**A THESIS**

**FOR THE DEGREE OF MASTER OF SCIENCE**

# Changes the expression profiles of digestive factors in red spotted grouper (*Epinephelus akaara*) by the rearing water temperature

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# Abstract

The growth of fish is under the control of various environmental factors especially the water temperature (WT). It is suggested as one of the main factors in the eating behavior. Previously we explored the positive effects of high WT in the growth rate and feed efficiency in the red spotted grouper (110 DAH). The red spotted grouper (110 DAH) are faster growth and better feed efficiency in high WT (24℃ and 28℃) than natural WT.

In this study, we studied the relationships between the water temperature and the expression profiles of digestive factors in red spotted groupers.

Seventeen month-old juveniles were divided randomly into 3 groups and adjusted for 2 weeks with 3 different WT; natural condition (15±1.0℃), 20±0.5℃ and 25±0.5℃. Commercial pellet diet was supplied at 11:00 (0 h) once a day to satiety. After 2 weeks, the fish were randomly sacrificed at 0 h, 3 h, 6 h and 21 h in all groups. They were applied to analyze the activity of goblet cell and the mRNA expression levels of cholecystokinin (CCK), leptin-a (LepA), leptin-b (LepB) and neuropeptide Y (NPY).

The average lengths of intestinal villus were 220.92±14.80 μm, 247.08±23.09 μm and 356.66±12.71 μm (NC, 20 and 25℃ respectively). The numbers of intestinal goblet cells per tissue section were significantly many at 25℃; NC (462.3±67.8), 20℃ (461±91.2) and 25℃ (766±144.3). The mRNA levels of CCK, and NPY were significantly high at 25℃ compared with other temperature but not Leptin.

Based on them, it is suggested that the water temperature is a key factor for

feeding and digestive activity. It is considered that the faster growth of the red spotted grouper at high water temperature have correlate with the digestive activity through the increased expression of digestive factors and the histological changes in intestine.

# 1. Introduction

Among internal and external factors affecting the growth of fishes, external factors include habitation environmental factor such as the light, water temperature, and food, and internal factors of physiological responses such as immune, digestion, stress and sexual maturity.

In the external factors, water temperature has the greatest influence on the growth and feeding activity of fish, and the optimal water temperature for rearing is species specific (Jobling, 1983; Pepin, 1991; Burel et al., 1996). The digestion system that is responsible for digestion of the food play a role in supplying energy for fish growth from the internal factors. Neuropeptide Y (NPY), cholecystokinin (CCK) and leptin are produced by endocrine cells present in the digestive tract. The hormones and peptides are involved in intake regulation and feeding behavior of the fish. In mammals, neuropeptide Y (NPY) is a potent appetite inducing factor, which also play a function as an appetite-related factor in fish. NPY mRNA tends to be upregulated in hypothalamus upon deprivation of food while downregulated by refeeding (De Pedro et al., 2001; Volkoff et al., 2005). Cholecystokinin (CCK) is present in the hypothalamus and the gastrointestinal tract, where it confers satiety in the brain and also promotes enzyme secretion of pancreas, peristalsis and gallbladder contraction in the gastrointestinal tract (Volkoff et al., 2005; Dockray, 2009). Leptin is mostly secreted from white adipose tissues in mammals, suppressing appetite. Besides, leptin is involved in lipid metabolism, hematopoiesis, reproduction and bone formation (Copeland et al., 2011). In fish however, it is mainly secreted in the

liver due to absence of adipose tissues. Leptin in fish mostly regulates appetite and is involved in energy metabolism. Leptin has different regulatory functions for appetite depending on the fish species unlike the mammals. In terms of energy metabolism, suppression of food supply reduces leptin in *Schizothorax prenanti*, and striped bass (Won et al., 2012; Yuan et al., 2014), whereas it increases leptin level in orange spotted grouper and fine flounder (Fuentes et al., 2012; Zhang et al., 2013). In addition, food deprivation had no effect on leptin level in goldfish (Tinoco et al., 2012). In terms of appetite regulation, leptin increases after a while from diet, and the time is also dependent on species.

Red spotted grouper (*Epinephelus akaara*) is a semitropical fish living in southern China, Taiwan, the East China Sea, Korea and southern Japan. While red spotted groupers have a high commercial value in Asia, the growth rate is relatively lower than other groupers. Thus, it is required that establish rearing management to enhance growth and comprehend physiological characteristics of red spotted grouper. To do this, various study about red spotted grouper is conducted (Han et al., 2014; Lee et al., 2014; Kim et al., 2015; Wang et al., 2016).

In one study, when red spotted groupers were reared in various water temperatures (Natural condition, 20℃, 24℃ and 28℃) for 11 months, the highest growth rates were found at 24℃ and 28℃. In addition, the amount of feed intake and feed efficiency were also higher. These results suggest that rearing water temperature have effect on the growth of red spotted groupers. In addition, the amount of food and feeding efficiency were different by water temperature. Thus, additional studies are required on the relationship between the water temperature and the digestive activity.

Therefore, this study aimed to investigate the effect of rearing water temperature on feeding activity and digestion-related factors in red spotted groupers.

