A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of cholecystokinin and mucus-secreting goblet cell in longtooth grouper, *Epinephelus bruneus*

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Chapter I

Ontogenetic development of the digestive system of the longtooth grouper

Abstract

The longtooth grouper, *Epinephelus bruneus* is a commercially and recreationally valuable species and a promising candidate for marine aquaculture in Korea. Studies related to the ontogenetic development of the digestive system of marine fish larvae are an initial step in the identification and implementation of new feed alternatives for marine fish larvae. The purpose of this study was to describe the ontogenetic development of the digestive system of longtooth grouper larvae, and to characterize its cholecystokinin (CCK) and goblet cells in the hope of better understanding their organization and functionality in order to improve the rearing efficiency of longtooth grouper larvae. The larvae were kept in 35-metric-ton concrete tanks for 55 days. They were fed rotifer (*Brachionus plicatilis*) from 0 days after hatching (DAH) to 30 DAH. *Artemia* nauplii were given from 15 DAH to 55 DAH, and an artificial diet was used from 20 DAH onward.

At the time of hatching, the digestive system consisted of an undifferentiated straight tube laying over the yolk sac and an unopened mouth and anus. At 2 DAH, the digestive tract became wider and the anus opened. The epithelium of the intestine consisted of a single layer of columnar cells. CCK mRNA was first expressed at and was continually expressed until the end of the experiment. The mouth was open at 4 DAH. The liver and pancreas were observed, and the intestinal valve was formed by columnar epithelium in the posterior intestine. Mucosal folds were formed in the rectum, and goblet cells appeared in the buccopharyngeal cavity. At 5 DAH, the digestive tract started coiling and the yolk sac was almost depleted. At 10 DAH, the pancreas was completely diffused around the liver and intestine as observed in

juveniles. At 20 DAH, goblet cells appeared in the anterior intestine. At 25 DAH, the stomach was morphologically differentiated and separated from the anterior intestine by the pyloric sphincter. At 30 DAH, the stomach was still changing in shape and increasing in size, and a number of gastric glands were observed. The pyloric ceca appeared as invaginations of the anterior intestine surrounding the stomach. At 35 DAH, canine-like teeth and taste buds were observed to protrude from both the oral valve and the posterior region of the buccopharyngeal cavity. The stomach size and the number of gastric glands increased continually.

Longtooth grouper larvae develop a well-differentiated digestive tract with a functional stomach at about 30 DAH. The weaning of longtooth grouper larvae to formulated feeds was started at 30 DAH at 25 ℃ once the functional stomach, pyloric sphincter, and pyloric ceca had formed.

1. Introduction

Studies related to the ontogenetic development of the digestive system of marine fish larvae are an initial step in the identification and implementation of new feed alternatives for them. The larval stage constitutes a critical period during the fish life cycle in which ontogeny causes important structural and functional changes in the body tissues, organs, and system (Sánchez-Amaya et al., 2007). Successful development of the digestive system is crucial for the survival and growth of fish larvae because an efficient digestive system enables them to capture, ingest, digest, and absorb foods. For these reasons, knowledge about the structural development of the digestive system is essential to understand the digestive physiology and determine the appropriate time to wean fish larvae.

Ontogenetic development of the digestive system has been studied in several commercially important species such as European turbot (Cousin and Baudin-Laurecin, 1985; Cousin et al., 1987; Segner et al., 1994), European seabass (Beccaria et al., 1991; Delpano et al., 1991), Atlantic cod (Kjørsvik et al., 1991; Morrison, 1993), gilthead seabream (Guyot et al., 1995; Sarasquete et al., 1995; Calzada et al., 1998), Japanese eel (Kurokawa et al., 2004), and redbanded seabream (Sánchez-Amaya et al., 2007). In the rearing of fish larvae, knowledge of digestive physiology, including the role of hormones and digestive activation in the early life stage, is essential. However, there is no available information about the development of the digestive system of the longtooth grouper during its early life stage.

The longtooth grouper is a commercially valuable Serranidae species and a promising candidate for marine aquaculture in Korea (Lee et al., 2008). The purposes

of this study were to describe in detail the ontogenetic development of the digestive system of the longtooth grouper right from hatching to metamorphosis, and to characterize its cholecystokinin (CCK) and goblet cells in the hope of better understanding their organization and functionality in order to improve the rearing efficiency of longtooth grouper larvae.

2. Materials and Methods

2.1. Larvae and rearing condition

Fertilized eggs of the longtooth grouper were kept in 35-metric-ton concrete tanks supplied with underground seawater (temperature, 25 ± 0.5 °C and salinity, 28 ± 0.5 ppt). Newly hatched larvae were fed enriched rotifer (*Brachionus plicatilis*) from 0 days after hatching (DAH) to 30 DAH (Fig. 1). Microalgae were added to the rearing tanks as food for the rotifers. Enriched *Artemia* nauplii were introduced to the rearing tanks from 15 to 55 DAH (Fig. 1). Larvae were fed commercial pellet feeds from 25 DAH to the end of the experiment (55 DAH) (Fig. 1).

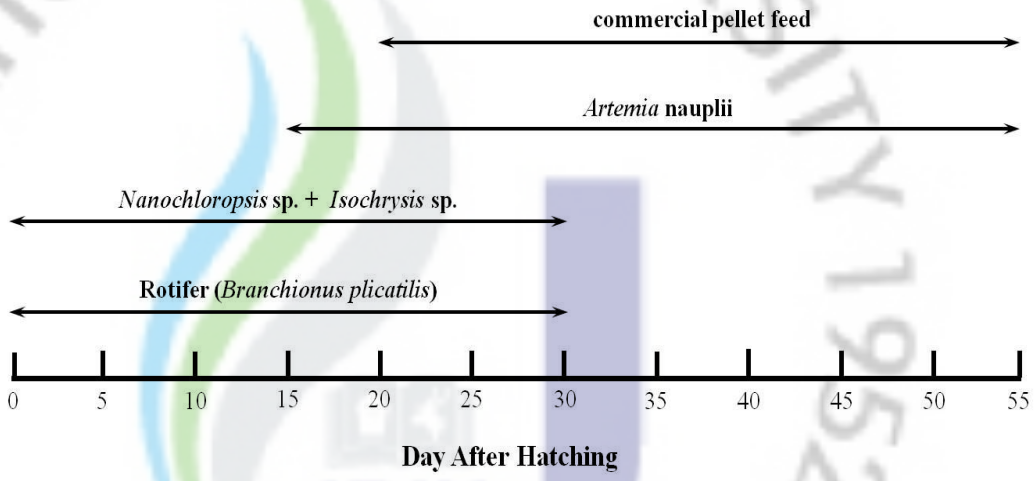

Fig. 1. Feeding scheme during larval rearing of the longtooth grouper.

2.2. Fish sampling and growth measurement

Fifteen larvae were randomly sampled from the rearing tanks each day from hatching to 15 DAH, and were then sampled at 20, 25, 30, 35, 40, 45, 50, and 55 DAH to examine development of the digestive system. Larvae were transferred into a sampling tube filled with rearing tank water and kept at 4 ℃ for growth measurement and histological observation. Total length (TL) was measured to the nearest 0.1 mm using a light microscope (HBO 50; Carl Zeiss) and Image scope 2.3 (Image Line, Inc.) software.

2.3. Histological observation

On each sampling day, the sampled larvae were anesthetized with 2-phenoxyethanol, fixed in Bouin's solution, dehydrated in a graded series of ethanol, embedded in paraffin, and then sectioned in 5-μm sagittal serial sections using a Leica RM 2135 rotary microtome. From 0 to 10 DAH, the days when the larvae were transparent, they were directly observed under the light microscope (HBO 50; Carl Zeiss).

Slides were stained with Harris hematoxylin and 0.5% eosin for general histological observation, and with Alcian blue (AB) at pH 2.5 and periodic acid-Schiff (PAS) for observation of the mucus-secreting goblet cells. The slides with sections were permanently mounted using Canada balsam (Junsei, Japan). The slides were observed using a light microscope (HBO 50; Carl Zeiss) with Image scope 2.3

(Image Line, Inc.) software. Photographs were taken using a Canon digital photomicrographic system.

2.4. Sampling for the ontogenetic expression of CCK

The ontogeny of CCK in longtooth grouper larvae was determined using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Ten longtooth grouper larvae were regularly sampled during daytime hours at 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 13, 15, 17, 20, 22, 26, 29, 32, 35, 38, 41, and 48 DAH. Total RNA was isolated from the whole larvae using RNAiso Plus (Takara, Kyoto, Japan) and chloroform extraction with TRI Reagent following the manufacturer's protocol. Total RNA concentrations were measured by optical density reading at 260 nm using NanoVue (Ver.1.0.1; GE Healthcare, UK). The total RNA sample quality was assessed by measuring the ratio of sample absorbance at 260 and 280 nm. Only RNA samples with ratios of 1.7–2.1 were used. The isolated total RNA (2 μg) was incubated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) at 37 °C for 60 min to prevent DNA contamination and was stored at -80 °C until use. First-strand cDNA synthesis was performed using 0.5 μg of total RNA using a PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan). Reverse transcription was performed at 37 °C for 15 min and the samples were incubated at 85 °C for 15 s for inactivation of reverse transcriptase. Double-stranded rapid amplification of cDNA ends (RACE)-ready cDNA were made using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Purified DNA was diluted by the addition of 40 μL of nuclease-free water

and stored at -20 °C until further analysis. Primers for real-time quantitative RT-PCR of CCK were designed in the obtained CCK nucleotide sequence. For standardization of the analysis, an 18S rRNA gene was used. The PCR mixture (total volume, 20 μL) contained 7 μL of cDNA, 0.5 μL of each forward and reverse primer, 5 μL of nuclease-free water, and 7 μL of SYBR premix Ex™ Taq II (Takara, Kyoto, Japan). Reactions were conducted in 96-well plates and samples were run in triplicate. Amplification and detection of all samples were performed using a CFX96™ Real-Time System (Bio-Rad, CA, USA) with the following thermal cycling conditions: 95 °C for 30 s (1 cycle), 95 °C for 5 s, and 60 °C for 30 s (40 cycles). After each PCR reaction, 1 cycle of melting curve from 65 to 95 °C at a transition rate of 0.5 °C/s with continuous fluorescence detection was performed.

3. Results

3.1. Growth

The average TL of longtooth grouper larvae was 2.06 ± 0.03 mm at hatching (0 DAH), and increased to 34.84 ± 0.78 mm at 55 DAH (Fig. 2).

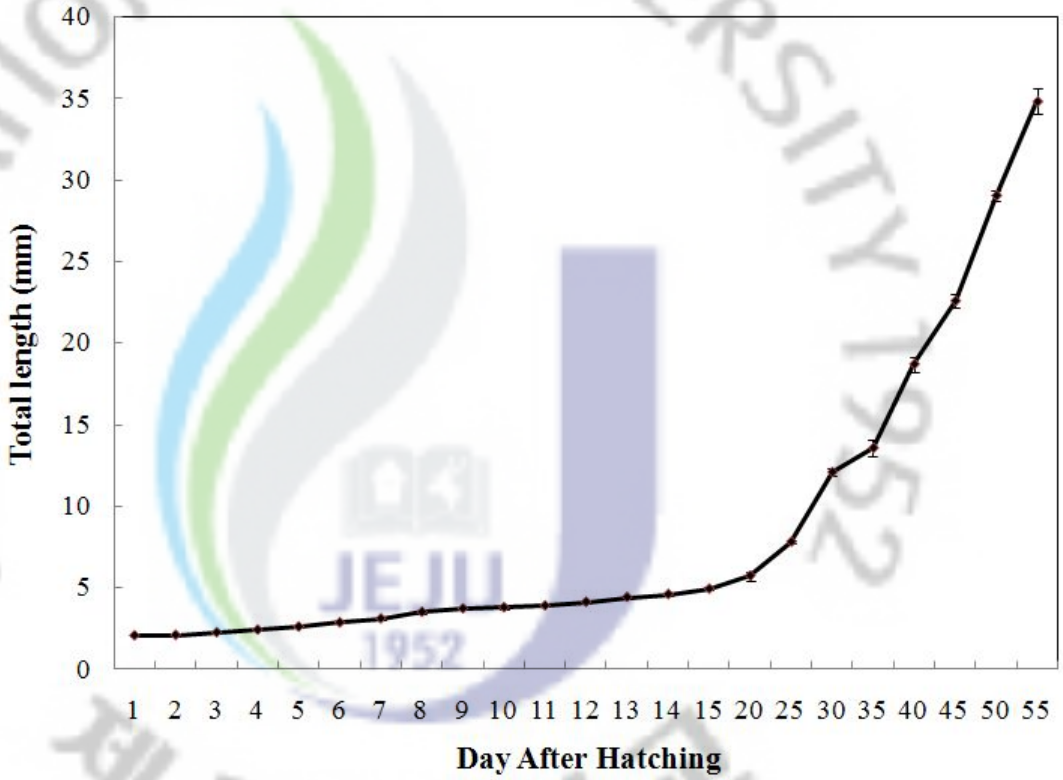

Fig. 2. Growth in total length (㎜) of longtooth grouper larvae from hatching to 55 DAH. Vertical bar denotes standard error of mean. Each point corresponds to the mean of 10 larvae.

3.2. General development of the digestive system

At hatching (0 DAH, TL = 2.06 ± 0.03 mm, n = 10), the digestive tract of each longtooth grouper larvae consisted of a straight tube lying dorsal to the yolk sac and bent ventrally to the posterior intestine portion (approximately 90°) (Fig. 3A and B). The lumen of the digestive tract was observed in the posterior intestine portion (Fig. 3B). The mouth and anus were closed. There was no sign of an incipient liver or pancreas.

At 2 DAH (TL = 2.10 ± 0.04 mm, n = 10), the anus opened, yolk sac volume decreased, digestive tract elongated, and gut lumen increased in size (Fig. 3C).

At 4 DAH (TL = 2.06 ± 0.03 mm, n = 10), the intestinal valve was formed and the mouth opened (Fig. 3D). The yolk sac and oil globule were reduced in size (Fig. 3D). The liver and pancreas, and mucosal folds in the rectum were visible (Fig. 4A). At this time, the bile ducts appeared connected to the hepatopancreas, and numerous lipid vacuoles appeared in the liver (Fig. 4A). Goblet cells appeared in the buccopharyngeal cavity and the rectum (Fig 4B and C).

At 5 DAH (TL = 2.60 ± 0.06 mm, n = 10), the intestinal curve formed in the anterior intestine portion and the size of the digestive tract substantially increased (Fig. 3E). Most of the oil globule in the yolk sac was depleted (Fig. 3E).

At 6 DAH (TL = 2.86 ± 0.03 mm, n = 10), the intestine was divided into a mid-intestinal portion and a posterior intestinal portion (Fig. 4D). Goblet cells appeared in the posterior intestine portion (Fig. 4D). The pancreas was located in the intestinal loop and diffused throughout the abdominal cavity (Fig. 4D). The liver and pancreas

substantially increased in size without any visible morphological and histological changes compared with the ontogeny development.

At 7 DAH (TL = 3.10 ± 0.08 mm, n = 10), absorption of the yolk sac and oil globule was complete (Fig. 3F). Food particles were easily identified in the intestine, and evacuation was observable (Fig. 3F).

At 10 DAH (TL = 3.79 ± 0.12 mm, n = 10), goblet cells appeared at the border of the esophagus and in the anterior intestine portion (Fig. 5A). The anterior intestine portion was lined with a single layer of columnar epithelial cells and several mucosal folds were observed (Fig. 5A). Goblet cells appeared in the esophagus at 15 DAH (TL = 4.93 ± 0.10, n = 10).

At 20 DAH (TL = 5.73 ± 0.29 mm, n = 10), goblet cells were observed in the epithelium of the mucosal folds in the anterior intestine portion (Fig. 5C). Mucosal folds in the anterior intestine portion increased in size and number (Fig. 5C).

At 25 DAH (TL = 7.82 ± 0.11 mm, n = 10), a presumptive stomach appeared at the end of the esophagus but the gastric glands were not differentiated (Fig. 6A). At this time, goblet cells were observed in the epithelium of the mucosal folds of the mid intestine portion (Fig. 5D).

At 30 DAH (TL = 12.09 ± 0.22 mm, n = 10), the number of gastric glands and pyloric ceca were observable (Fig. 6B). The gastric glands consisted of single layers of cubic epithelium and were located in the intermediate and posterior region of the stomach (Fig. 6B). The muscular layer of the posterior stomach was increased in size and formed the pyloric sphincter, which separated the stomach from the anterior intestine (Fig 6C). The mucosal folds of the stomach were formed by simple cubic epithelium (Fig. 6C).

At 35 DAH (TL = 13.58 ± 0.52 mm, n = 10), the canine-like pharyngeal tooth and taste buds occurred in the buccopharyngeal cavity (Fig. 6D). The stomach size and the numbers of gastric glands increased continually, but no histological or histochemical changes were observed during 35–55 DAH.

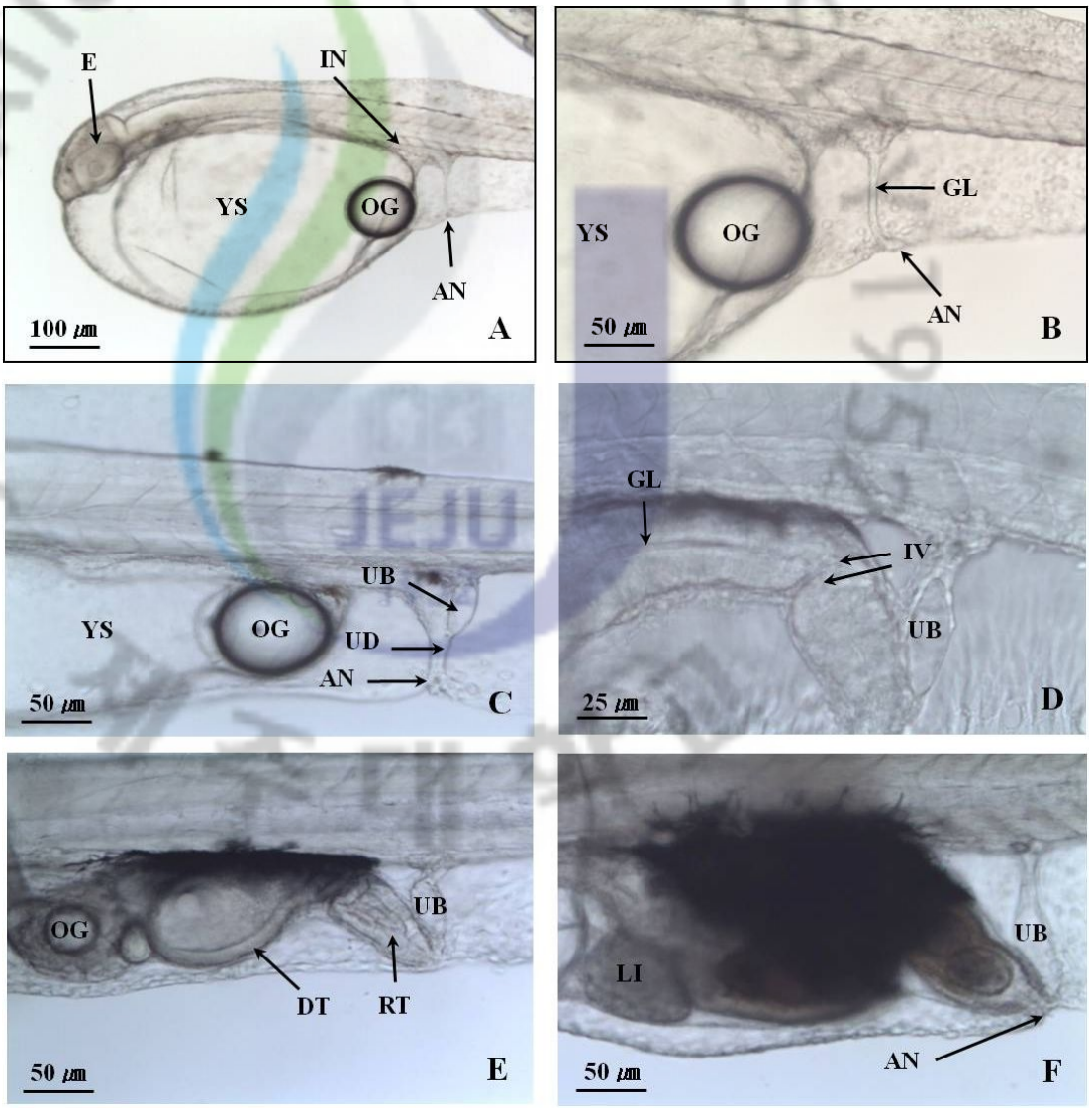

Fig. 3. Morphological observations of the digestive system during the development of longtooth grouper larvae.

