larva, showing enterocytes with infranuclear lipid droplets and nucleus, with magnification = ×1200, where LD = lipid droplets, MV = microvilli and N = nucleus.

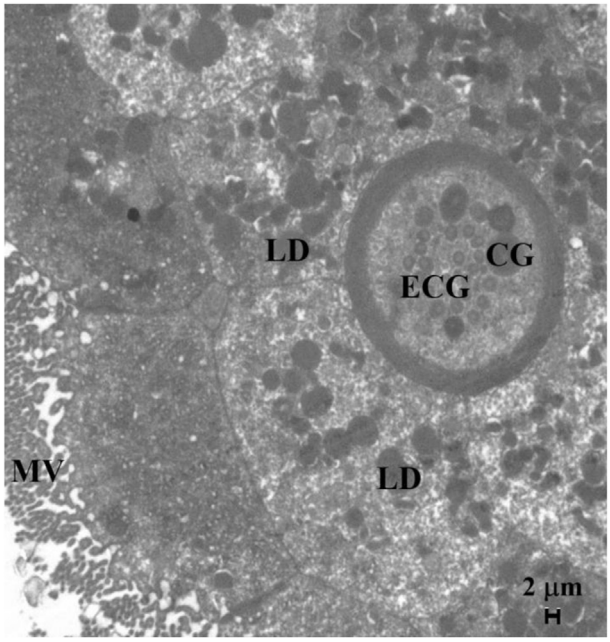

Fig. 10. Transmission electron micrograph of the intestine of the 42 d after hatching *E. fuscoguttatus*, a section showing an eosinophilic granule cell with dark-stained cytoplasmic granules and surrounding lipid droplets, with magnification = ×2000, where CG = cytoplasmic granules, EGC = eosinophilic granule cell, LD = lipid droplets and MV = microvilli.

assimilate the first exogenous food when their yolk reserves have been completely absorbed (Micale et al., 2006). These morphological changes are directed to increase the absorption and digestion of exogenous nutrients (Qu et al., 2012).

Noteworthy is that the time of mouth opening varies among grouper species being for example, at 2 DAH in *E. fuscoguttatus* (the present study; Aripin, 2010), *P. leopardus* (Qu et al., 2012), while it was 3 DAH in *E. coioides* (Quinitio et al., 2004a; Primavera-Tirol et al., 2014) and *M. rosacea* (Martínez-Lagos et al., 2014). These differences probably reflect variations in the factors affecting larval development, such as the egg size, incubation temperature, breeding conditions or genetic origin (Blaxter, 1988).

Stage III began from 3 DAH when the larvae started complete external feeding; two regions were anatomically differentiated in the intestine by the appearance of an internal valve, as previously reported in *E. fuscoguttatus* (Aripin, 2010; Qu et al., 2012), and in other species, such as sea bream *S. aurata* (Elbal et al., 2004) and white seabass *Atractoscion nobilis* (Galaviz et al., 2011). At 5 DAH, large acidophilic supranuclear vacuoles which were a consequence of intracellular digestion were observed in the posterior region of the intestine (Govoni et al., 1986). The supranuclear vacuoles of the tiger grouper disappeared when the gastric glands differentiated, similar to the coral trout (Qu et al., 2012) and gilthead sea bream (Elbal et al., 2004). The mechanism of digestion and absorption which changed from pinocytosis and intracellular digestion to extracellular digestion and membrane transport as indicated by the development of gastric glands, was a typical development pattern of fish larvae (Govoni et al., 1986). The presence of lipid droplets

and chylomicrons in the enterocytes demonstrated functional lipid metabolism in fed 3 DAH larvae (Primavera-Tirol et al., 2014) and thus, indicated that the larvae undergo the transition from endotrophy to exotrophy. This change may somehow correlate with vulnerability of the larvae at this stage which is known as a critical period for the survival of grouper larvae (Duray et al., 1997; Quinitio et al., 2004b; Aripin, 2010; Qu et al., 2012). In addition, the sign of lipid metabolism observed in the same period may reflect the importance of essential fatty acid on larvae survival.

The enterocyte at 6 DAH showed increased height with large lipid droplets. The accumulation of lipid droplets in the intestinal mucosa of the 6 DAH larvae may reflect normal growth and survival as has been documented for early *E. coioides* larvae (Qu et al., 2012). Generally, the early stage fish larvae have limited ability to biosynthesize phospholipids which are necessary for lipoprotein synthesis, lipid transport from the intestine and lipid utilization (Cahu et al., 2003, 2009; Tocher et al., 2008). Therefore, the lipid droplets found in the enterocytes may be a result of exogenous feeding. The goblet cells were first observed at 3 DAH which coincided with first feeding. This was similar to other fish larvae such as *S. aurata* (Elbal et al., 2004), *Paralichthys californicus* (Gisbert et al., 2004) and *E. coioides* (Primavera-Tirol et al., 2014) in which the goblet cells were observed in connection with the first external feeding. The number of the goblet cells increased as the larvae developed and became very abundant by 42 DAH.