# 2. Materials and Methods

## 2.1. Animals

Artificially fertilized and reared in the Marine Science Institute of Jeju National University *Epinephelus akaara* was selected as the experimental fish of red spotted grouper. Fishes at 90±5 g in weight were fed twice a day at natural water temperature (15±2℃) until the experiment.

## 2.2. Experimental design

Investigation of digestion-related factors in red spotted groupers with water temperature Fishes were divided into three groups with 30 fish per group in recirculating aqua culture systems depending on the water temperature (natural water temperature, 20℃ and 25℃) and reared for 2 weeks. Food was fully provided once a day at 11 AM, and feeding intensity was checked every day. Photoperiod was used for the natural photoperiod, and rearing water temperature was maintained with a boiler.

After two weeks, red spotted groupers were collected at four different time points based on the feeding time: immediately after food intake (+0 h), after 3 h (+3 h), after 6 h (+6 h) and 3 h before feeding of the next day (-3 h) (n=24). In order to eliminate reduction of feed intake caused by sampling stress before intake, sampling at 3 h before feeding was performed on the next day. Sampled tissues include the

brain with pituitary gland, liver and the midgut. Collected tissues were frozen at -80℃ until total RNA extraction for genetic analysis, and fixed for histological analysis after fixation with Bouin's solution.

## 2.3. Histological analysis

Of the collected tissues from the gastrointestinal tract, only the midgut region was used for analysis. Tissues fixed in Bouin's solution were fixated again in ethanol after washing with water. The fixed tissues underwent dehydration steps, followed by embedding in paraffin, which was then made as blocks. Tissues were sliced into 5 μm in thickness to make specimens. Sliced tissues were stained in Alcian blue (AB), pH 2.5 and periodic acid Schiff (PAS) to examine the goblet cells that secret mucosubstances. Stained tissues were sealed with Canada balsam, followed by microscopy.

To observe the goblet cells and intestinal villi, we used an optical microscope (BX53, Olympus) and Imaging Software (Olympus cellSens™ Microscope Imaging Software).

## 2.4. Total RNA extraction and cDNA synthesis

Collected tissues were frozen at -80℃ until total RNA extraction. Total RNAs were isolated from the brain and gonadal tissues extracted from each experimental group. Each tissue was mixed with 600 µl RiboEx$^{TM}$ LS (GeneAll, Korea) in an 1.7 ml tube, followed by homogenization with a homogenizer. 0.2 µl chloroform per 1 µl RiboEx$^{TM}$ LS was added and incubated at room temperature for 5 min, followed by centrifugation at 12,000 x g for 15 min, resulting in total RNA. Thereafter, total RNA was mixed with 500 µl isopropanol in a fresh tube, incubated at room temperature, and RNAs were precipitated by centrifugation at 12,000 x g at 4℃ for 10 min. After discarding the supernatant, precipitated RNAs were washed with 75% ethanol; 100% ethanol was diluted in diethyl pyrocarbonate (DEPC) treated $H_2O$, followed by washing with 95% ethanol. After washing, ethanol was removed, and then precipitated RNAs were resuspended in DEPC $H_2O$, resulting in total RNA. For quantitative and qualitative analyses of total RNAs, a spectrophotometer (Nano Vue) was used to measure the optical density at A260 and A280 nm, and then samples with 1.7-2.1 in ratio of A260/A280 nm were selected.

For cDNA synthesis, total RNAs from brain tissues were treated with DNase using RQ1 RNase-Free DNase Kit (Promega, USA), followed by synthesis using PrimeScript™ 1$^{st}$ strand cDNA synthesis Kit (Takara, Japan). In detail, DNase-treated RNAs were mixed with 8 µl RNase-free $H_2O$, 1 µl random hexamers and 1 µl dNTP mixture, followed by incubation at 65℃ for 5 min. Then a total of 20 µl of a reaction mixture containing 4 µl 5X PrimeCript Buffer, 0.5 µl RNase inhibitor, 10 µl

PrimeCript RTase and 4.5 µl RNase free $dH_2O$ was added, which was incubated at 30℃ for 10 min, 42℃ for 60 min and 95℃ for 5 min, resulting in synthesized cDNA. Each cDNA was diluted with 30 µl RNase free $dH_2O$ to make 50 µl in the final volume.

## 2.5. Tissue specific gene expression

In order to investigate tissue specific gene expression patterns, adult fish were sampled. Extracted tissues include three areas of the brain (fore, mid and hind), pituitary gland, eyes, kidney, liver, gonad, muscles, stomach, pyloric caeca, foregut, midgut and the hindgut, which were stored at -80℃ until analysis. From those tissues, total RNAs were isolated, followed by cDNA synthesis and RT-PCR analysis. Primers were designed to amplify about 120-200 bp of PCR products using the primer3 plus program based on DNA sequences registered in the National Center for Biotechnology Information (NCBI) (Table 1.).

PCR mixture was composed of 13 µl EmeraldAmp GT PCR Master Mix (2X Premix) (TaKaRa Bio), 0.6 µl 10 pM Primers (Forward and Reverse), and 2 µl cDNA (50 ng/µl), and PCR cycle was as follows: denaturation (45 s, 94℃), annealing (45 s, 58℃) and extension (1 min, 72℃) with 34 cycles. Amplified PCR products were analyzed by electrophoresis on 2% agarose gel.