(A) General view of the newly hatched larvae on 0 DAH. (B) Posterior region of the incipient intestine of newly hatched larvae on 0 DAH. (C) Posterior region of the incipient intestine of newly hatched larvae on 2 DAH. (D) Incipient intestine on 4 DAH. (E) Incipient intestine on 5 DAH. (F) Incipient intestine on 7 DAH. Abbreviations: AN, anus; DT, digestive tract; E, eye; GL, gut lumen; IN, intestine;

IV, intestinal valve; LI, liver; OG, oil globule; RT, rectum; UB, urine bladder; UD, urine duct; YS, yolk sac.

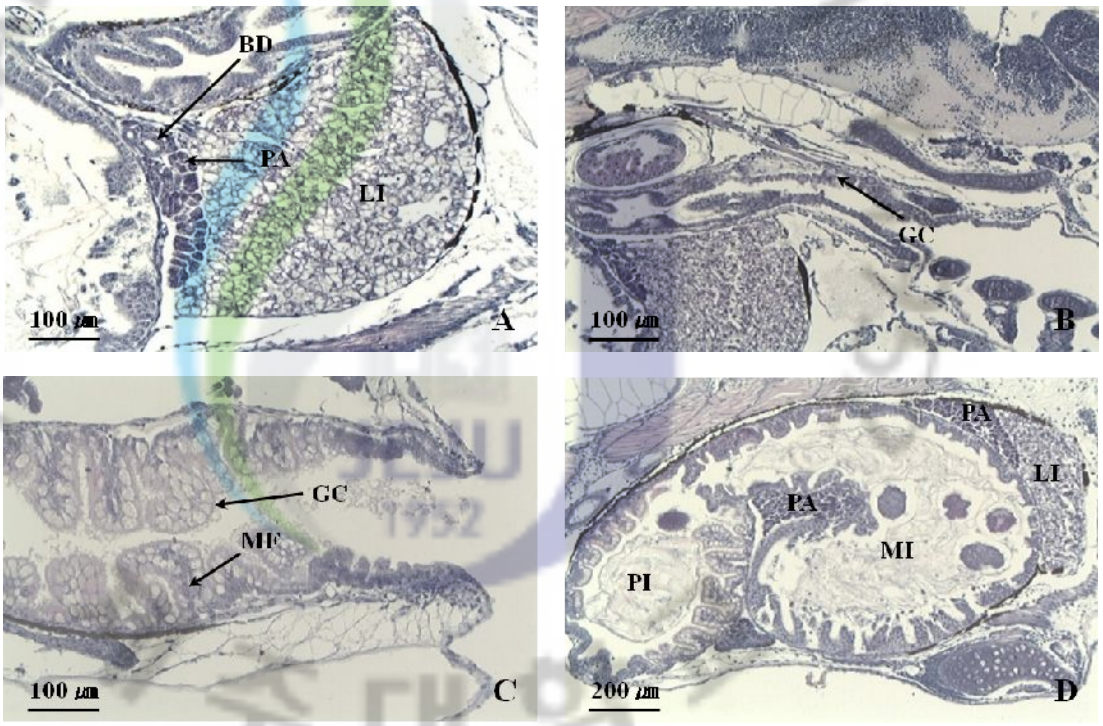

Fig. 4. Sagittal section of the digestive system during the development of longtooth grouper larvae. (A) Larvae of 4 DAH, showing the liver and pancreas; HE. (B) Larvae of 4 DAH, showing the goblet cells in the buccopharyngeal cavity; HE. (C) Larvae of 4 DAH, showing the goblet cells in the mucosal folds of the rectum; HE (D) Larvae of 6 DAH, showing the goblet cells in the posterior intestine portion; HE. Abbreviations: BD, bile duct; GC, goblet cell; HE, hematoxylin-eosin; LI, liver; MI, mid intestine portion; MF, mucosal fold; PA, pancreas; PI, posterior intestine portion.

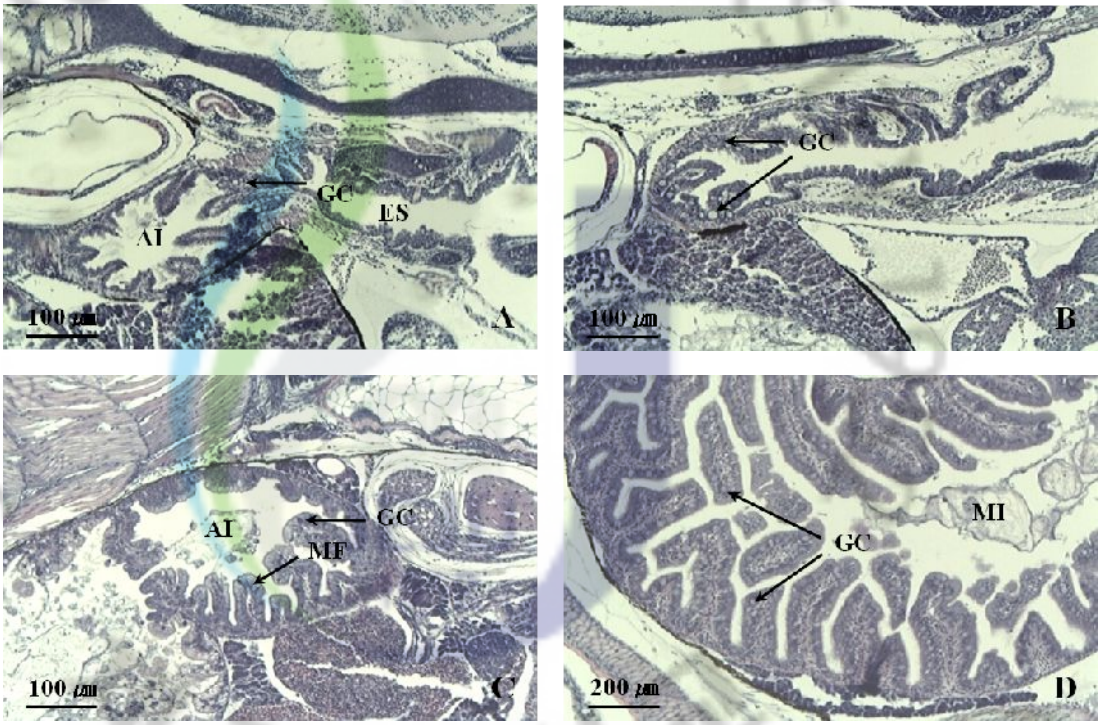

Fig. 5. Sagittal section of the digestive system during the development of longtooth grouper larvae. (A) Larvae of 10 DAH, showing the goblet cells in the border of the esophagus and the anterior intestine portion; HE (B) Larvae of 15 DAH, showing the goblet cells in the esophagus; HE. (C) Larvae of 20 DAH, showing the goblet cells in the mucosal folds of the anterior intestine portion; AB-PAS (D) Larvae of 25 DAH, showing the goblet cells in the mucosal fold of the mid intestine portion; HE. Abbreviations: AB-PAS, Alcian blue-periodic acid schiff; AI, anterior intestine portion; ES, esophagus; GC, goblet cell; HE, hematoxylin-eosin; MI, mid intestine portion; MF, mucosal fold.

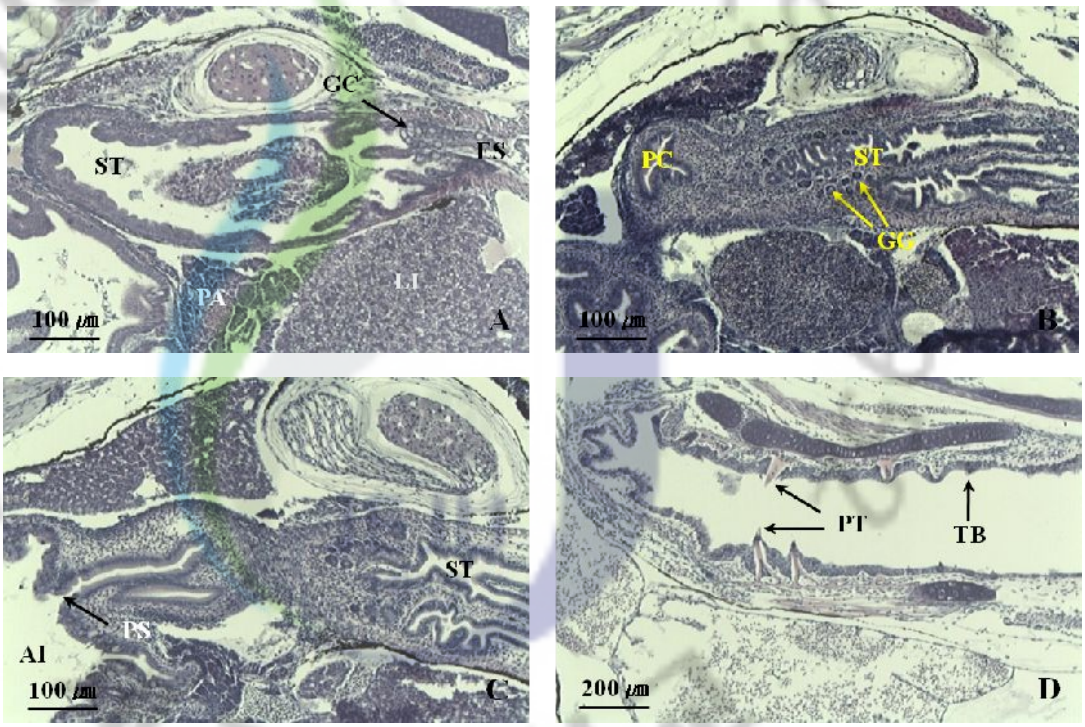

Fig. 6. Sagittal section of the digestive system during the development of longtooth grouper larvae. (A) Larvae of 25 DAH, showing the presumptive stomach; HE (B) Larvae of 30 DAH, showing the gastric glands in the stomach and pyloric ceca; AB-PAS. (C) Larvae of 30 DAH, showing the pyloric sphincter; AB-PAS (D) Larvae of 35 DAH, showing the pharyngeal teeth and taste buds in the buccopharyngeal cavity; HE. Abbreviations: AB-PAS, Alcian blue-periodic acid schiff; AI, anterior intestine portion; ES, esophagus; GC, goblet cell; GG, gastric gland; HE, hematoxylin-eosin; LI, liver; PA, pancreas; PC, pyloric ceca; PT, pharyngeal teeth; TB, taste bud.

3.3. Ontogenetic expression of CCK

CCK mRNA of longtooth grouper larvae were not expressed until 1 DAH. CCK mRNA was first expressed at 2 DAH and was continually expressed until the end of the experiment (Fig. 7).

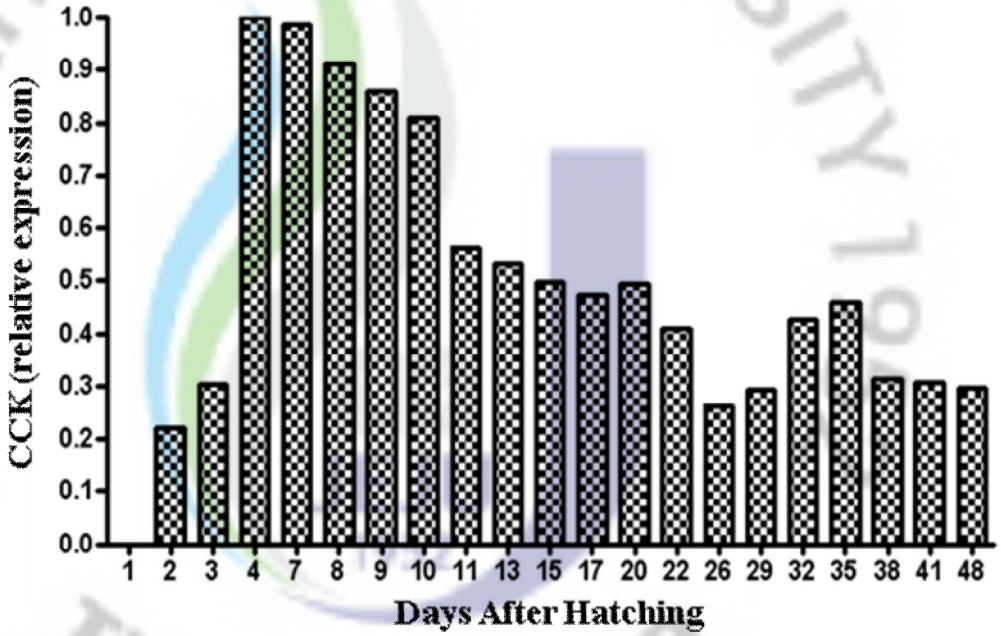

Fig. 7. CCK mRNA levels measured with quantitative real-time RT-PCR assay in the longtooth grouper from hatching to 48 DAH.

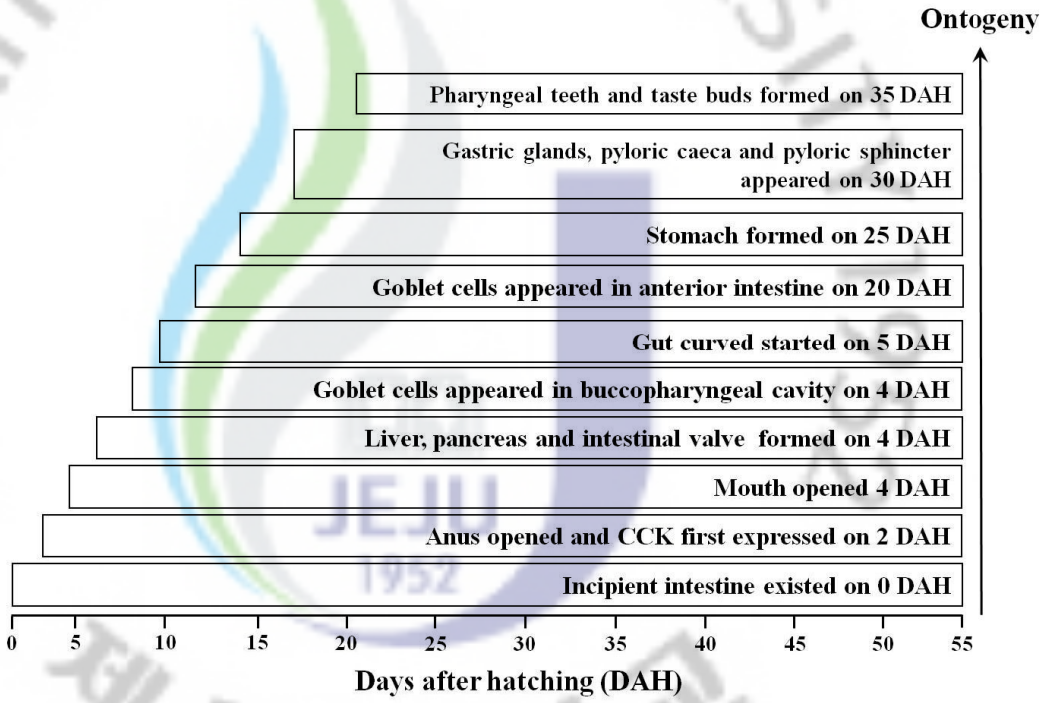

Fig. 8. Summary of the main developmental events of the digestive system in the longtooth grouper from hatching to 55 DAH (see text for details).

4. Discussion

Ontogenetic development of the digestive system in most fish is generally divided into 3 major phases (Buddington, 1985; Chen et al., 2006). The first phase starts at hatching and ends at the completion of endogenous nutrition absorption. In this stage, newly hatched larvae depend on the energy stored in the yolk sac and oil globule (Chen et al., 2006). The second phase starts at the onset of exogenous feeding and lasts until formation of the gastric glands in the stomach. In this stage, larvae depend on pinocytosis and intracellular digestion and absorption (Watanabe, 1982, 1984; Buddington, 1985). The last phase starts when the functional stomach (differentiation of gastric glands in the stomach) develops. Differentiation of the gastric glands indicates functional maturation of the digestive system (Tanaka, 1971; Gisbert et al., 2004).

Like other marine teleosts, the ontogenetic development of the digestive system of the longtooth grouper was divided into 3 major phases based on its morphological and histological features. The first phase extends from hatching to 7 DAH. In this phase, the mouth and anus were opened and the digestive system was ready to accept live foods. At hatching (0 DAH), the digestive tract of longtooth grouper larvae was an undifferentiated straight tube lying dorsal to the yolk sac, similar to the sevenband grouper (Song, 2004), California halibut (Gisbert et al., 2004), gilthead seabream (Elbal et al., 2004), and red porgy (Roo et al., 1999). At this stage (phase I), many species of larvae start exogenous feeding before the yolk is depleted even though the digestive system is only a short and simple tube (Gawlicka et al., 2000). Newly hatched larvae, with their incomplete digestive systems, must be able to ingest and

digest exogenous foods once they have exhausted their yolk reserves. Most larvae usually possess the morphological features needed for feeding by the end of the yolk sac stage (phase I). The presence of mouth opening is a prerequisite for feeding activities such as finding and capturing live foods (Kjørsvik et al., 1991; Kjørsvik and Reiersen, 1992). The mouths of longtooth grouper larvae were opened at 4 DAH, similar to sevenband grouper (Song, 2004), gilthead seabream (Elbal et al., 2004), common dentex (Santamaría et al., 2004), turbot (Segner et al., 1994), and European seabass (Deplano et al., 1991).

The mucus secreted by goblet cells in the buccopharyngeal cavity and esophagus protect the digestive tract against chemicals, parasites, hypertonic media, and stomach acidity, and act as a lubricant for ingested food particles (Smith, 1989, Scocco et al., 1996; Shephard, 1994; Pabst, 1987; Simmoneaux et al., 1987; Hirji and Courtney, 1983). In the longtooth grouper larvae, goblet cells first appeared at 4 DAH in the buccopharyngeal cavity and the esophagus when the larvae could first ingest rotifers. Similar results were observed in the redbanded seabream (Sánchez-Amaya et al., 2007), California halibut (Gisbert et al., 2004), common dentex (Santamaria et al., 2004), white seabream (Ortiz-Delgado et al., 2003), Senegal sole (Ribeiro et al., 1999), yellowtail flounder (Baglole et al., 1997), gilthead seabream (Sarasquete et al., 1995), turbot (Segner et al., 1994), and common sole (Bouhlic and Gabaudan, 1992). At 4 DAH, coinciding with the first exogenous feeding of live food (rotifers), the liver and pancreas appeared and lipid storage in the liver was observable. At this phase, longtooth grouper larvae start pinocytotic absorption and intracellular protein digestion. Pinocytosis and intracellular digestion are the main mechanisms for lipid and protein absorption in the larvae in the absence of a

functional stomach (Govoni et al., 1986). The intestinal valve divided the intestine into the mid intestine and rectum by 4 DAH. Epithelial cells were observed in the mucosal folds of the rectum.