The gastric glands were first observed at 24 DAH. This indicated improvement of extracellular protein digestion (Chen et al., 2006; Qu et al., 2012). Concurrently, the supranuclear inclusions in the absorptive cells of the posterior intestinal segment disappeared due to a transition from intracellular digestion to extracellular digestion (Govoni et al., 1986). Differentiation of the gastric glands was simultaneous with the appearance of pyloric caeca on 24 DAH, which indicated the beginning of Stage IV, the juvenile stage (Tanaka, 1971). It is assumed that before the full development of the digestive mechanism at the juvenile stage, more energy could have been used for growth. Pyloric caeca are considered to increase the surface area of the digestive tract to enable the absorption of more nutrients (Baglole et al., 1997). In the present study, the transmission electron microscopy micrographs showed that 24 DAH *E. fuscoguttatus* larvae showed a sign of suboptimal nutritional condition indicated by some apoptotic enterocytes. Generally, fish larvae increased assimilation and energy transformation efficiencies during metamorphosis (Elbal et al., 2004). This might relate to the presence of apoptotic enterocytes in this stage, as has been reported in 19 DAH *E. coioides* larvae (Primavera-Tirol et al., 2014). The irregular mitochondrial morphology found in the present study may relate to exogenous phospholipids obtained from feed (MacQueen Leifson et al., 2003).

Some studies have indicated that weaning should start around the day the gastric glands and pyloric caeca appear (Chen et al., 2006; Qu et al., 2012). For the tiger grouper, the gastric glands and pyloric caeca were observed at 24 DAH, as such it is suggested that the tiger grouper larvae should be weaned around 24 DAH. The lipid accumulations were observed in the hepatocytes of 36 DAH larvae. In the hepatocytes, numerous cytoplasmic organelles were found, such as mitochondria, rough endoplasmic reticula and free ribosomes, which indicated that the liver was very metabolically active (Qu et al., 2012). The development of the endoplasmic reticulum and Golgi complex at this stage suggested that lipoprotein synthesis was stronger than in the previous phases (Senger et al., 1993; Elbal et al., 2004).

The ultrastructural feature of the anterior intestine enterocytes at 42 DAH, showed large lipid droplets with a few mitochondria which related to the direct role of mitochondrial β-oxidation in the catabolism of fatty acids (Frøyland et al., 1997) in enterocytes of metamorphic grouper larvae (Primavera-Tirol et al., 2014). The number of mucus granules increased as the larvae developed and became very abundant by 42 DAH. The secretion of mucus was reported to facilitate food transportation and to protect the digestive mucosa against physiochemical damage and bacterial and viral attack (Hachero-Cruzado et al., 2009; Sanchez-Amaya et al., 2007). The thinning and irregular microvilli structures found in some enterocytes merit further study as these may lead to an induced pathogenic state where the epithelial barrier is weakened (Olsen et al., 1999, 2000). The EGCs observed in the intestinal epithelia of metamorphic grouper larvae in the present study signified increased immunity capability at this time (Mulero et al., 2007; Primavera-Tirol et al., 2014).

In conclusion, the ontogeny of the digestive tract of the tiger grouper in the present study appeared normal and followed a similar pattern as reported in other marine teleosts, consisting of: Stage I (0–2 DAH), the digestive tract has not differentiated; Stage II (2–3 DAH), the mouth has opened but with an undeveloped digestive tract; Stage III (3–24 DAH), the digestive tract has developed; and Stage IV (25–42 DAH), gastric glands are observed in the stomach and pyloric caeca are well-developed.

Regarding the critical period for larval survival (3–8 DAH) according to Duray et al. (1997), Quinitio et al. (2004b), Aripin (2010) and Qu et al. (2012), the present study showed substantial changes involving the change from endotrophy to exotrophy and the commencement of lipid metabolism. Thus, it is surmised that essential fatty acids are required at this stage as has been reported by Primavera-Tirol et al. (2014) that larval growth and survival improved when fed diets contained appropriate levels of phospholipid supplementation. In the present study the enriched *Artemia*- and copepod nauplii were supplied to the larvae as a source of fatty acid. However, whether they were readily digested and assimilated is still unknown.

Regarding the feeding regime used in the present study, the overall regime was appropriate except for the early stage when the larvae should be supplied with the appropriate source of essential fatty acids. Furthermore, the weaning stage should be done soon after 24 DAH (for example, 30 DAH) when the gastric glands developed instead of at 35 DAH as a delay in weaning would result in increased live-feed cost. However, in order to determine a precise feeding regime, the digestive enzyme activities during larval development of the tiger grouper have also been studied by the present authors our group and will be published elsewhere.

Conflict of interest

The authors declare that there are no conflicts of interest.

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