**Table 1** Primer sequence for PCR

| Gene | Primer information | | | |
|---|---|---|---|---|
| | Primer | 5'-3' sequence | Melting temp (℃) | Amplicon size (bp) |
| NPY | NPY F | TGCATCCTAACTTGGTGAGC | 88.5 | 204 |
| | NPY R | TGGACCTCTTCCCATACCTC | | |
| CCK | CCK F | GACACCCACACCCTAGGAGA | 87.0 | 186 |
| | CCK R | TCCGTTGACTCTGCTGTTTG | | |
| LepA | LepA F | CCGTCAGAGACGAGATGTCA | 84.0 | 224 |
| | LepA R | TTGTGGTGCCACTGACTCTT | | |
| LepB | LepB F | GCTCTGCAGTTCATTGTCCA | 84.0 | 207 |
| | LepB R | GGGTGCTCAAGTCTTCCAAC | | |
| B-actin | B-actin F | GAGCGTGGCTACTCCTTCAC | 87.0 | 390 |
| | B-actin R | AGGAAGGAAGGCTGGAAGAG | | |

NPY: Neuropeptide Y; CCK: Cholecystokinin; LepA: Leptin-a; LepB: Leptin-b

## 2.6. Real-time quantitative PCR

Expressions of each gene was analyzed by quantitative real-time PCR with EvaGreen 2X qPCR MasterMix-Rox Kit (abm, Canada) using BioRad CFX96™ Touch™ Real Time PCR (BioRad, Hercules, CA). Primer sequences were designed to result in about 120-200 bp of PCR products using the primer3 plus program based on DNA sequences registered in the National Center for Biotechnology Information (NCBI) (Table 1.). A total 10 µl volume of PCR mixture composed of 2 µl cDNA (50 ng/µl), 5 µl EvaGreen 2X qPCR MasterMix, 0.4 µl forward primer (10 pM), 0.4 µl reverse primer (10 pM), and 2.2 µl RNase free $H_2O$ were subjected to 40 cycles of denaturation (45 s, 94℃), annealing (45 s, 58℃) and extension (1 m, 72℃) for amplification. Expression level of each gene was tested by two replications, in which B-actin gene was used as an internal control for relative quantification and mean values of experiments were used for further analyses.

## 2.7. Statistics

All results of this study was presented in mean ± SEM. Expression patterns of each gene was subjected to significance test with One-Way analysis of variance (ANOVA) using Duncan's multiple range test (Duncan, 1955). Only when significant difference was $P < 0.05$, statistical significance was accepted.

# 3. Results

## 3.1. Differences between villi and goblet cells by the water temperature

The result of feeding intensity according to WT is that the temperature for the highest mean feed intake amount was 25℃, followed 20℃ and NC in order (Fig. 1, Table 2.). The lengths of villi in the midgut tended to become longer as water temperature went higher. At 25℃, the value was 365.66±12.71 μm, which was significantly higher than those of other WT (Fig. 2A, B, Table 2.). The numbers of goblet cells that produce mucosubstances in the midgut of red spotted groupers by various water temperatures were counted by a histological approach, which found the highest number at 25℃, though there was no significant difference. The numbers were 462.3±67.8 at natural water temperature, 461.2±901 at 20℃ and 766.0±144.3 at 25℃. (Fig. 2A, C, Table 2.).

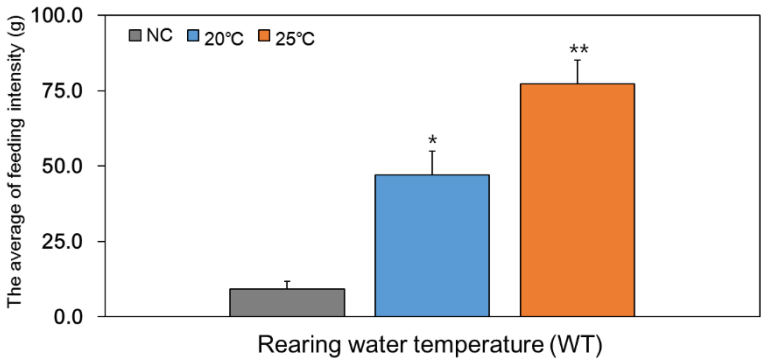


Fig. 1. The average amount of feeding intake for 2weeks.