In other fish larvae with immature digestive systems, the presence of epithelial cells is indicative of pinocytosis and intracellular digestion (Govoni et al., 1986; Watanabe, 1982). Protein ingestion and intracellular digestion by epithelial cells has been studied in the Atlantic halibut (Kjørsvik and Reiersen, 1992; Luizi et al., 1999), Japanese eel (Kurokawa et al., 1996), Atlantic cod (Kjørsvik et al., 1991), and delta smelt (Watanabe, 1984). This mechanism may enable the larva to compensate for its immature extracellular protease activity (Govoni et al., 1986), and it may play an important role in nutrition (Watanabe, 1984). The secretion of mucus in vertebrates including fish plays important roles in the absorption of easily digestible substances (Osman and Caceci, 1991) and in pregastric digestion (Baglole et al., 1997). At 6 DAH, the digestive tract of longtooth grouper larvae was divided into the mid-intestine and the posterior intestine. There are many well-developed mucosal folds and goblet cells in the posterior intestine. In addition, first evacuation was observed at 7 DAH. At this phase, the digestive and absorptive actions of longtooth grouper larvae mainly occur in the posterior intestine and the rectum. In the present study, complete absorption of endogenous nutrition (yolk sac) occurred at 7 DAH, indicating the existence of combined endogenous and exogenous feeding.

Phase II in the development of the digestive system of the longtooth grouper extends from 8 DAH to 29 DAH. This phase is critical because the larvae need to develop a digestive system to adapt to exogenous feeding. During this phase, the digestive system of the longtooth grouper larvae further develops in structure and

function for successful ingestion, digestion, and assimilation of exogenous foods. During this phase, the digestive tract elongated substantially and the intestine lumen increased in size. We observed that at 10 DAH, the anterior intestine was lined with a single layer of columnar epithelial cells. Several mucosal folds were observed in the anterior intestine. The digestive and absorptive processes continue to develop with the appearance of mucosal folds and mucus in the intestine (Sánchez-Amaya et al., 2007; Ortiz-Delgado et al., 2003). A few goblet cells were observed in the anterior intestine at 10 DAH, and they gradually increased in number at 15 DAH.

As development continued, goblet cells in the mucosal folds of the anterior intestine increased in number and developed further, indicating that the digestive and absorptive processes are more pronounced in the anterior intestine in phase II. The stomach was not present at the onset of the first feeding. In fact, the stomach was not differentiated after the artificial diet feeding period (20 DAH). The lack of a stomach at the onset of exogenous feeding implies that there are functional limitations in processing the ingested foods. Storage capacity and gastric digestion are still absent; therefore, food mincing and protein denaturation are low (Rojas-García, 2002). Fish larvae without a functional stomach thus rely on an immature digestive mechanism. In most teleost larvae, a completely differentiated functional stomach appears several weeks after exogenous feeding starts (Ribeiro et al., 1999; Segner et al., 1994; Stroband and Kroon, 1981). These features indicate that most of the digestive processes of longtooth grouper larvae take place in the whole intestine until the gastric glands are fully developed in the stomach.

Phase III of ontogenetic development of the digestive system starts at 30 DAH. The development of gastric glands in the stomach is a crucial event for extracellular

digestion, as they secrete HCl and digestive enzymes, primarily pepsin. The change from intracellular to extracellular digestion is a prerequisite for complex digestive processes, such as protein digestion, in order to be able to assimilate more food to meet the increasing energy demands of the growing larvae (Zaiss et al., 2006). The appearance of gastric glands in the stomach marks the formation of a functional stomach (Stroband and Kroon, 1981), and it is also a histological criterion to differentiate larvae from juveniles (Sarasquete et al., 1995; Tanaka, 1971).

Gastric glands increase digestion efficiency, but the duration of differentiation of gastric glands varies greatly among studied fish species (Chen et al., 2006). The period from hatching to phase III spans 36 days in the yellowtail flounder (Baglole et al., 1997), 33 days in the haddock (Hamlin et al., 2000), 30 days in the redbanded seabream (Sánchez-Amaya et al., 2007), 27 days in the shi drum (Parillo et al., 2004), 27–30 days in the California halibut (Gisbert et al., 2004), and 60 days in the gilthead seabream (Elbal et al., 2004), all of which are considered slow-growing species. In contrast, gastric glands appeared at 15 DAH in the yellowtail kingfish (Chen et al., 2006), 16 DAH in the spotted sand bass (Peña et al., 2003), and 11 DAH in the Pacific bluefin tuna (Kaji et al., 1996), all of which are considered fast-growing species. In the present study, gastric glands of longtooth grouper larvae appeared in the intermediate and posterior region of the stomach at 30 DAH. Compared to the fast-growing species, the time spans of phases II and III of the longtooth grouper were much longer, indicating its slow-growing nature.

Formation of the pyloric ceca in fish larvae indicates the last major change in the digestive system (Hamlin et al., 2000; Bisbal and Bengston, 1995). The pyloric ceca of some species are sac-shaped extensions found immediately adjacent to the pyloric

sphincter, but in other species, they can extend for several centimeters along the intestine. The pyloric ceca have the same histological features and functions as the anterior intestine, and are necessary for increasing the surface area for digestion (Baglole et al., 1997; Buddington and Diamond, 1987; Cataldi et al., 1987, 1988). It is generally accepted that the presence of the pyloric ceca mark the transition from the larval to the juvenile period (Hamlin et al., 2000; Sarasquete et al., 1995; Bisbal and Bengston, 1995; Govoni et al., 1986). The present study showed that the pyloric ceca appeared at the border between the stomach and the anterior intestine at 30 DAH. The appearance of the first pyloric ceca is simultaneous with the differentiation of the gastric glands. This result is similar to that in the gilthead seabream (Elbal et al., 2004), whereas in the European seabass and yellowtail kingfish, the pyloric ceca appeared prior to gastric gland differentiation (Chen et al., 2006; García Hernández et al., 2001).

At 30 DAH, the pyloric sphincter appeared simultaneously with differentiation of the gastric glands and pyloric ceca. The pyloric sphincter, when closed, physically separates the stomach from the intestine and prevents the passage of food items. The nervous system and hormones influence the contraction of the pyloric sphincter and thereby regulate the movement of food into the intestine (Buddington et al., 1997). This is critical to ensure that the hydrolytic and absorptive capacities of the intestine are not exceeded and that the foods are efficiently digested.

Most fish have teeth. The teeth can be associated throughout the mouth and the buccopharyngeal cavity, including the lips and tongue. The shape of the teeth is highly variable among fish and corresponds with the diversity in feeding habits. In the present study, longtooth grouper larvae developed canine-like pharyngeal teeth in

the buccopharyngeal cavity at 35 DAH. These canine-like pharyngeal teeth are well adapted to seizing and holding food items (Buddington et al., 1997).

CCK is one of the most important hormones of gastrointestinal function (Liddle, 1995; Bently, 1998; de Pedro and Björnsson, 2001). The physiological roles of CCK in digestion include stimulating pancreatic enzyme secretion, gallbladder contraction, and intestinal motility regulation (Liddle, 1995). CCK is secreted in response to the presence of digestive products (fats, proteins, and amino acids) in the intestine (Lewis and Williams, 1990; Herzig, 1998). In most teleosts, the pancreas develops prior to the differentiation of the stomach (Tanaka, 1969; Govoni et al., 1986; O'Connell, 1981). The pancreas is the sole organ responsible for the secretion digestive enzymes during the larval phase (Kurokawa et al., 2000). In the present study, CCK expression started at 2 DAH, 2 days prior to the first feeding. This indicates that CCK-producing cells are differentiated and functional in the intestine at 2 DAH.

The digestive system ontogeny of longtooth grouper larvae follows a general pattern similar to those in other species described to date. However, species-specific differences in the differentiation of goblet cells, gastric glands, and pyloric ceca were noticed. In longtooth grouper larvae, the time span of each developmental phase was much longer, indicating that it is a slow-growing species. Longtooth grouper larvae developed a well-differentiated digestive system with a functional stomach at about 30 DAH.

In conclusion, weaning of longtooth grouper larvae to formulated feeds was started on 30 DAH at 25 °C when the functional stomach, pyloric sphincter, and pyloric ceca were formed. Nevertheless, future work must focus on the ontogeny of

enzymatic secretions and digestive enzyme activity to provide precise informationabout the functionality of the digestive system of the longtooth grouper.

Chapter II

Effects of light spectrum on growth performance, goblet cell activation, and CCK expression in the longtooth grouper

Abstract

Light characteristics are very specific in the aquatic environment. Fish vision and different light spectra perception are related to each species' natural habit. Light is one of the main environmental conditions and can be easily manipulated in artificial rearing settings. Cholecystokinin (CCK) and mucus-secreting goblet cells are the main regulators of digestion. In this study, we established whether the light spectrum (natural condition, full spectrum: green, 520 nm; red, 590 nm, and blue, 480 nm) influences growth performance and digestive activity related to CCK mRNA expression and mucus-secreting goblet cell activity in order to develop a good management protocol and optimal rearing system for the longtooth grouper. For each light spectrum, fish were reared 12 weeks under a flow-through system and fed commercial pellet diets once daily. At the end of the experiment, the final body weights differed among the fish reared under different light spectra. The highest growth performance value was observed in fish reared under the green light condition. On the other hand, the growth performances of fish in the natural and blue light conditions were drastically decreased in last 3 weeks of the experiment. CCK mRNA expression and mucus-secreting goblet cell activity were significantly higher in the fish under green light condition than in the fish under the natural, red, and blue light conditions. Rearing of the longtooth grouper under the green light condition had positive effects on fish growth performance and digestion.

We recommend that the appropriate light spectrum for the artificial culture of the longtooth grouper is the green light condition from the perspective of growth performance and the synergistic effects of CCK and mucus-secreting goblet cells.

However, longer light treatment periods are needed in future investigations to clarify the effects of light spectrum on the longtooth grouper. Together with the findings of the present study, such studies would result in better understanding of the digestive physiology and contribute to the development of optimal rearing management for commercial production of the longtooth grouper.

1. Introduction

The digestive tract is basically a tube that courses through the body. However, the structural and functional characteristics of the digestive system vary widely among species (Suyehiro, 1941), and are established by genetic determinants such that they are tailored to a wide diversity of feeding habits and environmental conditions (Buddington and Krogdahl, 2004).

Light is one of the main environmental conditions that can be easily manipulated in artificial rearing settings. The fish visual system is adequately equipped to respond to different wavelengths (Neumeyer, 1992; Flamarique and Hawryshyn, 1996; Cheng and Flamarique, 2004). Depending on the fish species' natural habitat characteristics and specific visual abilities, the light spectrum affects multiple physiological aspects such as growth, neurohormonal system, reproduction, and behavior (Downing, 2002; Bayarri et al., 2002; Naor et al., 2003; Ruchin, 2001,2004; Karakatsouli et al, 2007; Villamizar et al., 2009). However, till date, the effects of different wavelengths of light on growth performance and the activation of digestion in the longtooth grouper have not been investigated. In addition, no studies have been conducted with the longtooth grouper to determine optimum light spectra conditions during artificial rearing. As mentioned above, light can be easily manipulated and controlled in indoor aqua farms. The provision of adequate light conditions for artificial rearing of fish species is important to optimize production success and ensure fish welfare.

The longtooth grouper is a commercially important marine species in which the activation of digestion involving cholecystokinin (CCK) and mucus-secreting goblet cells has never been examined. CCK and mucus-secreting goblet cells are the main regulators of the physiological activation of digestion. In this study, we establish

whether light spectrum influences growth performance and digestion using CCK mRNA expression and mucus-secreting goblet cell activity in order to develop a good management protocol and an optimal rearing system for the longtooth grouper.

2. Materials and Methods

2.1. Specimens and rearing condition

Thirty-six longtooth grouper specimens with an initial body weight of 349.2 ± 38.4 g and total length (TL) of 28.8 ± 0.2 cm were reared under green, red, blue, or natural light. Specimens were individually marked via insertion of a micro-tag (Trovan Electronic Identification System, USA) into the dorsal skin, and were acclimated to the experimental conditions for 2 weeks before the trial. Nine specimens were randomly distributed into each rearing tank (fiberglass reinforced plastic; length × height × width, 150 × 150 × 150 cm), and were reared 12 weeks under a flow-through system. To prevent the effects of background light on the experiment, the internal wall of each rearing tank was colored white, and each tank side was isolated by a black opaque plastic cloth. In the different spectrum trials, lamps containing light-emitting diodes (LEDs) were used (green, red, and blue LEDs). The light source (125 W; Savener, Korea) intensity and foot-candles (ft-cd) were measured using a digital illumination meter (DX-200, Taiwan). The light intensity of the green, red, and blue treatments was adjusted 150 lx and 15.2 ± 1 ft-cd and controlled by adjusting the distance from 15 to 75 cm between the water surface and the LED bulbs (Fig. 1). During the experimental period, the natural photoperiod was controlled by an electronic clock.

Fish were fed commercial pellet diets (moisture, 10.00%; crude protein, 52.00%; crude fat, 8.00%, crude fiber, 1.30%; ash, 11.90%; phosphorous, 1.70%; Le Gouessant, France) once daily at a feeding level of 7% body weight that was reduced

to 5% body weight for the last 3 weeks of the experiment. Water temperature and dissolved oxygen were measured once a day during the entire experimental period.

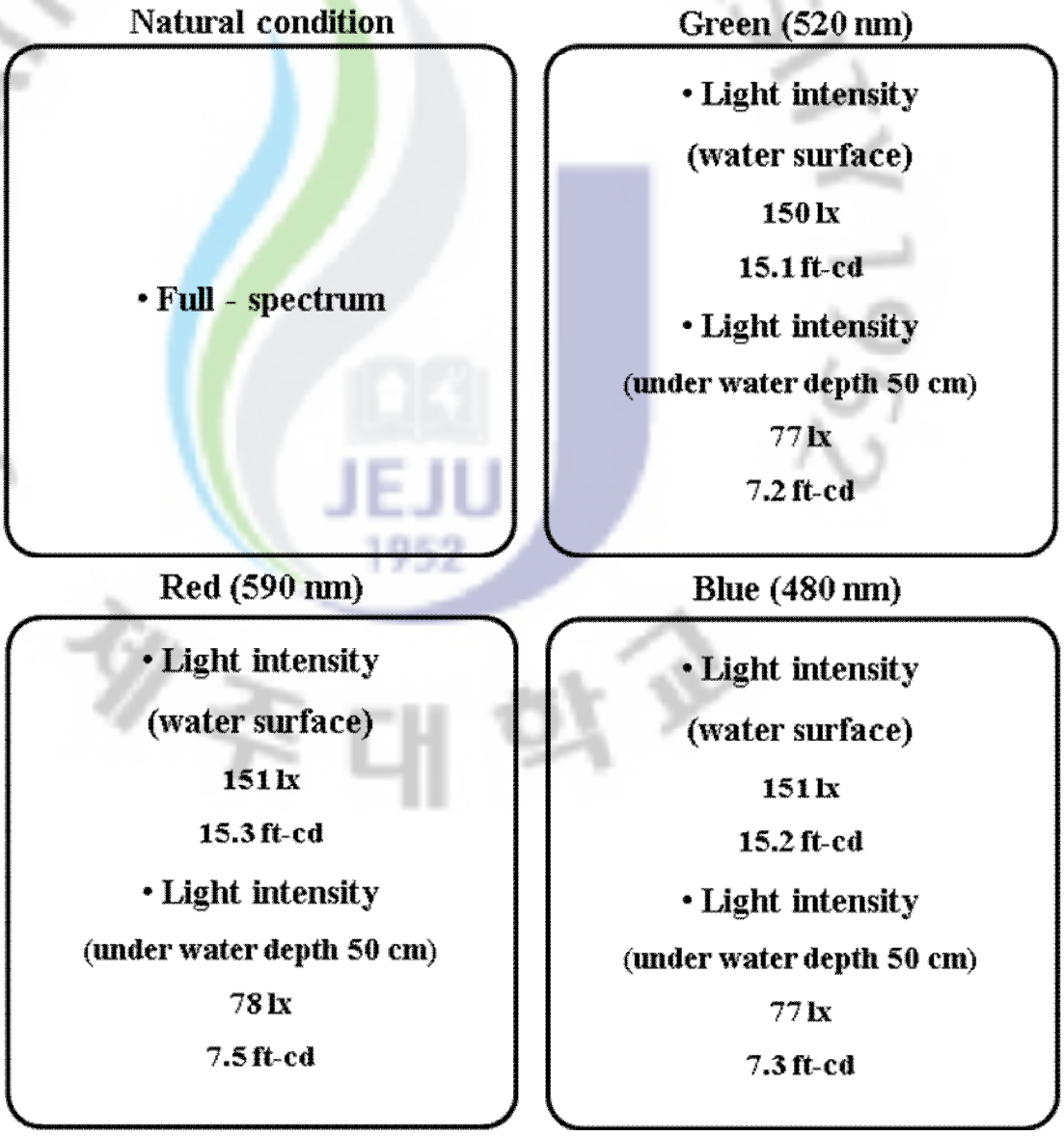

Fig. 1. The lighting layout in each experimental rearing tank to generate different light spectra condition.

2.2. Measurements and sampling

All fish populations were individually measured for body weight (BW) and TL every 3 weeks. At the end of the experiment, 6 fish from each experimental group were sampled for histochemical and gene expression analyses. For histochemical analysis, fish were anesthetized with 2-phenoxyethanol (Sigma, MO, USA), and the entire digestive tract from the esophagus to the anus was removed from the abdominal cavity. Sampled digestive tracts were fixed in Bouin's solution. For gene expression analysis, the whole brain and the digestive tract (anterior intestine portion and pyloric ceca) were rapidly removed from the abdominal cavity. Immediately after dissection, the whole brain and the digestive tract were immersed into RNAiso Plus (Takara, Kyoto, Japan) and stored at -80 ℃ until RNA isolation was performed.

2.3. Histochemical analysis

Sampled digestive tracts were divided into 6 parts (anterior intestine portion, mid intestine portion, posterior intestine portion, rectum, and pyloric ceca), re-fixed in Bouin's solution, dehydrated in a graded series of ethanol, embedded in paraffin, and then cut into 5-μm longitudinal and cross-sections. Slides were stained with Gill's hematoxylin and 0.5% eosin for histological observation, and with Alcian blue (AB) at pH 2.5 and periodic acid-Schiff (PAS) for observation of mucus-secreting goblet cells. The AB-PAS staining method proposed by Pearse (1985) was used.

Microscopy of the mucus-secreting goblet cells was carried out using a light microscope (HBO 50, Carl Zeiss) with Image scope 2.3 (Image Line, Inc.) software.

The characteristics and the number of mucus-secreting goblet cells from different regions of the digestive tract were noted.