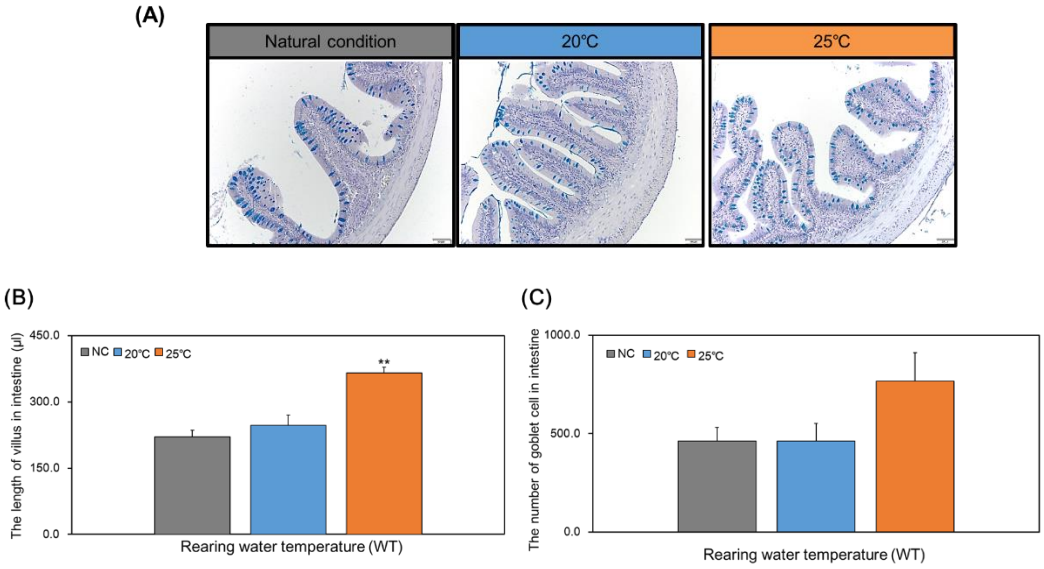


Fig. 2. The histological features in mid-intestine by AB-PAS staining.

A, Goblet cells in the mid intestine of red spotted grouper reared under different water temperature; B, The difference of villus length in intestine by the water temperature (WT); C, The difference of goblet cell number intestine by the WT;

**Table 2** Feeding intensity and histological analysis

| Water Temperature (℃) | Feeding intensity (g) (mean ± S.E.) | Villi length (㎛) (mean ± S.E.) | Number/tissue section Goblet cells (mean ± S.E.) |
|---|---|---|---|
| Natural condition | 9.3 ± 2.5 | 220.92 ± 14.80 | 462.3 ± 67.8 |
| 20 | 47 ± 8* | 247.08 ± 23.09 | 461 ± 91.2 |
| 25 | 77.3 ± 7.9** | 356.66 ± 12.71* | 766 ± 144.3 |

* 'significance level: p <0.05; ** p <0.01 (ANOVA).

## 3.2. Tissue specific gene expression

Gene expression patterns in tissues including central nerve tissues and their peripheral tissues of red spotted groupers were tested through RT-PCR (Fig. 3).

NPY mRNA levels were high in all tissues including brain, pituitary gland, the eyes, kidney, liver, gonad, muscle, stomach, pyloric caeca and intestine. CCK mRNA level was also found in all tissues, and in particular relatively stronger in the fore brain and the mid brain. LepA mRNA band was found in brain, liver, gonad and muscle, whereas LepB mRNA expression was detected in brain, the eyes, liver, muscle, pyloric caeca and foregut, of which the brain and the liver had relatively higher levels of LepB mRNA.

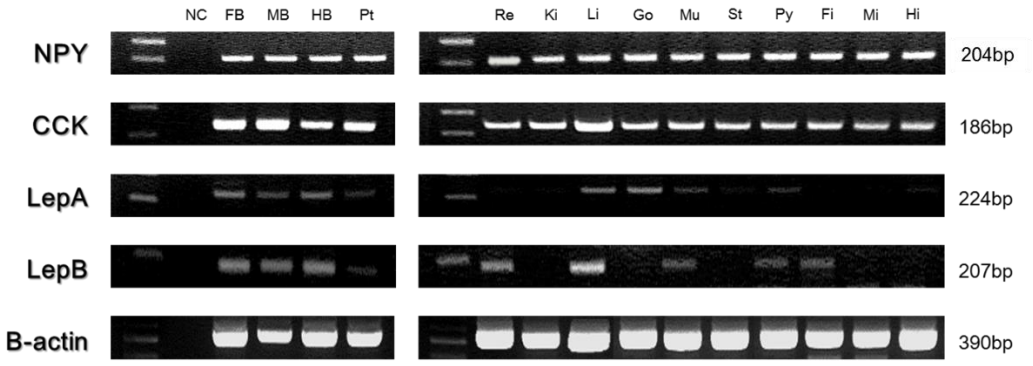


Fig. 3. Tissue distribution of red spotted groper digestive related genes.

RT-PCR analysis of NPY, CCK, LepA and LepB in different tissues as shown in a 2% agarose electrophoresis gel with ethidium bromide and 100bp molecular marker. Abbreviation: NC, negative control; FB, forebrain; MB, midbrain; HB, hindbrain; Pt, pituitary; Re, retina; Ki, kidney; Li, liver; Go, gonad; Mu, muscle; St, stomach; Py, pyloric ceca; Fi, fore-intestine; Mi, mid-intestine; Hi, hind-intestine;

## 3.3. Changes in expression of digestion-related factors depending on water temperature

### Brain (NPY, CCK, LepA and LepB)

Changes in expression level of NPY, CCK, LepA and LepB mRNA in the brain of red spotted groupers were investigated (Fig. 4, Table 3.).

In the brain, the highest NPY mRNA level was found at -3 h when it was 3 h before feeding in all experimental groups. On the other hand, the highest values of NPY mRNA level were found at 25℃ throughout all time points, which was statistically significant ($p < 0.05$).

CCK mRNA accumulation were found to increase only after feeding at 20℃ and 25℃. However, there was difference in time point when the level started to increase, in which the level started to increase 3 h after feeding at 20℃ and 6 h after feeding at 25℃. When CCK mRNA level was compared by experimental group, its levels, like NPY mRNA, were significantly higher at 25℃ at all-time points ($p < 0.05$).

LepA mRNA level increased only at 25℃ after feeding. When expression value was compared between experimental groups, NC group had a higher value at +0 h, whereas the group at 25℃ tended to become higher at -3 h, though there was no significant difference. For LepB mRNA, all experimental groups had no significant difference in expression level, and between experimental groups.

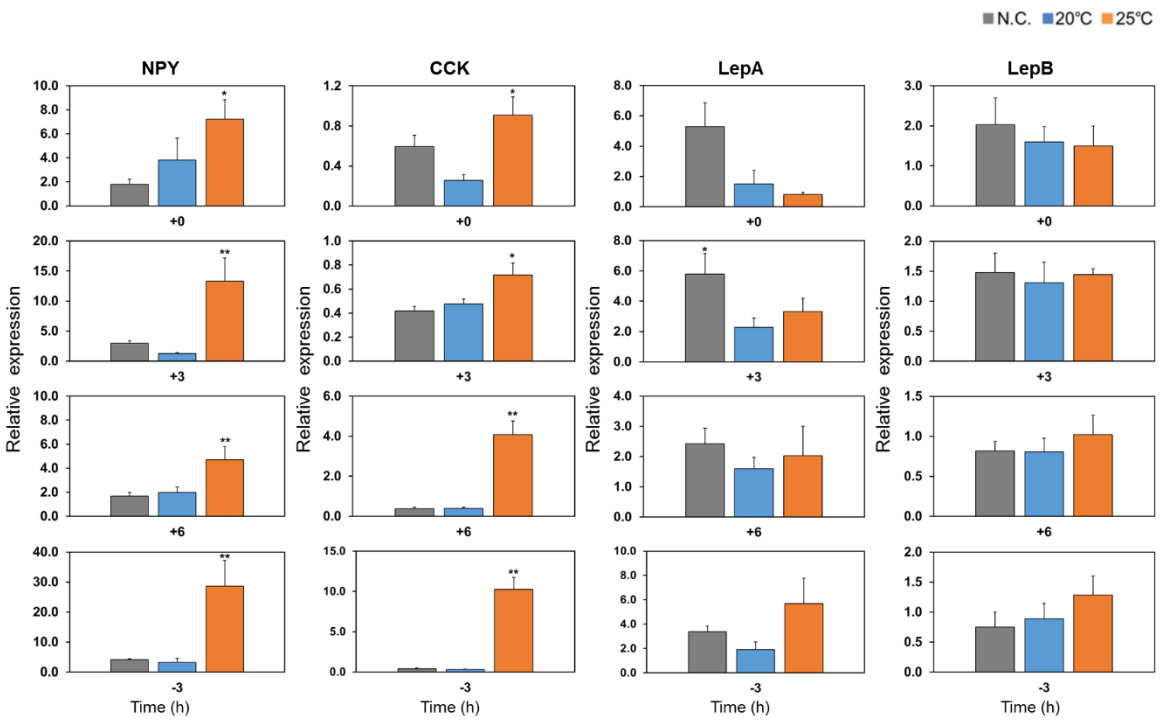


Fig. 4. Relative expression of digestive related genes in brain.

Relative mRNA expression was quantified using real-time PCR and normalized against B-actin as a housekeeping gene. All values represent the mean ± S.E.M. (n=6)

### Liver (LepA, LepB)

Changes in expression level of LepA mRNA and LepB mRNA in the liver of red spotted groupers were investigated (Fig. 5, Table 3.).

In the liver, LepA mRNA level significantly increased only at 20℃ after feeding, whereas there was no significant difference between experimental groups at all time points.

For LepB mRNA, all three groups showed different expression patterns. At 20℃, the level tended to decrease after feeding, whereas it increased at 25℃ after feeding.