2.4. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) for CCK gene expression

The expression of CCK mRNA in the brain and digestive tract under different light spectra was studied using real-time quantitative RT-PCR. cDNA for CCK gene expression analysis was used from the whole brain and the distinct digestive tract regions (anterior intestine portion and pyloric ceca). Primers for real-time quantitative RT-PCR of CCK were designed from the obtained CCK nucleotide sequence. The 18R rRNA gene was used to standardize the analysis. The PCR mixture (total volume, 14 μL) contained 2 μL of cDNA, 0.5 μL of each forward and reverse primer, 4 μL nuclease-free water, and 7 μL of SYBR premix Ex™ Taq II (Takara, Kyoto, Japan). Reactions were conducted in 96-well plates and samples were run in triplicate. Amplification and detection of all samples were performed using a CFX96™ Real-Time System (Bio-Rad, CA, USA) using the following thermal cycling conditions: 95 °C for 30 s (1 cycle), 95 °C for 5 s, and 60 °C for 30 s (40 cycles). After each PCR reaction, 1 cycle of melting curve from 65 to 95 °C at a transition rate of 0.5 °C/s with continuous fluorescence detection was performed.

2.5. Statistical analysis

All real-time quantitative RT-PCR data are expressed as mean ± standard error of the mean. All data were subjected to one-way analysis of variance. Duncan's multiple comparisons test was conducted using SPSS 12.0 statistical software (SPSS, IL, USA).

3. Results

3.1. Growth

At the end of the experiment, the growth rate of the fish varied according to the different light spectra (Fig. 2). The highest growth rate was observed in fish reared under the green light condition (Fig. 2). The growth rate of the green light condition was higher than those in the natural, red, and blue light. Such differences were more noticeable in the last 3 weeks of the experiment. Although growth rate of the fish reared under the red light condition was lower than those of the fish reared under the green and natural conditions, it continuously increased at the end of experiment (Fig. 2). The growth rates of the fish reared under the natural and blue light conditions were drastically decreased in the last 3 weeks of the experiment (Fig. 2). On the other hand, the body lengths of the fish reared under different light spectra showed similar patterns and no significant differences.

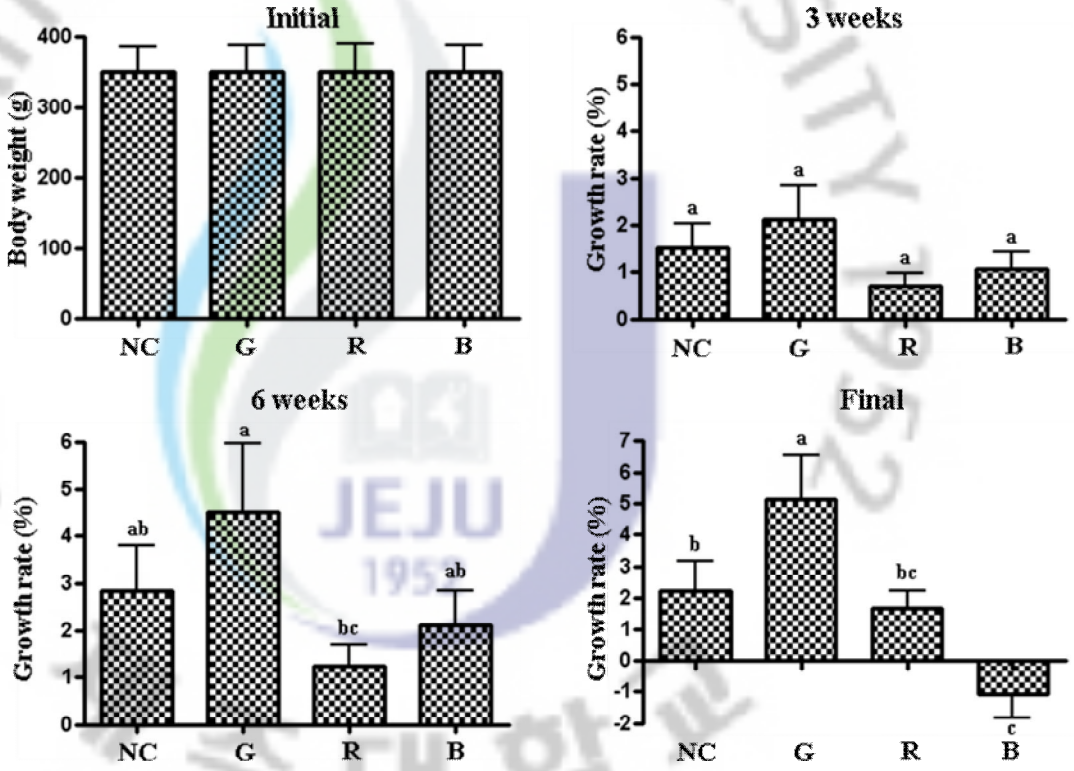

Fig. 2. Initial body weight (g) and growth rate (%) of longtooth grouper reared under different light spectra for 12 weeks. Abbreviation: B, blue light condition; G, green light condition; NC, natural condition; R, red light condition. Different superscript on the bars are significantly different (P<0.05).

3.2. Effects of different light spectra on mucus-secreting goblet cells

According to the location of the digestive tract and the situation in those regions, different regional distributions and relative frequencies of mucus-secreting goblet cells among different light spectrum were observed. These differences are shown in Table 1 and Fig. 3.

The number of mucus-secreting goblet cells differed significantly among the different light spectra. Fish reared under green light condition had significantly higher numbers of mucus-secreting goblet cells compared to fish reared under the natural, red, and blue light conditions. Histologically, the mucosal folds in the digestive tract of fish raised in the green light condition were more developed and contained more abundant numbers of goblet cells (Fig. 5). Mucus-secreting goblet cells of fish in the green light condition were detected in the anterior intestine portion (2332 ± 242), mid intestine portion (1958 ± 190), posterior intestine portion (2078 ± 105), rectum (2337 ± 182), and pyloric ceca (661 ± 40) (Table 1 and Fig 5). Mucus-secreting goblet cells of fish in the natural condition were detected in the anterior intestine portion (1312 ± 175), mid intestine portion (888 ± 81), posterior intestine portion (1015 ± 48), rectum (1311 ± 125), and pyloric ceca (237 ± 31) (Table 1 and Fig 4). Mucus-secreting goblet cells of fish raised in the red light condition were detected in the anterior intestine portion (1608 ± 96), mid intestine portion (1233 ± 83), posterior intestine portion (1193 ± 60), rectum (1217 ± 110), and pyloric ceca (250 ± 45) (Table 1 and Fig 6). Mucus-secreting goblet cells of fish in the blue light condition were detected in the anterior intestine portion (1248 ± 105), mid intestine portion (809 ± 86), posterior intestine portion (992 ± 67), rectum (1197 ± 180), and pyloric ceca (197 ± 27) (Table 1 and Fig 7).

Table 1. Number of mucus-secreting goblet cells in the digestive tract of longtooth grouper reared under different light spectra for 12 weeks.

Region	Number of goblet cells/Tissue section			
	Natural condition (mean±S.E.)	Green (mean±S.E.)	Red (mean±S.E.)	Blue (mean±S.E.)
Anterior intestine portion	1312±175^{b}	2332±242^{a}	1608±96^{b}	1248±105^{b}
Mid intestine portion	888±81^{b}	1958±190^{a}	1233±83^{b}	809±86^{b}
Posterior intestine portion	1015±48^{b}	2078±105^{a}	1193±60^{b}	992±67^{b}
Rectum	1311±125^{b}	2337±182^{a}	1217±110^{b}	1197±180^{b}
Pyloric ceca	237±31^{b}	661±40^{a}	250±45^{b}	197±27^{b}

Values are mean ± S.E. in same column superscripted with different letters are significantly different (P<0.05).

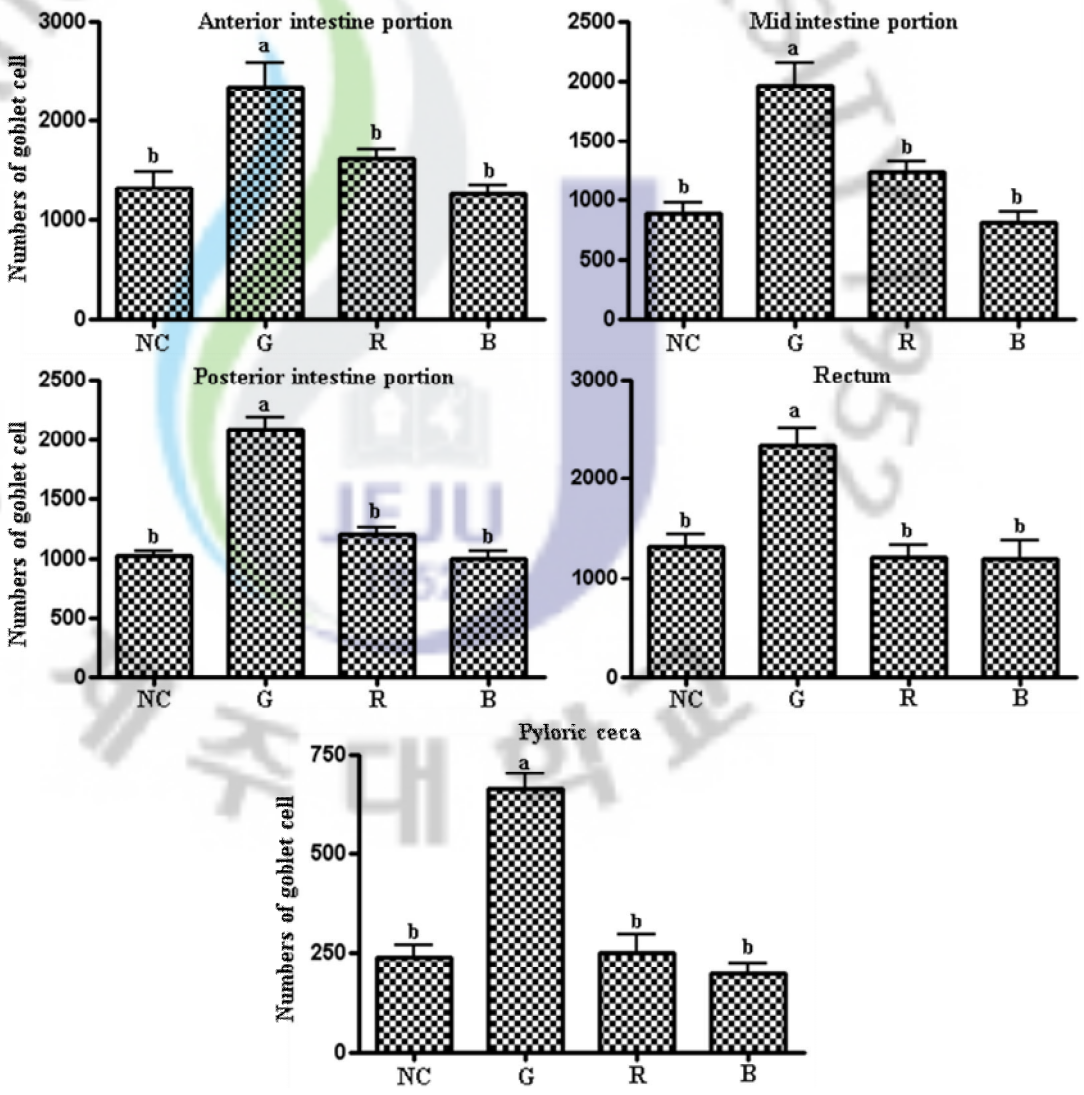

Fig. 3. Changes of mucus-secreting goblet cells in the digestive tract of longtooth grouper reared under different light spectra for 12 weeks. Abbreviation: B, blue light condition; G, green light condition; NC, natural condition; R, red light condition. Different superscript on the bars are significantly different (P<0.05).

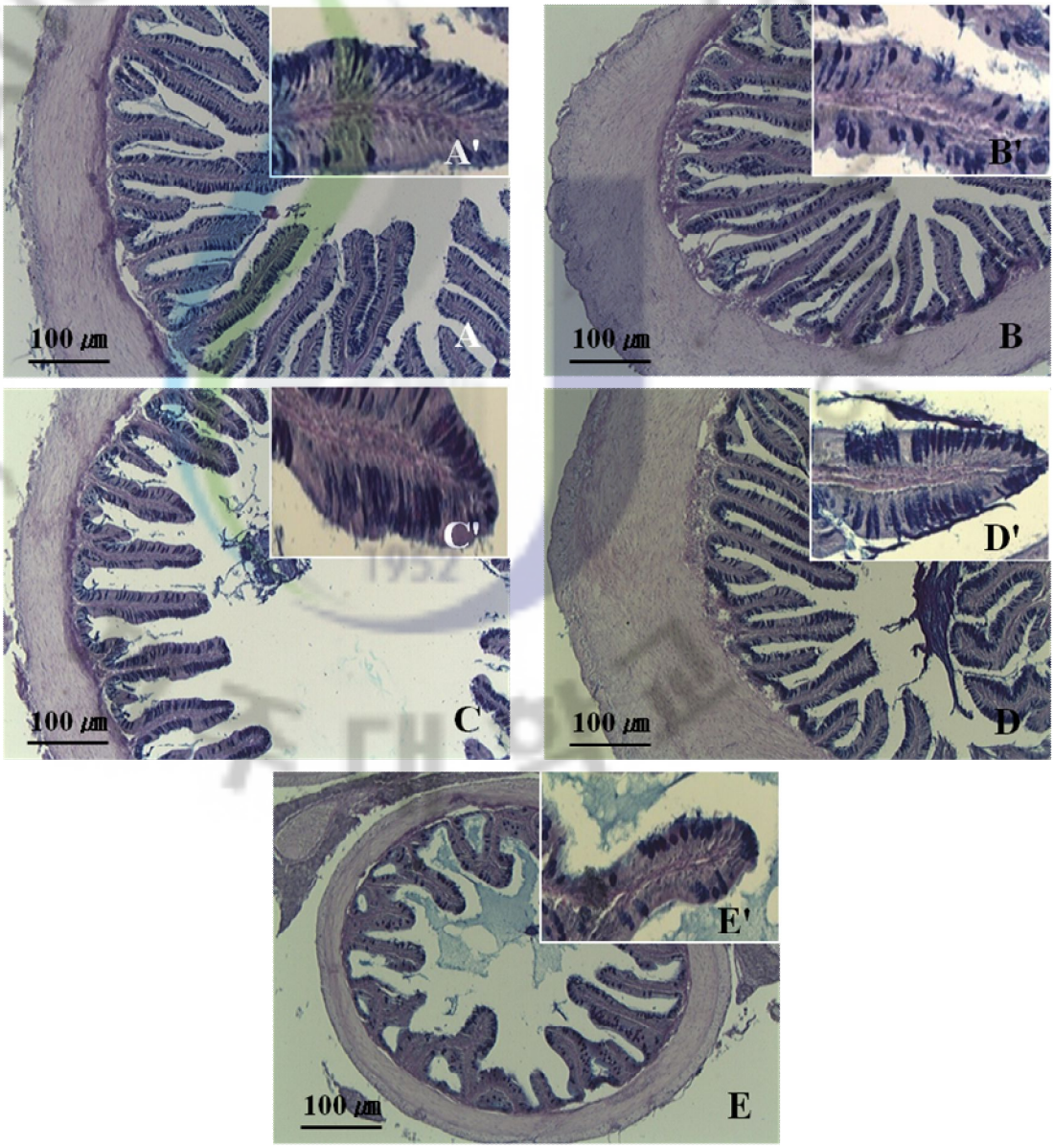

Fig. 4. Photomicrographs of goblet cells in the digestive tract of longtooth grouper reared under natural condition.

A: esophagus, B: anterior intestine portion, C: Mid intestine portion, D: posterior intestine portion, E: pyloric ceca. Magnified goblet cells in (A-E) were shown in (A'-E').

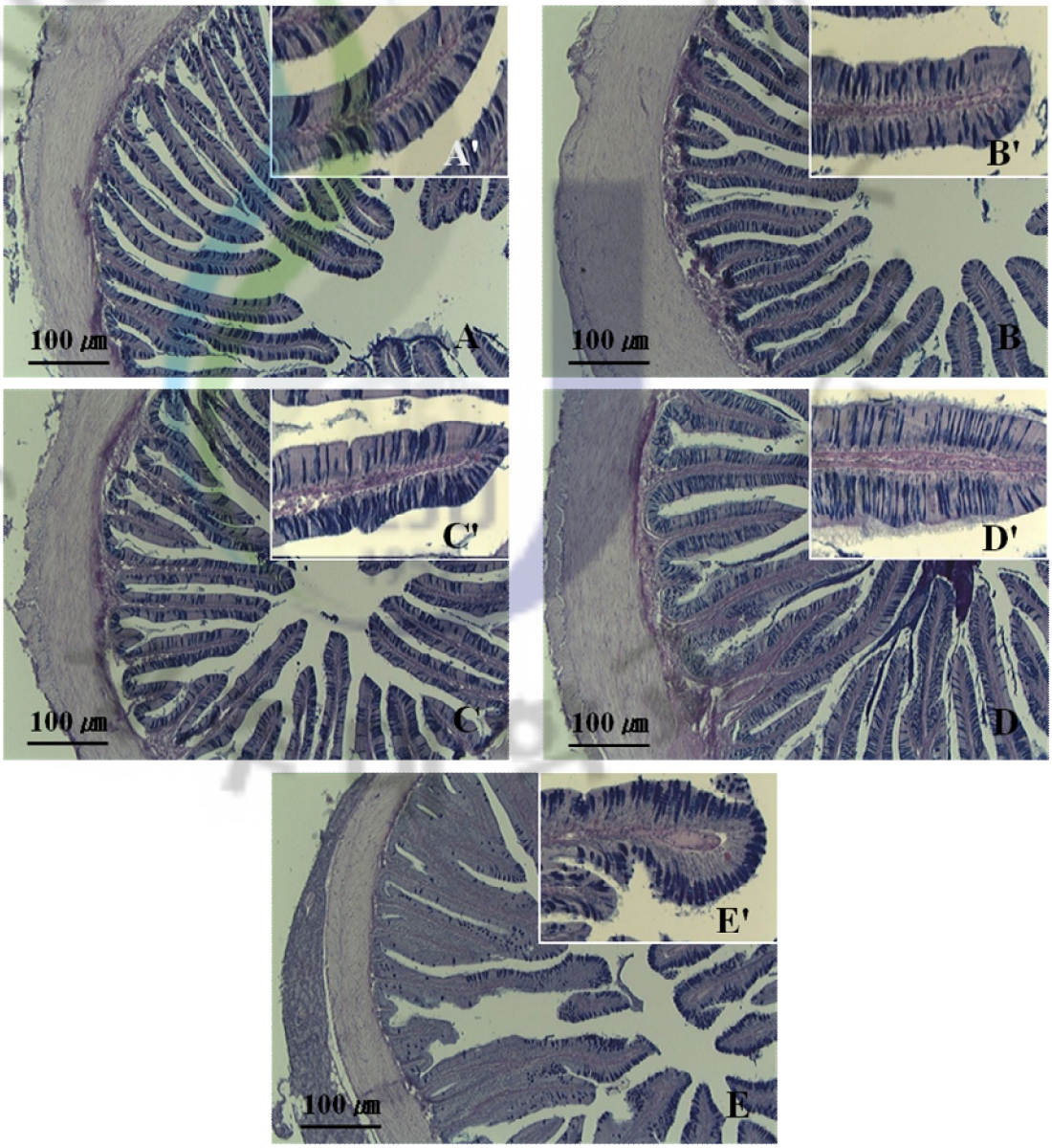

Fig. 5. Photomicrographs of goblet cells in the digestive tract of longtooth grouper reared under green light condition.

A: esophagus, B: anterior intestine portion, C: Mid intestine portion, D: posterior intestine portion, E: pyloric ceca. Magnified goblet cells in (A-E) were shown in (A'-E').

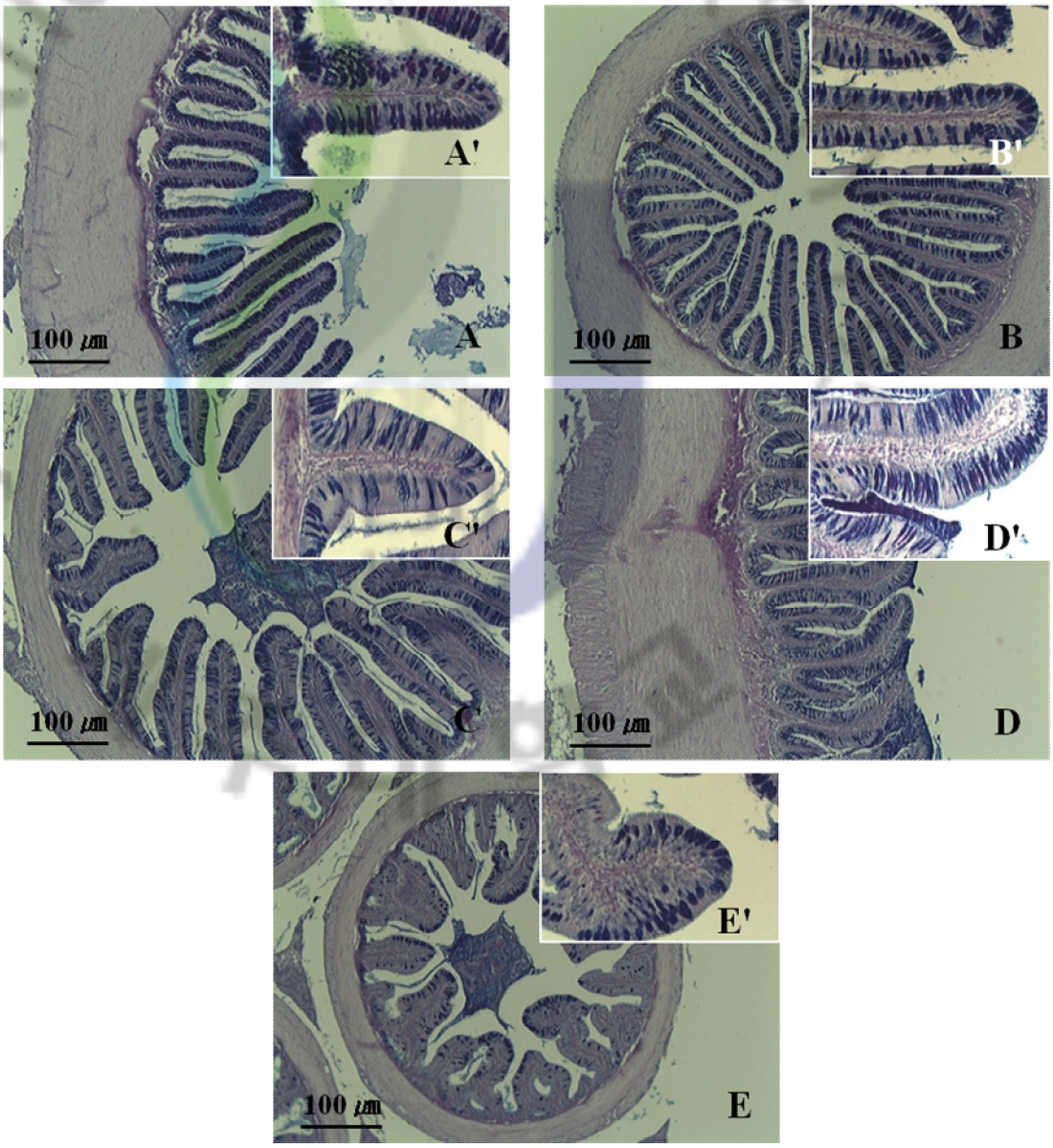

Fig. 6. Photomicrographs of goblet cells in the digestive tract of longtooth grouper reared under red light condition.

A: esophagus, B: anterior intestine portion, C: Mid intestine portion, D: posterior intestine portion, E: pyloric ceca. Magnified goblet cells in (A-E) were shown in (A'-E').

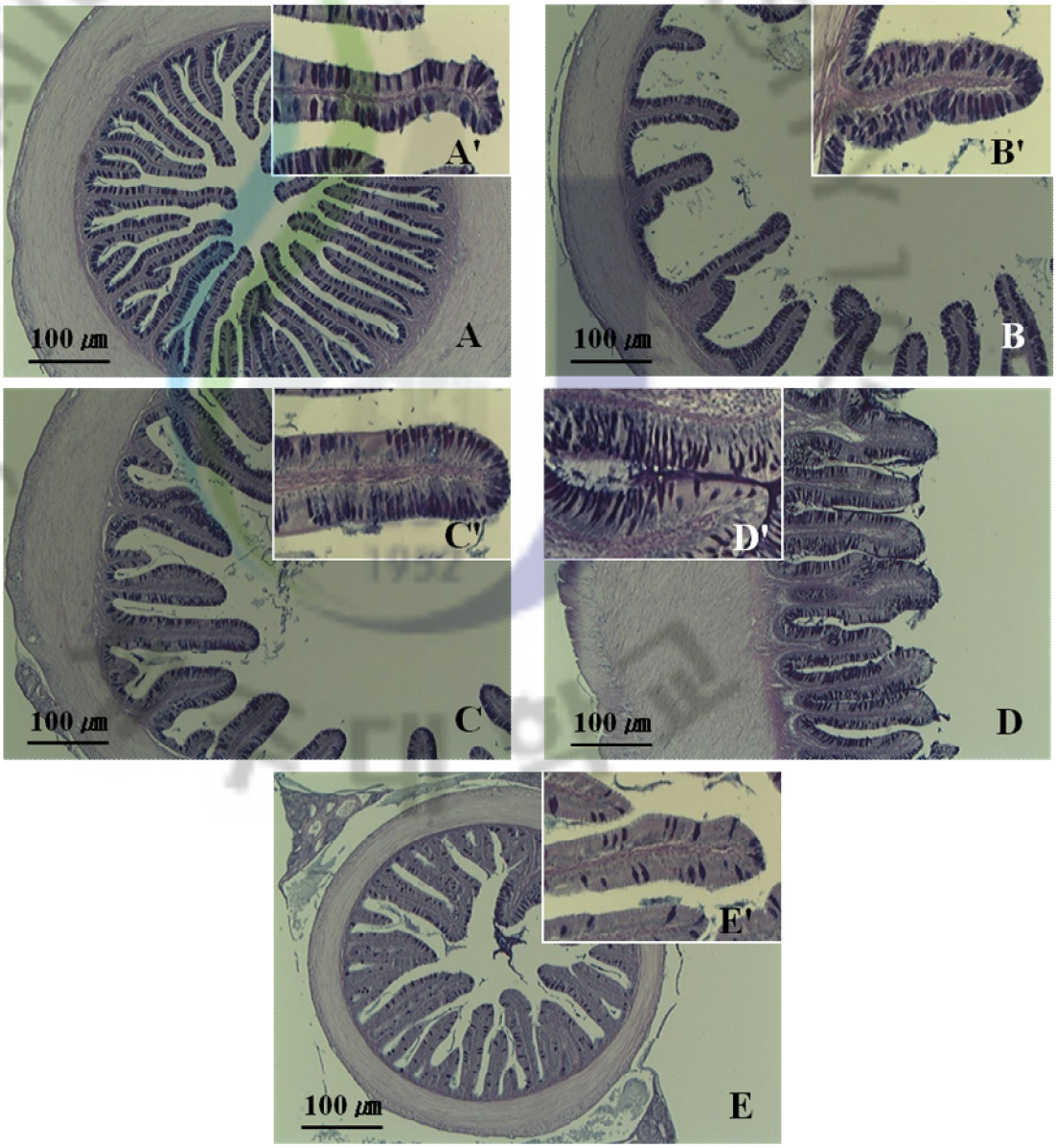

Fig. 7. Photomicrographs of goblet cells in the digestive tract of longtooth grouper reared under blue light condition.

A: esophagus, B: anterior intestine portion, C: Mid intestine portion, D: posterior intestine portion, E: pyloric ceca. Magnified goblet cells in (A-E) were shown in (A'-E').

3.3. Effects of different light spectra on CCK gene expression

The brain, anterior intestine portion, and pyloric ceca were used to measure CCK mRNA expression under the different light spectra. There were significant changes in CCK mRNA expression in the brain and distinct digestive tract areas (anterior intestine portion and pyloric ceca). CCK mRNA levels in the brains of fish raised in the green light condition were significantly higher than those of fish raised in natural, red, and blue light conditions (Fig. 8). CCK mRNA expression in the anterior intestine portion and pyloric ceca were both significantly higher in fish of the green light condition than in those of the natural, red, and blue light conditions (Fig. 9 and 10).

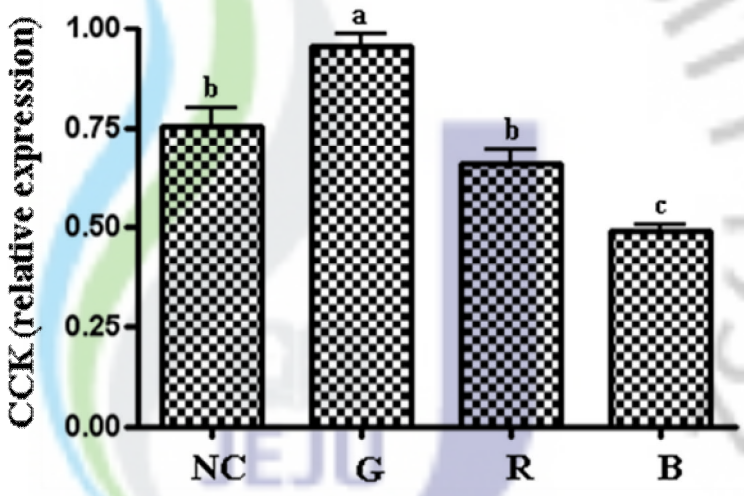

Fig. 8. CCK mRNA expression in the brain of longtooth grouper reared under different light spectra for 12 weeks. Abbreviation: B, blue light condition; G, green light condition; NC, natural condition; R, red light condition. Different superscript on the bars are significantly different (P<0.05).

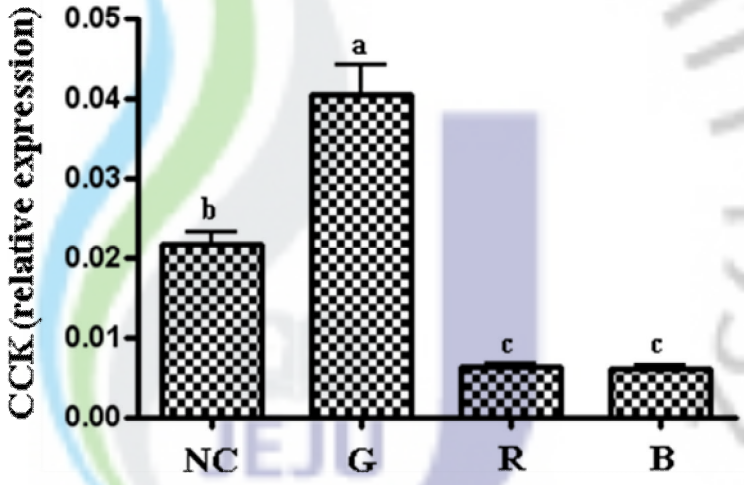

Fig. 9. CCK mRNA expression in the anterior intestine portion of longtooth grouper reared under different light spectra for 12 weeks. Abbreviation: B, blue light condition; G, green light condition; NC, natural condition; R, red light condition. Different superscript on the bars are significantly different (P<0.05).

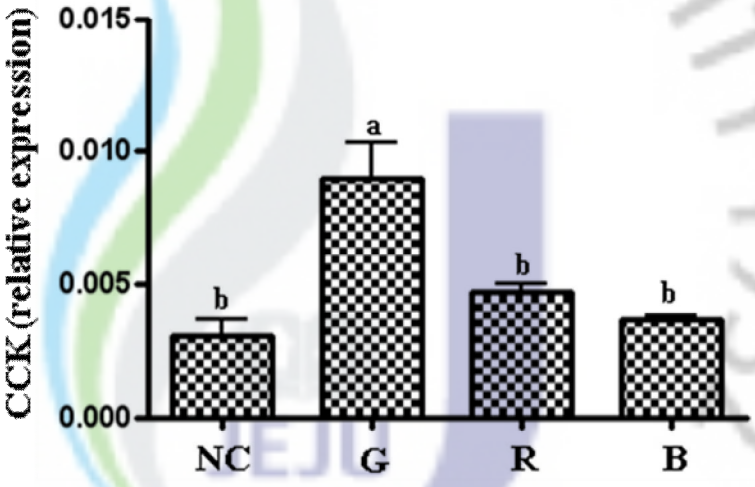

Fig. 10. CCK mRNA expression in the pyloric ceca of longtooth grouper reared under different light spectra for 12 weeks. Abbreviation: B, blue light condition; G, green light condition; NC, natural condition; R, red light condition. Different superscript on the bars are significantly different (P<0.05).

4. Discussion

Fish should respond optimally under the spectral condition encountered most frequently in their natural ecological setting (Downing and Litvak, 2001). Fish move within their environment and often their environment moves around them, affecting the light received (Sumpter, 1992).

The effects of light intensity and spectrum on fish growth, behavior, and feeding activities have been well studied (Blaxter, 1968; Dabrowski and Jewson, 1984; Gehrke, 1994; Biswas et al., 2004, 2005; Karakatsouli et al., 2010). The reported effects of different light wavelengths on fish growth suggest a positive effect of the blue-green light condition on the gilthead seabream (Naor et al., 2003; Karakatsouli et al., 2007); common carp, crucian carp, Chinese sleeper (Ruchin, 2001, 2004); and haddock (Downing and Litvak, 2001). On the other hand, the red light condition has been found to increase survival rates and total biomass of the wallago catfish (Giri et al., 2002) and yellow perch (Head and Malison, 2000). These studies suggest that the effect of different wavelengths are species-specific, and reflect the great variability of the visual ability of fish.

The different light spectra used in the present study appeared to affect growth performance. The longtooth grouper was affected differently by different light spectra. Steadily increasing growth rates were observed under green and red light conditions. The highest growth rate was observed in fish reared under the green light condition. This result is similar to those observed in the barfin flounder (Yamanome et al., 2009), yellow perch (Head and Malison, 2000), and salmon (Dabrowski and Jewson, 1984). Although the observed growth rate of the fish in the red light

condition was lower than those of the fish in the green and natural conditions, it continuously increased at the end of experiment. On the other hand, growth rates of the fish raised in the natural and blue light conditions were drastically decreased in last 3 weeks of the experiment simultaneous with the decrease in water temperature. These results suggest that water temperature has a partially negative growth effect on longtooth grouper reared under natural or blue light conditions.

Light spectrum and intensity can contribute to fish stress (Boeuf and Le Bail, 1999; Papoutsoglou et al., 2000, 2005, Bayarri et al., 2002), which may affect their behavior (e.g., swimming performance, activity levels, and habitat utilization) (Mesa and Schreck, 1989; Schreck et al., 1997; Marchesan et al., 2005). The physiological and behavioral responses of fish change according to a wide variety of physical, chemical, and biological stresses (Ashley, 2007). Stress may cause reduced growth rate and feeding efficiency.

In the present study, longtooth grouper reared under the blue light condition displayed very rigid swimming performance and low feeding behavior (data not shown). This result seen in the fish reared under blue light condition may be a manifestation of physiological stress in the longtooth grouper. However, our data are not yet sufficient to prove this hypothesis. Light spectrum effects on growth and stress response are probably related to neurohormonal mechanisms, and possible interaction among several hormones affected by both stress and lighting conditions cannot be excluded (Karakatsouli et al., 2008). Plasma and pineal melatonin have been studied and reported to be influenced by lighting conditions (Boeuf and Le Bail, 1999; Szisch et al., 2002; Van der Salm et al., 2004) and acute stressors (Kakizawa et al., 1995; Reddy et al., 1995; Ruane et al., 1999). Further studies of the relationship

between different light spectra and fish stress response, plasma cortisol levels and neurohormonal mechanisms need to be carried out.

Digestive capability and endocrine functions lead to numerous physiological effects and can enhance somatic growth and development. These processes are controlled at the molecular and cellular levels of biological organization. Regulation of gene expression and the subsequent molecular processes lead to developmental and physiological changes (Kortner et al., 2011). Hormones can affect fish growth in this manner. However, information with regard to their relation with the influence of light, is lacking. In the present study, we have used molecular and cellular approaches to characterize digestive activity and growth performance. CCK and mucus-secreting goblet cells play a major role in the digestive process. CCK is synthesized by intestinal endocrine cells and influence digestion. CCK plays a crucial role in the regulation of pancreatic enzymes secretion (Jensen et al., 1985; Einnarsson and Davies, 1996; Einnarsson et al., 1997), gallbladder contraction (Liddle et al., 1985; Aldman et al., 1992; Einnarsson et al., 1997), amino acid and sugar transport regulation (Verspohl and Ammon, 1987), and intestinal peristalsis regulation (Olsson et al., 1999). CCK seems to account for the secretion of all enzymes (Solomon et al., 1984). In the present study, the brain, anterior intestine, and pyloric ceca were used to measure CCK mRNA expression under different light spectra. We found significant differences in CCK expression in the brain and in distinct digestive tract regions (anterior intestine portion and pyloric ceca) of the longtooth grouper under different light conditions. CCK mRNA expression in the brain and the distinct digestive tract regions was significantly higher under the green light condition than under the natural, red, and blue light conditions.

In this study, we observed apparent changes in both the intestine mucosal folds and the number of mucus-secreting goblet cells. The mucus secretion of goblet cells in vertebrates including fish plays important roles in the absorption of easily digestible substances (Osman and Caceci, 1991; Domeneghini et al., 2005) and produces a lubricant for the mucosal surface to prevent the mucous membrane from being damaged by physical or chemical substances and protects it from the actions of digestive enzymes (Allen et al., 1986). The number of mucus-secreting goblet cells differed significantly among the fish reared under different light spectra. Fish reared under the green light condition showed significantly higher numbers of mucus-secreting goblet cells compared to fish reared under natural, red, and blue light conditions. CCK and mucus-secreting goblet cells are 2 of the key physiological regulators of the digestive process, contributing to efficient digestion and absorption and high pancreatic activity. These results suggest that digestive processes in the longtooth grouper reared under the green light condition are more pronounced than those in the longtooth grouper reared under other light conditions.

Based on the CCK mRNA expression and mucus-secreting goblet cell activation results of the present study investigated in combination with growth performance, we recommend that the green light condition is the most appropriate for artificial rearing of the longtooth grouper from the perspective of growth performance and the synergistic effects of CCK and mucus-secreting goblet cells. However, our results concerning the physiological effects of different light spectra on growth regulation and digestive processes of the fish are limited.