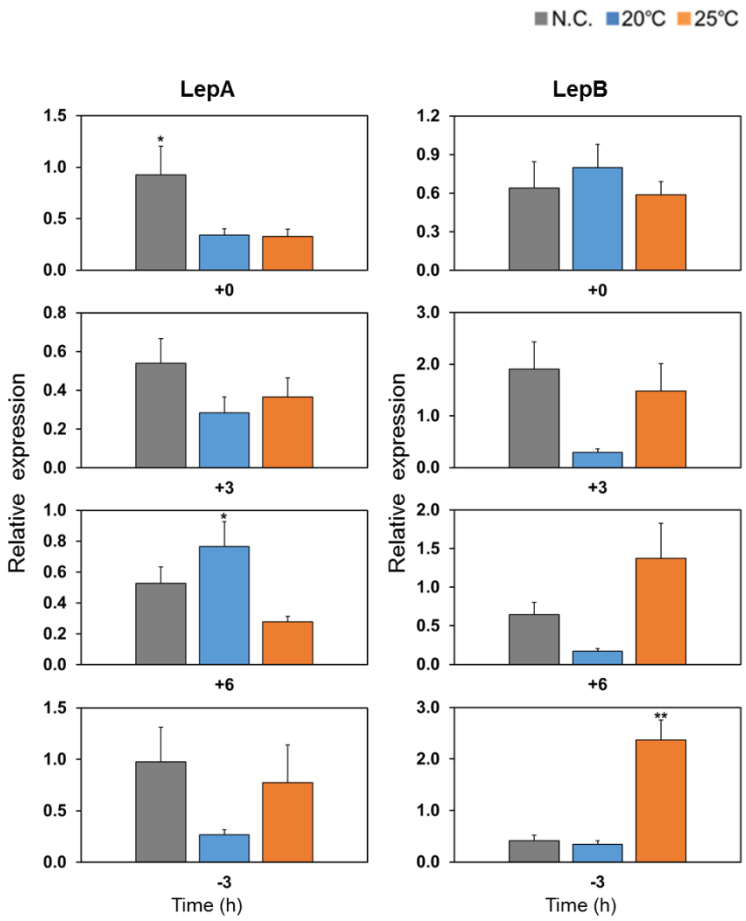


Fig. 5. Relative expression of leptin genes in Liver.

Relative mRNA expression was quantified using real-time PCR and normalized against B-actin as a housekeeping gene. All values represent the mean ± S.E.M. (n=6)

## Intestine (CCK)

When CCK mRNA expression levels were tested in the midgut, all three groups showed no significant difference by time point. While there was no significant difference at 25℃ by time point, the group at 25℃ was found to have significantly higher expression levels ($p<0.01$) at all time points than other groups (Fig. 6, Table 3.).

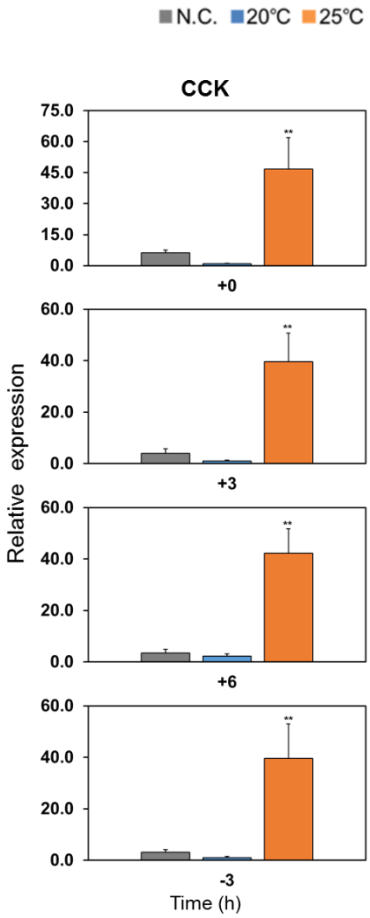


Fig. 6. Relative expression of CCK mRNA in Intestine.

Relative mRNA expression was quantified using real-time PCR and normalized against B-actin as a housekeeping gene. All values represent the mean ± S.E.M. (n=6)

**Table 3** Digestive related gene epxression according to the water temeprature and feeding time

| Tissue | Gene | Water teperature | Gene expression | | | |
|---|---|---|---|---|---|---|
| | | | +0h | +3h | +6h | -3h |
| Brain | NPY | N.C. | 1.8±0.4 | 2.9±0.4 | 1.7±0.3 | 4.1±0.3* |
| | | 20℃ | 3.8±1.8 | 1.2±0.2 | 2.0±0.5 | 3.2±1.3 |
| | | 25℃ | 7.2±1.6 | 13.3±3.9 | 4.7±1.1 | 28.6±8.7* |
| | CCK | N.C. | 0.6±0.1 | 0.4±0.0 | 0.4±0.1 | 0.4±0.1 |
| | | 20℃ | 0.3±0.1 | 0.5±0.0* | 0.4±0.1 | 0.3±0.0 |
| | | 25℃ | 0.9±0.2 | 0.7±0.2 | 4.1±0.7* | 10.3±1.5** |
| | LepA | N.C. | 5.3±1.6 | 5.8±1.3 | 2.4±0.5 | 3.4±0.5 |
| | | 20℃ | 1.5±0.9 | 2.3±0.6* | 1.6±0.4 | 1.9±0.6 |
| | | 25℃ | 0.8±0.1 | 3.3±0.9 | 2.0±1.0 | 5.7±2.1 |
| | LepB | N.C. | 2.0±0.7 | 1.5±0.3 | 0.8±0.1 | 0.8±0.3 |
| | | 20℃ | 1.6±0.4 | 1.3±0.3 | 0.8±0.2 | 0.9±0.3 |
| | | 25℃ | 1.5±0.5 | 1.4±0.1 | 1.0±0.2 | 1.3±0.3 |
| Liver | Lep-a | N.C. | 0.9±0.3 | 0.5±0.1 | 0.5±0.1 | 1.0±0.3 |
| | | 20℃ | 0.3±0.1 | 0.3±0.1 | 0.8±0.2* | 0.3±0.0 |
| | | 25℃ | 0.3±0.1 | 0.4±0.1 | 0.3±0.0 | 0.8±0.4 |
| | Lep-b | N.C. | 0.6±0.2 | 1.9±0.5 | 0.6±0.2 | 0.4±0.1* |
| | | 20℃ | 0.8±0.2* | 0.3±0.1 | 0.2±0.0 | 0.3±0.1 |
| | | 25℃ | 0.6±0.1 | 1.5±0.5 | 1.4±0.5 | 2.4±0.4 |
| Intestine | CCK | N.C. | 6.2±1.3 | 4.0±1.8 | 3.5±1.4 | 3.0±1.2 |
| | | 20℃ | 0.9±0.2 | 1.1±0.4 | 2.2±0.9 | 1.1±0.4 |
| | | 25℃ | 46.7±15.3 | 39.7±11.1 | 42.2±9.5 | 39.5±13.5 |

NPY: Neuropeptide Y; CCK: Cholecystokinin; LepA: Leptin-a; LepB: Leptin-b; N.C.: Natural conditon
* 'significance level: p <0.05; ** p <0.01 (ANOVA).

# 4. Discussion

## 4.1. Histological characteristics in gastrointestinal tract depending on water temperature

As fish is poikilothermal animals, fish metabolism rate is determined by water temperature. So, water temperature is a major environmental factor that regulates food intake and digestion (Cyrino, 2008). Studies on feeding activity depending on water temperature were performed for other fishes. Rainbow trout had a higher food intake as water temperature became higher between 6-15℃ (Azevedo et al., 1998), and Atlantic salmon had the highest feed intake amount at 14℃ between 6-18℃ of water temperature, while intake amount decreased at 18℃ (Handeland et al., 2008). In addition, grass carp had a higher feed intake amount as water temperature became higher between 12.8-29.4℃ (Kilambi and Robison, 1979). In the present study, when red spotted groupers were reared in various water temperatures (natural water temperature, 20℃ and 25℃), the highest feed intake amount was found at 25℃. In addition, previous studies had the highest intake at 28℃ when reared between 20-28℃, so that these indicate that feed intake amount of red spotted groupers should increase as water temperature becomes higher within a certain range. However, it is considered necessary to further study on threshold water temperature that feed intake and metabolism start to be suppressed.

It is known that goblet cells that secret mucosubstances play critical roles in nutrient uptake of vertebrates including fishes (Osman and Caceci, 1991;

Domeneghini et al., 2005), and protect mucous from injury by physiological and chemical materials (Allen et al., 2009).

This study found that goblet cells were affected by water temperature. Goblet cells in the gastrointestinal tract were examined histologically, which found the highest number of goblet cells at 25℃, corresponding to the highest water temperature among the three groups. In addition to goblet cells, the group at 25℃ also had significantly longer villi, which was speculated to be related with feed intake amount. It seems that villi development was promoted for effective digestion and uptake as feed amount increased, and number of goblet cells increased to protect the gastrointestinal tract from ingested feeds, playing the roles as a lubricant.

## 4.2. Gene expression in tissues

Neuropeptide Y (NPY), cholecystokinin (CCK), LepA and LepB mRNA levels were examined in different tissues, which found different expression patterns in four genes.

NPY mRNA showed a high expression levels in all tissues, which was similar to that of Brazilian flounders (Campos et al., 2010). NPY mRNA showed various accumulation patterns in tissues depending on species. Blunt snout breams had higher expression levels only in the brain and the pituitary gland, whereas other tissues showed either no or low expression values (Ji et al., 2015). All tissues of winter skate showed expression, while the brain had a relatively higher level (MacDonald and Volkoff, 2009). Atlantic cod had a higher expression level in the

endbrain and the kidney (Kehoe and Volkoff, 2007). These results indicate that NPY mRNA show different tissue-specific expression patterns depending on species.

In this study, CCK mRNA expression was detected in most tissues, and particularly higher in the brain. This result was similar to dourado that had a higher expression in all tissue including brain (Volkoff and Cyrino, 2016). And in *Schizothorax prenanti*, the CCK mRNA was highly expressed in brain and intestine (Yuan et al., 2014).

LepA mRNA level was relatively higher in the brain, the pituitary gland, the liver and the gonad, whereas LepB mRNA level was relatively higher in the brain and the liver. *Schizothorax prenanti* had high expression level in liver (Yuan et al., 2014), orange spotted grouper had high expression level of LepA mRNA in cerebellum and liver and had high expression level of LepB mRNA in brain and ovary (Zhang et al., 2013).

## 4.3. Changes in gene expression depending on water temperature

In this study, we investigated the impact of rearing water temperature on changes of digestive related genes expression in red spotted grouper.

NPY mRNA, an appetite inducing factor, plays a role in promotion of appetite in fishes (Volkoff, 2006). In this study, all experimental groups showed elevation of NPY mRNA level at 3 h before feeding. However, comparison of expression levels between experimental groups found that the group at 25℃ of water temperature had significantly higher expressions at all time points. These results indicate that a high

water temperature induces appetite of red spotted groupers. For Atlantic cods, NPY mRNA level was found to be unaffected by water temperature, which was explained that Atlantic cods live in a wide range of water temperature in nature (Kehoe and Volkoff, 2008). In contrast, red spotted grouper is a semitropical fish species that lives in water with a higher temperature, so that it seems that a higher water temperature induces appetite and induces NPY expression.