To summarize the present study, rearing of longtooth grouper under green light condition had positive effects on fish growth performance and the physiological state

of digestion. However, most differences among the different light conditions were observed during the last 3 weeks of the experiment, and longer different light treatment periods are needed to clarify the effects of light spectra on longtooth grouper. Together with the findings of the present study, such studies would result in better understanding of the digestive system of the longtooth grouper, and would contribute to the development of optimal rearing management for the commercial production of the longtooth grouper.

Chapter III

Identification and characterization of CCK, CCK-A receptor, and mucus-secreting goblet cells in the longtooth grouper

Abstract

Cholecystokinin (CCK) and mucus-secreting goblet cells play a key role in the digestive function of vertebrates including fish. CCK accelerates the release of pancreatic enzymes, contracts the gallbladder, and regulates intestinal peristalsis. The mucus secretion of goblet cells in vertebrates including fish plays important roles in the absorption of easily digestible substances and prevents the mucous membrane from being damaged by physical or chemical substances. The amino acid sequence of CCK is well conserved; the fish homology has only single substitution throughout vertebrates. Recently, the CCK gene has also been identified in several fish, and 2 major clusters of teleost CCK were reported after phylogenetic analysis. As a part of a molecular biological study on the longtooth grouper digestive tract, we have isolated CCK cDNA from the brain and the digestive tract using PCR. The cDNA of CCK was 535 bp in length. Phylogenetic analysis revealed that the longtooth grouper CCK subunit was a fish CCK2. Longtooth grouper CCK mRNA expression was detected in the brain (the telencephalon, optic tectum, medulla and hypothalamus, cerebellum, and pituitary) and the digestive tract except for the stomach, suggesting that longtooth grouper CCK could act as a central neuropeptide and as a neurotransmitter in the digestive tract. CCK-producing cells in the digestive tract of the longtooth grouper were scattered throughout the digestive tract. The highest frequency of CCK-producing cells was observed in the anterior intestine portion and pyloric ceca, with a very small number of cells distributed as far as the rectum. Mucus-secreting goblet cells in the digestive tract of the longtooth grouper were found to differ remarkably in their regional distributions and relative frequencies.

High frequencies of mucus-secreting goblet cells were found in the digestive tract of the longtooth grouper, mainly in the anterior intestine portion and pyloric ceca, but not the esophagus; the frequency decreased slightly toward the rectum.

Our result suggests that food digested by gastric acid in the stomach moves on and is then delayed in the anterior (including the pyloric ceca) and mid intestine portion, thereby ensuring effective stimulation of the CCK-producing cells. In addition, the distribution pattern of the CCK-producing cells closely resembled that of mucus-secreting goblet cells. In the longtooth grouper, CCK and mucus-secreting goblet cells seem to be well adapted to promoting optimal control of the digestive process.

1. Introduction

The digestive system comprises the largest endocrine organ in the vertebrate body (Holst et al., 1996). The wide diversity and amount of secreted hormones and signaling molecules secreted by numerous types of endocrine cells rapidly and reversibly alter the characteristics of the digestive system and other organ systems (Buddington and Krogdahl, 2004). Energy and nutrients are made available by a sequential process. Complex polymers are hydrolyzed into small molecules that are absorbed across the apical membrane of epithelial cells and transferred into the systemic circulation (Buddington et al., 1987; Collie and Stevens, 1985; Collie and Ferraris, 1995).

Digestive system functions are regulated by several digestive hormones and substances. Among these materials, cholecystokinin (CCK) and mucus-secreting goblet cells play important physiological roles in the intestines of vertebrates including fish. CCK, one of the gastrointestinal hormones, is the most abundant neurotransmitter peptide in the brain and the intestines. CCK plays a crucial role in the regulation of pancreatic enzymes secretion (Jensen and Holmgren., 1985; Einnarsson et al., 1997; Johnsen, 1998), gallbladder contraction (Liddle et al., 1997; Aldman et al., 1992; Einnarsson et al., 1997), amino acid and sugar transport regulation (Verspohl and Ammon, 1987), and intestinal peristalsis regulation (Olsson et al., 1999). The physiological functions of CCK are mediated by the CCK receptor. Two CCK receptor types (type A, pertaining to the alimentary canal and type B, pertaining to the brain) have been identified (Wank et al., 1995). The CCK-A

receptor is present in the pancreas, gallbladder, muscles, and brain. However, very little is known about the CCK-A receptor in fish other than the zebra fish.

The mucus-secreting goblet cells in the fish digestive tract produce a lubricant for the mucosal surface to protect it against damage induced by physical or chemical substances as well as digestive enzymes (Allen et al., 1986). The mucus secreted by the goblet cells in vertebrates including fish plays important roles in the absorption of easily digestible substances (Osman and Caceci, 1991; Domeneghini et al., 2005).

Longtooth grouper is one of the most commercially important marine aquaculture species in Korea (Song et al., 2005). As mentioned above, CCK and mucus-secreting goblet cells are the main regulators of the digestive processes in fish. To identify the characteristics of CCK, the CCK-A receptor, and mucus-secreting goblet cells in the longtooth grouper, we investigated the cDNAs that code for CCK, the CCK-A receptor, and the distribution and characteristics of mucus-secreting goblet cells and CCK-producing cells in order to provide a basis for understanding the digestive physiology and biology of the longtooth grouper.

2. Materials and Methods

2.1. Specimens

The RNA used to clone the CCK and CCK-A receptor genes originated from longtooth groupers (<3 years old) reared in an indoor tank at the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). Fish were kept in natural photoperiod and natural water temperature conditions. Fish were fed commercial pellets (moisture, 10.00%; crude protein, 52.00%; crude fat, 8.00%, crude fiber, 1.30%; ash, 11.90%; and phosphorous, 1.70%; Le Gouessant, France) once a day. Tissue samples were collected after the fish were anesthetized with 2-phenoxyethanol (Sigma, MO, USA).

2.2. Molecular cloning of CCK and CCK-A receptor

2.2.1. RNA extraction and cDNA synthesis

For the CCK and CCK-A receptor cloning, three longtooth groupers were dissected to obtain samples of the brain and the digestive tract (anterior intestine portion and pyloric ceca). Total RNA was isolated from the whole brain and the digestive tract (anterior intestine portion and pyloric ceca) using RNAiso Plus (Takara, Kyoto, Japan) and chloroform extraction using TRI Reagent following the manufacturer's protocol. Total RNA concentrations were measured by optical density reading at 260 nm using NanoVue (Ver.1.0.1; GE Healthcare, UK). The quality of the total RNA samples was assessed by measuring the ratio of sample

absorbance at 260 and 280 nm. Only RNA samples with ratios 1.7–2.1 were used. The isolated total RNA (2 μg) was incubated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) at 37 °C for 60 min to prevent DNA contamination and stored at -80 °C until use. First-strand cDNA synthesis was performed using 0.5 μg of total RNA using a PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan). Reverse transcription was performed at 37 °C for 15 min and incubated at 85 °C for 15 s for the inactivation of reverse transcriptase. Double-stranded rapid amplification of cDNA ends (RACE)-ready cDNA were made using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to manufacturer's protocol. Purified DNA was diluted by addition of 40 μL nuclease-free water and stored at -20 °C until further analysis.

2.2.2. Polymerase chain reaction (PCR)

The CCK and CCK-A receptor mRNA were amplified by PCR using Taq polymerase (Takara, Kyoto, Japan) and degenerated primers designed from the known conserved regions of CCK and CCK-A receptor genes in other vertebrates (CCK F1, CCK F2, CCK R1, and CCK R2 for CCK; CCK-Ar F and CCK-Ar R for CCK-A receptor, Table 1). Degenerate primers for CCK were designed using highly conserved CCK regions in the Japanese amberjack (GenBank accession no. AB205406), red drum (EU598150), bastard halibut (AB086399), and Atlantic salmon (NM001139521), available in the GenBank database. Degenerate primers for the CCK-A receptor were designed based on the conserved regions in the domestic chicken (NM001081501), brown rat (NM012688), and humans (NM000730), stored

in the GenBank database. PCR amplification was performed in a volume of 12.5 μL using EmeraldAmp PCR Mastermix (Takara, Kyoto, Japan). PCR amplifications were achieved on a thermal cycler (BIOER TECHNOLOGY, Korea) using the following cycling conditions: 94 °C for 2 min (1 cycle), 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min (35 cycles), and the final incubation at 72 °C for 3 min. All PCR products were electrophoresed in 1% agarose gel containing ethidium bromide, and bands of expected size were purified using a DNA Purification System (Promega, Madison, WI, USA), ligated into a pGEM-T Easy Vector System (Promega). The colonies formed by transformation were cultivated in LB medium (Sigma, MO, USA) and plasmid DNA was extracted. Extracted plasmid DNA was sequenced using a high throughput DNA analysis system (Genotech, Korea).

In order to obtain full-length longtooth grouper CCK, 3′ and 5′ RACE PCR was performed. Full-length sequences of cloned CCK products were obtained using gene-specific primers (CCK-GSP 1, CCK-GSP 2, CCK-NGSP 1, and CCK-NGSP 2 for CCK; Table 2). The PCR parameter included 35 cycles at 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s for both the first and second PCRs. The RACE PCR products were purified from the gel, cloned into a pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced using a high throughput DNA analysis system (Genotech, Korea).

2.2.3. Sequence analysis

Nucleotide sequences were compared with the GenBank database using the BLAST algorithm. Multiple alignment of amino acid sequences were performed

using the ClustalW software (www.ebi.ac.uk/clustalw/). After manual were subjected to the ClustalW for making the phylogenetic tree using neighbor-joining method (Saitou and Nei, 1987). The phylogenetic tree was constructed with bootstrap confidence values based on 1000 replicates.

Table 1. Primers used in the cDNA cloning analysis in the longtooth grouper

Primer name	Primer sequence (5'-3')	Use
CCK F1	STGYTGGCAGTCCTGTGTA	CCK cloning
CCK R1	GCACAGAACCTTTCCTGGAV	CCK cloning
CCK F2	CHCTCCTTGADGCBGACACC	CCK cloning
CCK R2	AARTCCATCCASCCCAWGTA	CCK cloning
CCK-GSP 1	CGTTGACTCTGCTGTTTGCTGTGGA	CCK cloning (3'-RACE)
CCK-GSP 2	TGTTGGCAGTCCTGTGTACGAGCTG	CCK cloning (5'-RACE)
CCK-NGSP 1	TGGAGGAGATGAGTCTTGCCAGCAG	CCK cloning (3'-RACE)
CCK-NGSP 2	TCTGAAGCTCTCCTCGAGGCTGACA	CCK cloning (5'-RACE)
CCK-Ar F	ATGCTCTGYCTCTTCTGCAT	CCK-A receptor cloning
CCK-Ar R	CTGAAGATGGGMRTCCAGCA	CCK-A receptor cloning
CCK-Ar GSP 1	GGCTCTGTTGGATGAGGACATGGTG	CCK-A receptor cloning (3'-RACE)
CCK-Ar GSP 2	ATTATGCTGCTGCTCGTGCTGTTCG	CCK-A receptor cloning (5'-RACE)
CCK-Ar NGSP 1	TCTTGCACATGGCAGCTCCAAAGAT	CCK-A receptor cloning (3'-RACE)
CCK-Ar NGSP 2	GGCTCTGTTGGATGAGGACATGGTG	CCK-A receptor cloning (5'-RACE)

A or G; R, C or T; Y, A or C; M, G or T; K, G or C; S, A or T; W, (A, T or C); H, (G, T or T); D, (A, C, G or T); N. Primer design was based on conserved amino acid and nucleotide sequences of CCK and CCK-A receptor available in the GenBank database.

2.3. Tissue distribution and expression of CCK and CCK-A receptor

2.3.1. RT-PCR for tissue distribution and expression analysis

The distribution of the CCK and CCK-A receptor in different tissues was studied by RT-PCR. Total RNA was isolated from the distinct brain regions (the telencephalon, optic tectum, cerebellum, medulla, hypothalamus, and pituitary), gonads, spleen, kidney, liver, and the distinct digestive tract regions (the esophagus, stomach, anterior intestine portion, mid intestine portion, posterior intestine portion, rectum, and pyloric ceca) using RNAiso Plus (Takara, Kyoto, Japan) and chloroform extraction with TRI Reagent following the manufacturer's protocol. Total RNA concentrations were measured by optical density reading at 260 nm using NanoVue (Ver.1.0.1; GE Healthcare, UK). The quality of total RNA samples was assessed by measuring the ratio of sample absorbance at 260 and 280 nm. Only RNA samples with ratios 1.7–2.1 were used. The isolated total RNA (2 μg) was incubated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) at 37 °C for 60 min to prevent DNA contamination and stored at -80 °C until use.

First-strand cDNA synthesis was performed using 0.5 μg of total RNA using PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan). Reverse transcription was performed at 37 °C for 15 min and incubated at 85 °C for 15 s for inactivation of the reverse transcriptase. Purified DNA was diluted by adding 40 μL of nuclease-free water and stored at -20 °C until future analysis. CCK and CCK-A receptor were amplified using gene-specific primers designed on the basis of cloned sequences. Longtooth grouper 18S rRNA was used as a control gene. RT-PCR amplifications were carried out for 30 cycles with each tissue's cDNA, and the RT-PCR products

were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized on UV transilluminator. Negative controls were performed for each primer whenever cDNA was omitted from the RT-PCR reactions.

2.3.2. Real-time quantitative RT-PCR for CCK

Expression of the longtooth grouper CCK in different tissues was studied by real-time quantitative RT-PCR. cDNA for expression analysis was used from distinct brain regions (the telencephalon, optic tectum, cerebellum, medulla, hypothalamus, and pituitary) and the gonads, spleen, kidney, liver, and distinct digestive tract regions (the esophagus, stomach, anterior intestine portion, mid intestine portion, posterior intestine portion, rectum, and pyloric ceca).

Primers for real-time quantitative RT-PCR of CCK were designed in the obtained CCK nucleotide sequence (Table 2). To standardize the analysis, 18S rRNA was used. The PCR mixture (total volume, 14 μL) contained 2 μL of cDNA, 0.5 μL of each forward and reverse primer, 4 μL of nuclease-free water, and 7 μL of SYBR Premix Ex™ Taq II (Takara, Kyoto, Japan). Reactions were conducted in 96-well plates and samples were run in triplicate. Amplification and detection of all samples were performed using a CFX96™ Real-time System (Bio-Rad, CA, USA) with the following thermal cycling conditions: 95 °C for 30 s (1 cycle), 95 °C for 5 s, 60 °C for 30 s (40 cycles). After each PCR reaction, 1 cycle of melting curve from 65 to 95 °C at a transition rate of 0.5 °C/s with continuous fluorescence detection was performed.

Table 2. Primers used in tissue distribution and expression in the longtooth grouper

Primer name	Primer sequence (5'-3')	Use
CCK sp F1	TTGGCAGTCCTGTGTACGAG	CCK tissue distribution
CCK sp R1	CTGTCTGCTATCCGGTGGTT	CCK tissue distribution
CCK sp F2	GGCAAGACTCATCTCCTCCA	CCK tissue expression
CCK sp R2	CACGAGCAGCAGCATAATGT	CCK tissue expression
CCK-Ar sp F	GAGCTGCCATGTGCAAGATA	CCK-A receptor tissue distribution
CCK-Ar sp R	GCTGGACGACGTTCACATAG	CCK-A receptor tissue distribution
18S rRNA F	AAACGGCTACCACATCCAAG	18S rRNA rtq-RT-PCR and quantitative real-time RT-PCR
18S rRNA R	CCTCCGACTTTCGTTCTTGA	18S rRNA rtq-RT-PCR and quantitative real-time RT-PCR

Primer design was based on conserved amino acid and nucleotide sequences of CCK and CCK-A receptor available in the GenBank database.

2.4. Characteristics of CCK-producing cells and mucus-secreting goblet cells

2.4.1. Immunohistochemistry

CCK-producing cells were visualized using the avidin-biotin complex method (Hsu et al., 1981). Microscope slides were coated with poly-L-lysine to promote tissue section adherence. Samples from seven regions of the digestive tract (the esophagus, stomach, anterior intestine portion, mid intestine portion, posterior intestine portion, rectum, and pyloric ceca) were fixed in Bouin's solution, dehydrated in a graded series of ethanol, embedded in paraffin, and then cut into 7-μm longitudinal and cross-sections.

After the sections were deparaffinized and rehydrated, they were incubated in 0.5 mM periodic acid to block the endogenous peroxides. After 3 rinses in 0.1 M phosphate-buffered saline (PBS; pH 7.2), nonspecific binding was blocked with 10% normal goat serum in PBS for 15 min. The solution was blotted off from the slides, primary CCK-8 antiserum (1:1000, Sigma, Israel) was added, and the slides were incubated for 26 h at 4 °C in a moist chamber. After 3 rinses in PBS, the sections were incubated for 50 min at room temperature (around 20 °C) in anti-rabbit goat serum (IgG; Vector, USA) diluted to 1:200 with PBS. After 3 more rinses in PBS, the sections were incubated for 1 h at room temperature (around 20 °C) with streptavidin-labeled peroxidase diluted to 1:100. After 3 rinses in PBS, the DAB substrate system (Vector) was added for the peroxidase reactions. All samples were prepared on a clean bench and incubated in a moist chamber. After immunostaining,

the sections were mounted in Canada balsam (Junsei, Japan) and the CCK-IR cells were observed using light microscopy (HBO 50; Carl Zeiss).

2.4.2. Histochemistry

Samples from 7 regions of the digestive tract (the esophagus, stomach, anterior intestine portion, mid intestine portion, posterior intestine portion, rectum, and pyloric ceca) were fixed in Bouin's solution, dehydrated in a graded series of ethanol, embedded in paraffin, and then cut into 5-μm longitudinal and cross-sections. The slides were stained with Alcian blue at pH 2.5 and periodic acid-Schiff for the observation of mucus-secreting goblet cells.

Characteristics of the mucus-secreting goblet cells from different regions of the digestive tract was carried out using a light microscope (Carl Zeiss, HBO 50) with Image scope 2.3 (Image Line, Inc.) software.

2.5. Statistical analysis

All real-time quantitative RT-PCR data are expressed as mean ± standard error of the mean. CCK mRNA in different tissues was analyzed by one-way analysis of variance followed by Duncan's multiple comparison test using SPSS 12.0 statistical software (SPSS, IL, USA).

3. Results

3.1. Cloning of the longtooth grouper CCK and CCK-A receptor

Multiple bands were generated in all cloning experiments using degenerate primers. All bands within the expected size were cloned and sequenced.

3.1.1. CCK and CCK-A receptor

The nucleotide sequence of longtooth grouper CCK is 535 bp in length and includes a 64-bp 5′-untranslated region (UTR) and a 60-bp 3′-UTR including a stop codon (TAA). The open reading frame (411 bp) contains a 137-amino-acid precursor peptide with a putative CCK-8 C-terminal (Fig. 1 and 3). The amino acid sequence of longtooth groupers was compared to those of humans and 8 teleosts found in the GenBank database. Longtooth grouper CCK showed high homology with red drum CCK (95%), Japanese amberjack CCK (91%), Atlantic salmon CCK (85%), and Japanese eel CCK (60%). Longtooth grouper CCK displayed very low amino acid similarity with rainbow trout CCK (47%), and the lowest similarity was with human CCK (42%) (Fig. 4). The putative CCK-8 (DYMGWMDF) was nearly identical to those of other species (Fig. 3). A partial length of cDNA (721 bp) encoding the longtooth grouper CCK-A receptor was isolated by PCR amplification using degenerate primers (Fig. 2).