It is known that CCK mRNA expression suppresses appetite in the brain, and increases after feeding. In other fishes, CCK mRNA level also increased after feeding (Volkoff, 2006). But, in part of fish like cavefish, there was not changes of CCK mRNA after feeding (Wall and Volkoff, 2013). In this study, CCK mRNA expression increased in groups at 20℃ and 25℃ of water temperature after feeding, and the level reached to the peak at 20℃ at +3 h, while it started to be elevated from +3 h in the 25℃ group continued to -3 h on the next day, This result seems that increased food intake by the 25℃ WT made the time of digestion longer and for that reason, the CCK mRNA expression continued to -3 h. Nevertheless, it seems to be necessary to be further studied. When CCK mRNA expression levels in the brain were compared between experimental groups, the 25℃ group had significantly higher expression levels at all time points. Nevertheless, it is unable to be concluded as appetite suppression at 25℃, because CCK mRNA expression level was markedly lower than that of NPY mRNA. Such expression patterns were also similar in the midgut, where the 25℃ group had significantly higher levels of CCK mRNA at all time points than other groups though there was no significant difference in the midgut between groups by time point. Thus, it can be interpreted that such a high

expression of CCK mRNA at 25℃ was attributable to activation of digestive activities (gastric empting and promotion of secretion for digestive enzyme as well as gallbladder contraction) rather than suppression of appetite.

While leptin generally plays a role in suppression of appetite in mammals (Ahima and Flier, 2000), it often has different roles in fishes depending on species. In general, fishes have paralogous leptin genes including LepA and LepB (Jaillon et al., 2004; Volff, 2005). Consistently, red spotted groupers also have two types of leptin genes, LepA and LepB, and orange spotted groupers also have same genes, of which LepA gene has the main function in this fish species (Zhang, 2013). LepA mRNA expression level increased in the brain only at 25℃ after feeding, while there was no significant difference in other groups by time point. However, LepA mRNA level became higher in the 25℃ group after feeding, and also tended to increase in the liver at 20℃ and 25℃ after feeding. These results suggest that LepA gene should have a role in suppression of appetite in red spotted groupers. Similarly, orange spotted groupers also showed elevation of LepA gene at 9 h after feeding (Zhang, 2013). However, comparisons of differences in expression values of LepA mRNA in the brain and the midgut between all experimental groups found no significant difference. Therefore, in red spotted grouper, the LepA mRNA confers satiety but, isn't affected by water temperature.

There was no difference in expression value for LepB mRNA expression in the brain by time point and also no difference between groups. In contrast, liver had different expression pattern in the three groups by time point, whereas there was no significant difference among experimental groups. These results suggest that LepA

gene functions as a main gene in red spotted groupers.

In conclusion, red spotted groupers induce expression of NPY, an appetite inducing factor, as water temperature becomes higher, which is followed by elevation of feed intake amount, subsequently resulting in elevation of CCK, a digestive activity factor. These results indicate that red spotted groupers have physiological characteristics for adaption to semitropical environments.

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# 국문요약

양식 어류의 성장은 다양한 환경 요인에 의해서 조절된다. 이 중에서도 사육수온은 어류의 성장과 먹이 활동에 많은 영향을 준다. 이전 연구에서 붉바리 종묘(110일)를 수온(자연수온, 20℃, 24℃, 28℃)에서 사육하였을 때, 수온(24℃, 28℃)에서 성장뿐 아니라 사료 효율 또한 높은 것을 확인할 수 있었다. 따라서 이번 연구는 사육중인 붉바리 종묘를 대상으로 사육수온조건에 따른 붉바리의 소화활성 양상을 탐색하여 성장과의 관련성을 보고자 했다.

연구는 붉바리 치어를 4개의 그룹(Natural condition group; 15±1.0℃, group1; 20±0.5℃, group2; 25±0.5℃)으로 나눠 2주간 순치 사육했다. 사료는 매일 오전 11시에 1일 1회 각 수조별 만복 급이를 실시했다. 2주 뒤 사료 공급 직후(+0), 밥 먹은 3시간 후 (+3), 6시간 후(+6), 다음날 사료 공급 3시간 전(-3) 이렇게 4번의 샘플링을 실시하였다. 먼저 조직학적 분석을 통해 점액분비세포인 배상세포의 활성을 조사하였다. 다음으로 소화호르몬인 Cholecystokinin (CCK)와 Leptin, 식욕 조절 인자인 Neuropeptide Y (NPY) mRNA의 발현 양상을 real-time PCR을 통해서 확인하였다.

조직학적 분석 결과, 평균 장의 융모 길이의 경우 NC (220.92±14.80 μm), 20℃ (247.08±23.09 μm), 25℃ (356.66±12.71 μm)로 25℃가 가장 길었다. 장내 배상세포의 개수를 확인한 결과 NC (462.3±67.8), 20℃ (461±91.2) and 25℃

(766±144.3)로 25℃의 개수가 가장 많았다. 유전자 발현의 결과를 보면, Leptin mRNA은 실험구 간의 유의적인 차이가 없었다. 하지만, NPY와 CCK mRNA의 경우 다른 그룹들에 비해 25℃에서 유의적으로 발현 값이 높았다.

이 결과들을 토대로 볼 때, 붉바리 사육에 있어서 수온은 먹이 활동과 소화활성에 영향을 미친다는 것을 알 수 있다. 또한 성장이 높았던 사육 수온인 24℃와 28℃에서처럼 25℃에서 소화활성이 높았던 것으로 보아, 소화활성이 성장에 영향을 미친 것으로 사료되며, 이러한 결과는 붉바리는 아열대성 적응생리특성을 가지는 것을 의미한다.