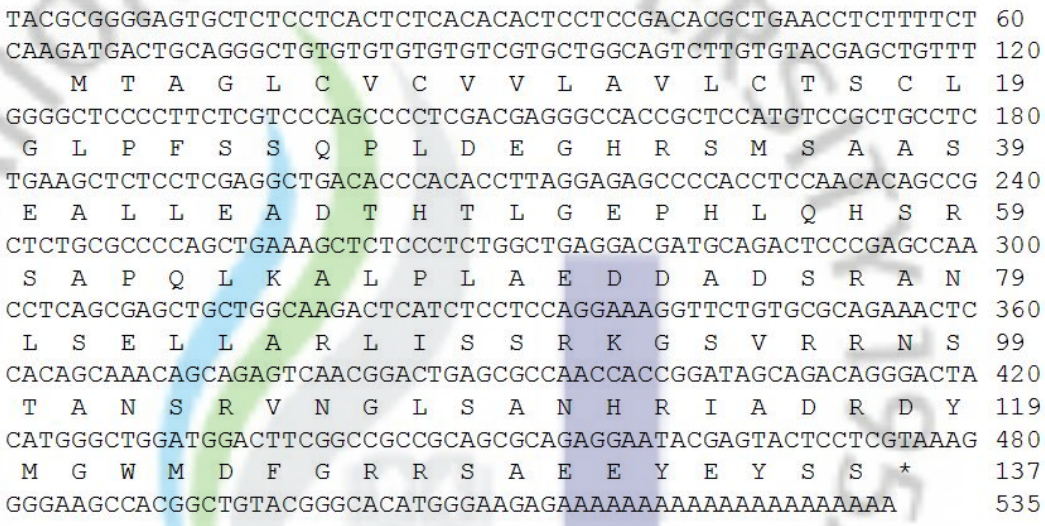

Fig. 1. The CCK nucleotide and deduced amino acid sequence of the longtooth grouper. The CCK cDNA was isolated by RACE. It was 535 base pairs long, contains 411 base pairs of ORF region encoding 137 amino acids, 64 base pairs of 5′ UTR and 60 base pairs of 3 ′ UTR regions. The stop codon is marked (*).

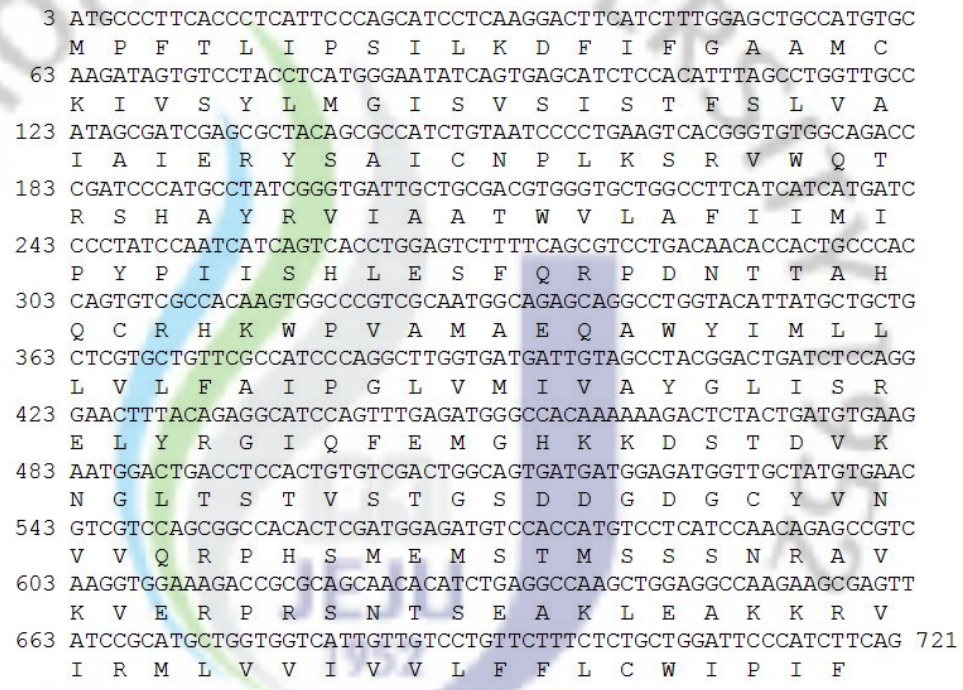

Fig. 2. The CCK-A receptor partial nucleotide and deduced amino acid sequence of the longtooth grouper. 721 base pairs long, encoding 239 amino acids.

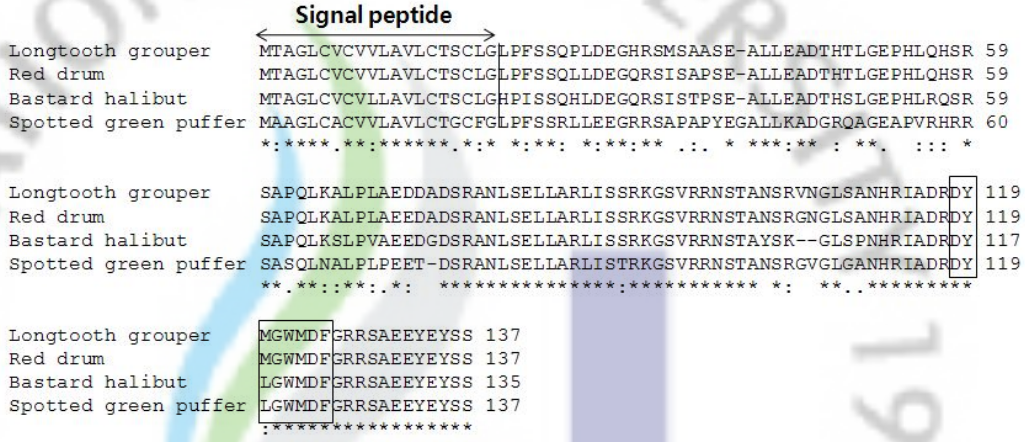

Fig. 3. Alignment of the CCK amino acid sequences without the signal peptide from other species. (*) indicated positions which have a single, fully conserved residue.

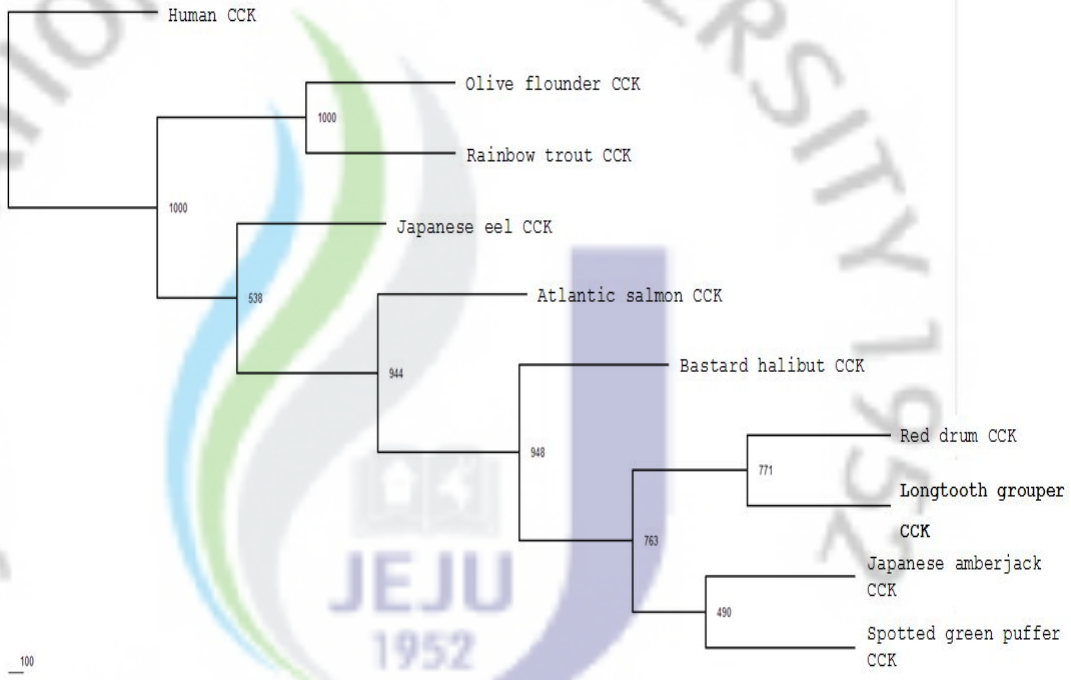

Fig. 4. Phylogenetic analysis of CCK from various species (without using the signal peptide. The phylogenetic tree was constructed using neighbor-joining method with the program clustalW. The scale bar indicates the substitution rate per residue. Node values represent an analysis of 1000 bootstrap trials.

3.2. Tissue distribution of the longtooth grouper CCK and CCK-A receptor

The tissue distribution of the longtooth grouper CCK and CCK-A receptor was investigated by RT-PCR. For all tissues examined, 18S rRNA gene-specific primers were used as controls and a 599-bp fragment of 18S rRNA was detected in all tissues. (Fig. 5 and 6). No band was observed in the negative control (no template).

In the brain, strong bands for CCK mRNA were observed in the telencephalon, optic tectum, medulla, and hypothalamus, while weaker bands were observed in the cerebellum and pituitary (Fig. 5). In the digestive tract, strong bands for CCK mRNA were detected in the anterior intestine portion, posterior intestine portion, and rectum, while weaker bands were detected in the esophagus, stomach, mid intestine portion, and pyloric ceca (Fig. 5). CCK mRNA was not detected in any peripheral organs (the gonads, spleen, kidney, or liver) (Fig. 5). CCK mRNA levels in different tissues were investigated using real-time quantitative RT-PCR. In particular, the telencephalon and the anterior intestine CCK mRNA levels were significantly higher than those in the other areas. (Fig. 5)

RT-PCR of the longtooth grouper CCK-A receptor was used to localize expression in the brain regions, digestive tract, and peripheral tissues. In general, CCK-A receptor mRNA were weakly expressed in the brain (Fig. 6). Weak bands were detected in the optic tectum and pituitary, and very low expression levels were seen in the telencephalon, cerebellum, and hypothalamus (Fig. 6). CCK-A receptor mRNA was not detected in the medulla, the distinct digestive tract regions, or any of the peripheral tissues examined (Fig. 6).

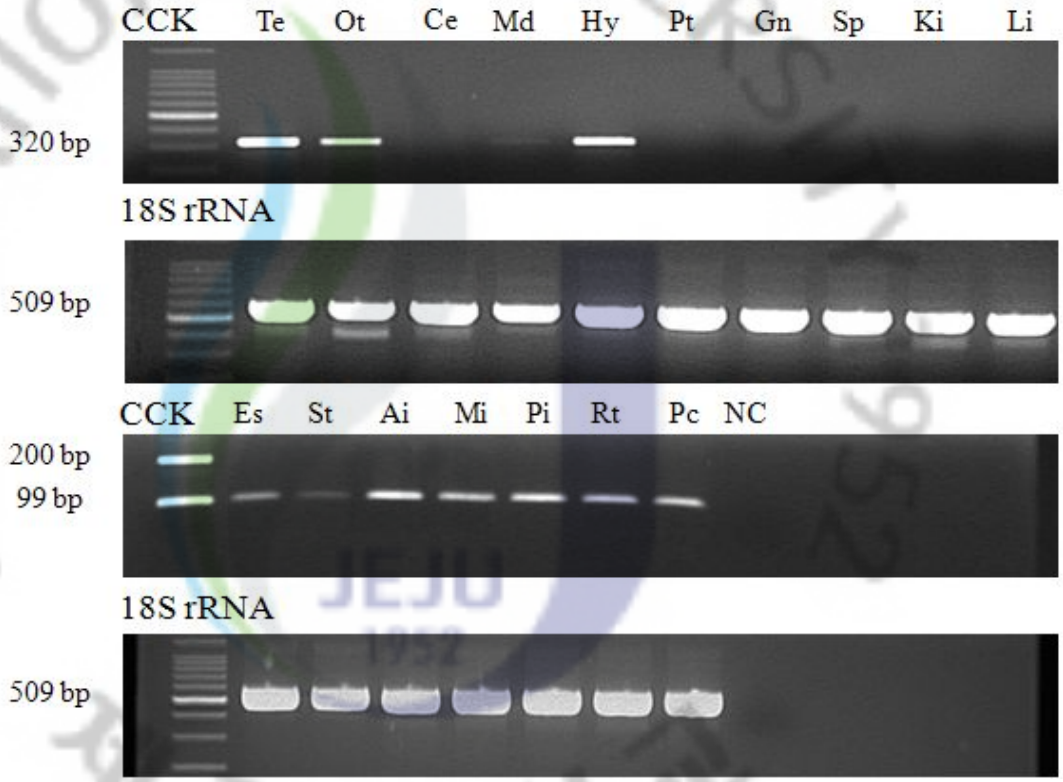

Fig. 5. RT-PCR distribution of CCK in different brain regions, peripheral tissues and different digestive tract regions of the longtooth grouper. Samples were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. Te, telecephalon; Ot, optic tectum; Ce, cerebellum; Md, medulla; Hy, hypothalamus; Pt, pituitary; Gn, gonad; Sp, spleen; Ki, kidney; Li, liver; Es, esophagus; St, stomach; Ai, anterior intestine portion; Mi, mid intestine portion; Pi, posterior intestine portion; Rt, rectum; Pc, pyloric ceca; NC, negative control.

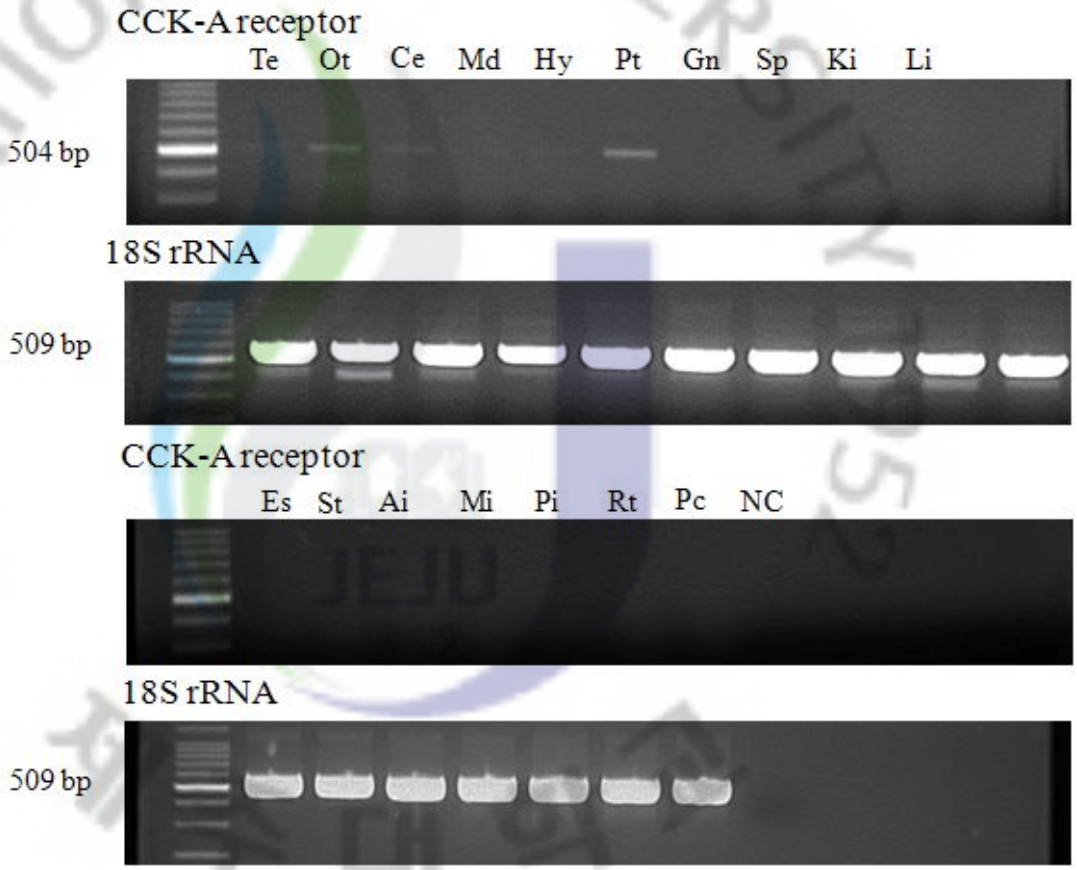

Fig. 6. RT-PCR distribution of CCK-A receptor in different brain regions, peripheral tissues and different digestive tract regions of the longtooth grouper. Samples were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. Te, telecephalon; Ot, optic tectum; Ce, cerebellum; Md, medulla; Hy, hypothalamus; Pt, pituitary; Gn, gonad; Sp, spleen; Ki, kidney; Li, liver; Es, esophagus; St, stomach; Ai, anterior intestine portion; Mi, mid intestine portion; Pi, posterior intestine portion; Rt, rectum; Pc, pyloric ceca; NC, negative control.

3.3. Characteristics of CCK-producing cells and mucus-secreting goblet cells

3.3.1. CCK-producing cells

CCK-producing cells of the longtooth grouper were not detected in the esophagus or stomach but were found at varying frequencies in the anterior intestine and extended to the rectum. The numbers of CCK-producing cells were recorded from the anterior intestine portion (10±3), mid intestine portion (4±2), posterior intestine portion (6±2), rectum (3±1), and pyloric ceca (12±4) (Table. 3). Thus, the highest frequency was observed in the anterior intestine portion (Table 3). The CCK-producing cells were typical endocrine-like cells, with a characteristic elongated spindle shape with a narrow apex pointing toward the intestinal lumen (Fig. 7). Spindle-shaped CCK-producing cells were dispersed among the epithelial cells of the mucosal folds of the intestine and pyloric ceca (Fig. 7).

3.3.2. Mucus-secreting goblet cells

Mucus-secreting goblet cells of the longtooth grouper were not detected in the stomach. They were, however, found with varying frequencies in the esophagus and extended to the rectum. The frequencies of the mucus-secreting goblet cells were recorded from the esophagus (1233±120), anterior intestine portion (903±53), mid intestine portion (516±56), posterior intestine portion (388±44), rectum (380±33), and pyloric ceca (154±44) (Table. 3). Mucus-secreting goblet cells in the esophagus were very densely distributed within the epithelium of the mucosal folds and were mainly large and oval in shape (Fig. 8). In other portions of the digestive tract, goblet cells were irregularly scattered from the upper to the lower parts of the mucosal folds and were mainly spherical and oval in shape (Fig. 8).

Table 3. Numbers of CCK-producing cells and goblet cells in different digestive tract regions of the longtooth grouper

	Numbers/tissue section	
	CCK-producing cells (mean±S.E.)	Goblet cells (mean±S.E.)
Esophagus	N·D	1233±120
Stomach	N·D	N·D
Anterior intestine portion	10±3	903±53
Mid intestine portion	4±2	516±56
Posterior intestine portion	6±2	388±44
Rectum	3±1	380±33
Pyloric ceca	12±4	154±44

"N·D" indicates not detected.

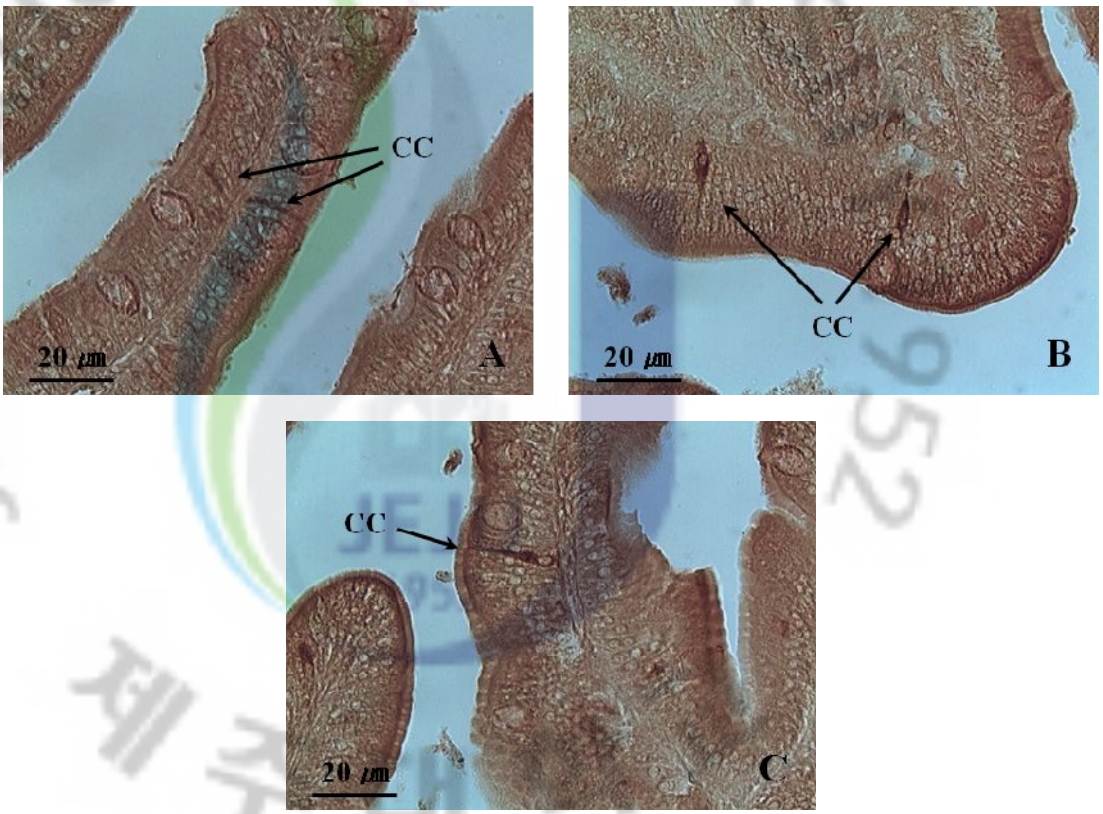

Fig. 7. Photomicrographs of CCK-producing cells in the digestive tract of the longtooth grouper.

A: anterior intestine portion, B: Mid intestine portion, C: pyloric ceca. Abbreviations: CC, CCK-producing cell

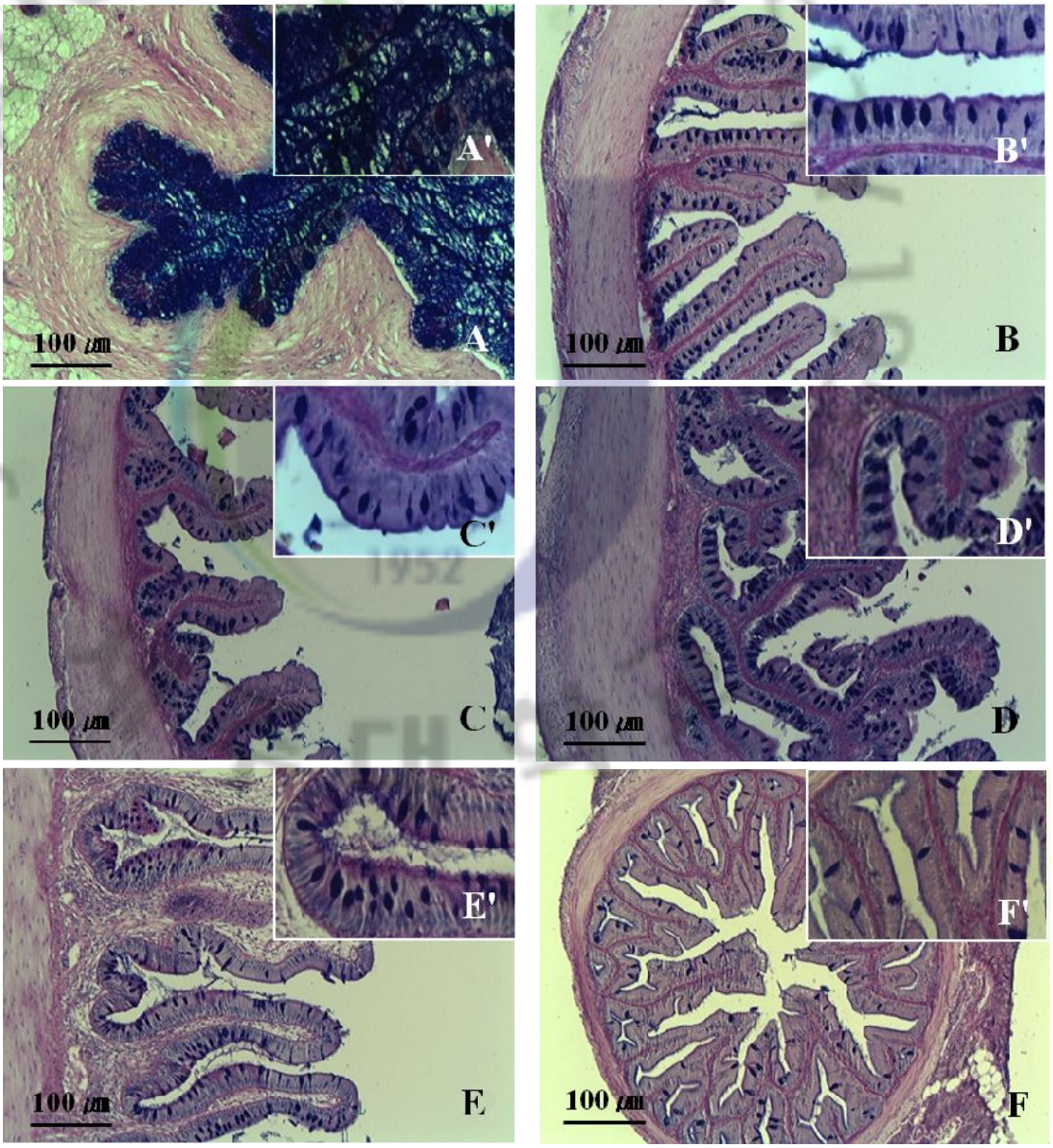

Fig. 8. Photomicrographs of goblet cells in the digestive tract of the longtooth grouper. A: esophagus, B: anterior intestine portion, C: Mid intestine portion, D: posterior intestine portion, E: rectum, F: pyloric ceca. Magnified goblet cells in (A-F) were shown in (A'-F').

4. Discussion

Using RT-PCR with degenerate primers and RACE complete cDNA sequences, CCK and CCK-A receptor were isolated from the longtooth grouper. GenBank comparison and alignment analysis of the longtooth grouper CCK gene clearly showed that it belongs to the CCK peptide hormone gene family. Longtooth grouper CCK sequence shows high degree of similarity with CCK in other teleosts (from 47–95%), having highest sequence similarity with the red drum (95%). CCK is well conserved in all vertebrates studied. CCK-8 in higher vertebrates is highly conserved as DYMGWMDF, with methionine (M) in the sixth position from the C-terminus (Johnsen, 1998). CCK sequences in several teleosts have been reported, and the amino acid sequence of CCK-8 has shown some variation in the sixth position; L in the goldfish and olive flounder (Pyeon et al., 1998; Suzuki et al., 1999; Kurokawa et al., 2003), L or N or T in the rainbow trout (Jensen et al., 2001), and L or V in the spotted river puffer (Kurokawa et al., 2003). Kurokawa et al. (2003) reported the phylogeny of CCK and found 2 major clusters in teleost CCK, naming them fish CCK1 and fish CCK2.

The present study characterized CCK mRNA of the longtooth grouper, and categorized it as fish CCK2. Its CCK-8 was revealed to be DYMGWMDF, belonging to that of higher vertebrates. These results are well conserved in the Atlantic herring and red drum (Kamisaka et al., 2005; Webb Jr et al., 2010). The presence of 2 types of CCK in teleosts as demonstrated in the olive flounder and spotted river puffer indicates that the longtooth grouper may have 2 types of CCK. In the present study, we were only able to confirm that the CCK gene belongs to fish

CCK2, although various PCR conditions were performed and the degenerate primers designed should have been able to pick up both CCK types.

RT-PCR and immunohistochemical analysis were performed in order to localize and characterize CCK and CCK-A receptor in the brain, peripheral tissues, and distinct digestive tract regions. The existence of CCK in the mammalian brain and digestive tract is widely studied (Walsh, 1994). In several species of fish, the existence of CCK mRNA has also been detected in the brain and the digestive tract (Kurokawa et al., 2004; Kurokawa et al., 2003; Kurokawa and Suzuki, 2002; Suzuki et al., 1999; Pyeon et al., 1998). Longtooth grouper CCK mRNA expression was detected in the brain (the telencephalon, optic tectum, medulla, hypothalamus, cerebellum, and pituitary) and in the digestive tract except for the stomach, suggesting that longtooth grouper CCK could act as a central neuropeptide and as a neurotransmitter in the digestive tract. The expression pattern of the longtooth grouper CCK, with its high abundance in the brain, was similar to that seen in the yellow tail and rainbow trout (Murashita et al., 2006; Jensen et al., 2001).

Using RT-PCR analysis, longtooth grouper CCK mRNA was detected in the esophagus. The longtooth grouper is the first species in which CCK expression has been detected in the esophagus. However, its role in the esophagus remains unclear. The digestive tract distribution patterns and characteristics of CCK-producing cells in the teleost digestive tract have been studied by immunohistochemistry and in situ hybridization methods (Fritsch et al., 1978; Noalillac-Depeyre and Hollande, 1981; Holmgren et al., 1982; El-Salhy, 1984a; El-Salhy, 1984b; Hansen et al., 1987; Garcia-Hernandez et al., 1994; Reinecke et al., 1997; Kurokawa et al., 2000). Most studies, however, have focused on the timing of CCK expression and its distribution

pattern in the early life stage. CCK-producing cells are concentrated in the anterior intestine. CCK-producing cells are found only in the anterior mid intestine portion of the olive flounder, Atlantic halibut, and bluefin tuna and in the early life stages of teleosts (Kurogawa et al., 2000; Kamisaka et al., 2001; Kamisaka et al., 2002). In the ayu and Atlantic cod, CCK-producing cells are distributed as far as the posterior intestine (Jösson et al., 1987; Kamisaka et al., 2003).

In our study, CCK-producing cells were scattered throughout the digestive tract of the longtooth grouper. However, the highest frequency of CCK-producing cells was observed in the anterior intestine portion and pyloric ceca, with a very small number of cells distributed as far as the rectum. This distribution pattern is quite similar to that of the ayu (Kamisaka et al., 2003), Atlantic herring (Kamisaka et al., 2005), and hairychin goby (Hur, 2004). Longtooth grouper CCK mRNA levels in the anterior intestine portion were the highest in the distinct digestive tract regions subjected to real-time quantitative RT-PCR, suggesting that CCK-producing cells are concentrated in the anterior intestine portion and produce more CCK than other regions of the digestive tract.

The mucus-secreting goblet cells in the fish digestive tract produce a lubricant for the mucosal surface that prevents the mucous membrane from being damaged by physical or chemical substances and even digestive enzymes (Allen et al., 1986). The mucus secretion of goblet cells in vertebrates including fish plays important roles in absorption of easily digestible substances (Osman and Caceci, 1991; Domeneghini et al., 2005). Mucus-secreting goblet cells in the digestive tracts of several fish species, including the catfish, file fish, and sea bream, have been studied with respect to mucus secretion (Jo et al., 1984). The histochemical characteristics of intestinal

mucus-secreting cells have been analyzed in the pitted stonefish and dark-banded rockfish (Byeon and Jo, 1985) as well as in the gilthead seabream and flower fish (Diaz et al., 2006). According to these studies, the shapes and sizes of the goblet cells in the digestive tract vary according to fish species and intestinal location.

The amount of mucus also depends on the fish species and the particular region of the digestive tract, with the number of mucus-secreting goblet cells generally increasing posteriorly (Reifel and Travill, 1979). However, goblet cells in the digestive tract seem to be highly specific to each teleost species (Pedini et al., 2001; Carrasson et al., 2006; Diaz et al., 2003, 2006, 2008a,b). In the present study, mucus-secreting goblet cells in the digestive tract of the longtooth grouper were found to differ remarkably in their regional distributions and relative frequencies. High frequencies of mucus-secreting goblet cells were found in the digestive tract of the longtooth grouper, mainly in the anterior intestine and pyloric ceca, but not the esophagus; the frequency decreased slightly toward the rectum. Their shape ranged from very oval to round.

To summarize the present study, the cDNAs for CCK and CCK-A receptor were cloned from the longtooth grouper brain and digestive tract. Real-time quantitative RT-PCR was used to measure mRNA levels, which revealed the tissue distribution of CCK and CCK-A receptor mRNA. In addition, characteristics of CCK-producing cells and mucus-secreting goblet cells of the longtooth grouper were investigated using immunohistochemical and histochemical techniques. Our result suggests that food digested by gastric acid in the stomach moves on and is then delayed in the anterior (including the pyloric ceca) and mid intestine, thereby ensuring effective stimulation of CCK-producing cells. In addition, the distribution pattern of CCK-

producing cells closely resembles that of mucus-secreting goblet cells. In the longtooth grouper, CCK and mucus-secreting goblet cells seem to be well adapted to promoting optimal control of the digestive process.

국문요약

자바리(*Epinephelus bruneus*)는 식용어로 기호도가 높으며 현재 우리나라에서 양식대상어종으로 선정되어 양식기술 개발과 산업화에 적극적인 연구가 이루어지고 있다. 이 연구는 자바리의 최적화 양식기술 개발의 기초자료로 활용하기 위해 소화기관의 초기 발달특징, 및 파장에 따른 성장 및 소화활성 특징, 소화호르몬 Cholecystokinin (CCK) 및 배상세포의 특징을 조사하였다.

자바리 소화기관의 초기발달 특징을 조사하기 위해 부화직후부터 부화 후 55 일까지의 자어를 조직학적 및 분자생물학적 방법을 이용하여 실험을 수행하였다. 자바리는 부화직후 큰 난황을 가지고 있었으며, 부화 후 4 일경 간과 각종 소화효소 분비에 관여하는 췌조직이 관찰되었다. 부화 후 5 일경 장이 꼬이기 시작하여 소화관 길이가 증가하고 장 내강의 폭이 급격히 넓어지기 시작하며, 점액물질을 분비하여 소화활성을 높여주는 배상세포가 직장에서 관찰되었다. 부화 후 20 일경 소화관 전장부와 중장부의 점막주름 상피층에서 배상세포가 관찰되었으며 부화일수가 경과할수록 배상세포의 수가 증가하는 특징이 관찰되었다. 부화 후 30 일경 위에서 위산을 분비하는 위선이 관찰되었으며 소화면적을 넓혀주고 소화속도를 조절해주는 유문수와 유문괄약근이 관찰되었다. 각종 소화효소 분비촉진 및 장 연동운동에 관여하는 주요소화호르몬인 CCK 의 경우 개구가 이루어지는 부화 후 2 일경

발현되기 시작하였으며 처음으로 먹이생물을 섭이하는 부화 후 4 일경 발현량이 급격히 증가하였다. 소화기관들의 기능적인 발달과 부화일수가 증가함에 따라 점차 CCK 발현양상이 안정화되는 특징이 관찰되었다.

빛 파장에 따른 자바리의 성장 및 소화활성 특징을 조사하기 위해 자연광조건 (full spectrum), Green (520 nm), Red (590 nm), Blue (480 nm) 조건하에 12 주간 사육 후 조직화학적 및 분자생물학적 방법을 이용하여 실험을 수행하였다. 성장률의 경우 Green 파장에서 유의적으로 높게 나타났으며, 소화활성에 관여하는 CCK 및 배상세포의 활성도 Green 파장에서 유의적으로 높게 관찰되었다.

자바리 고유의 CCK 및 배상세포의 특징을 조직화학적 및 분자생물학적 방법을 이용하여 조사하였다. 자바리 CCK cDNA 길이는 535 bp 였으며, 다른 경골어류와의 상동성 조사결과 Red drum 과 가장 높은 상동성 (95%)을 나타내었다. CCK 분비세포의 경우 소화관의 전장부와 유문수에 가장 많이 분포하였고 소수의 CCK 분비세포가 직장까지 분포하였다. 배상세포의 경우 식도를 제외한 소화관의 전장부와 유문수에서 가장 많이 분포하였으며 직장부로 갈수록 수가 감소하는 특징이 관찰되었다.

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실험을 수행하는데 많은 도움을 주신 제주대학교 해양과환경연구소의 강태연 선생님, 진찬경 선생님, 조창환 선생님, 임지일 선생님, 송경택 선생님, 고성대 선생님, 김선희 님, 윤태석 님에게도 고마움을 전합니다.

나의 곁에서 사랑과 믿음으로 묵묵히 바라보며 부족한 나를 이끌어준 사랑하는 나의 아내.. 우리 현주.. 만삭인 몸으로 많은 보살핌이 필요한 시기에 함께 많은 시간 보내지 못해 너무나 미안하고 그만큼 고마운 마음 전하고 싶습니다. 얼마전 우리에게 온 너무나 소중한 나의 딸.. 우리 채연이.. 그 누구보다 소중한 우리 현주.. 채연이.. 남편으로서 또한 친구로서 아빠로서 그 누구보다 최고인 사람이 될 수 있도록 항상 최선을 다하겠습니다. 또한 친아들 이상으로 많은 보살핌과 힘을 주고 격려해주신 장인어른, 장모님, 하나밖에 없는 우리 처제 계연에게도 죄송한 만큼 감사한 마음 전해드리고 싶습니다. 아버님, 어머님 고맙습니다.

지금 이 순간도 우리 가족과 저를 사랑으로 보호해주시고 무한한 사랑을 주고 계신 사랑하는 나의 어머니.. 힘든 시기 많은 위로와 사랑을 드리지 못한 마음이 들어 너무나 죄송할 따름입니다. 어머니.. 너무나 감사드립니다. 너무나 사랑합니다. 그리고 멀리 미국에 있는 큰누나, 작은누나, 브라이언, 작은 매형, 태근과 희근에게도 고마운 나의 마음을 전해드립니다. 모두 보고싶어요.

마지막으로 지금 이 순간 그 누구보다 기뻐하시고 자랑스러워하고 계실 너무나 그리운 나의 아버지.. 비록 지금은 잠시 멀리 떨어져 있지만 아버지 보시기에 부족함이 없도록 늘 겸손하고 나의 분야에서 최고이자 든든하고 푸근한 가장이 될 수 있도록 아버지께서 그러하셨듯이 변함없이 최선을 다하겠습니다. 아버지.. 끝까지 지켜드리지 못해 너무나 죄송해요. 아버지의 따뜻한 말씀과 미소가 너무나 그립습니다.

비록 많이 부족하지만 이 소중한 결실을 사랑하는 나의 가족과 가장 존경하고 그리운 나의 아버지 영전에 바치고자 합니다.

모두 감사드리고 사랑합니다.