Abstract
It takes quite a long time for the grouper to spawn. In the case of red spotted grouper (Epinephelus akaara), at least three to four years of rearing is usually required to reproduce them for the first time. Reproductive control techniques can be applied to repress, delay or advance the onset of puberty. Thus, they can be used to accelerate the process of selective breeding in this species. The present study investigated whether alterations of rearing water temperature (WT) can advance the onset of puberty in the red spotted grouper. Juvenile red spotted grouper (110 DAH, 7.25 ± 0.5 cm, 6.45 ± 1.5 g) were randomly divided into 4 groups and reared for approximately 10 months (from Nov. 2014 to Aug. 2015) at four different WT: natural condition (12.6-19.5°C), 20 ± 0.5°C, 24 ± 0.5°C and 28 ± 0.5°C treatment. When they were reared at 24 ± 0.5°C or 28 ± 0.5°C WT, sexually mature individuals appeared within 12 months after hatching during their breeding season (Jul. to Aug.). The mRNA levels of reproduction-related genes such as Kisspeptin, GnRH, FSHβ and LHβ were higher in the 24 ± 0.5°C and 28 ± 0.5°C treatment group than the other groups (P<0.05). Mature yolk stage oocytes (≥300 μm diameter) were found in the ovaries of female red spotted grouper reared at 24 ± 0.5°C or 28 ± 0.5°C groups, while only oogonia were found at natural condition and peri-nucleolus stage oocytes were observed at 20 ± 0.5°C group, respectively. The one-year-old mature females ovulated 6-10 ml of eggs that corresponded to 10% of their body weight. In artificial fertilization performed at 24 ± 0.5°C WT, the fertilization and hatching rates were determined to be 95% and 97%, respectively. This is the first report demonstrating that rearing at 24 or 28°C WT can significantly advance the onset of puberty in the red spotted grouper.

Keywords: Puberty; Red spotted grouper; Epinephelus akaara; Water temperature.

Introduction
The puberty of fish is the period when the capacity to reproduce gain for the first time in their lives after sexual differentiation [1,2], which makes the control of puberty critically important in aquaculture industry [3]. The puberty of fish is mainly associated with the activities of the brain-pituitary-gonadal axis (B-P-G axis) with the age and somatic growth [4,5]. In fish, the process of gametogenesis is activated or repressed by physiological factors and environmental factors. The most common physiological factors are nutrition, stress, and immunity; while the environmental factors include light, water temperature, water pressure, and tide.

Light and water temperature are the representative environmental factors involved in fish gametogenesis, playing an especially crucial role in the puberty of various farmed fish species including Atlantic cod [6]. Apart from the species whose onset of puberty is mainly influenced by the photoperiod, such as Eurasian perch and yellow perch [7,8], there are fish species whose puberty is most essentially influenced by water temperature, including Atlantic halibut (6 to 9°C) and grass carp (24°C) [9,10].

Such environmental factors stimulate the receptors in each organ and are summed up in the brain that lead to the activation of B-P-G axis. Previous studies on the puberty and sex maturation of seabream [11] and longtooth grouper [12] mostly focused on the activities of Gonadotropin-Releasing Hormones (GnRH) and Gonadotropic Hormones (GtHs). However, recent studies in fish (mackerels) have reported Kisspeptin (Kiss) and its receptor, G-protein receptor54 (GPR54), as the upstream factors that facilitate the secretion of GnRH [13]. There has been a recent investigation on the roles played by Kiss and Gpr54 in sex maturation during blacktip grouper [14].

Most grouper species inhabit subtropical and tropical regions. The red spotted grouper (Epinephelus akaara) is found in China, Korea and southern Japan. Spawning period of E. akaara that inhabits the coasts of Jeju Island in Korea is between July and August (summer breeder). The sex characteristic of red spotted grouper is a protogynous hermaphrodite and sex maturation occur three years after sexual differentiation into female, some of which (longer than 26 cm; over 5-year-old) reverses the sex to male [15-18]. The red spotted grouper is commercially important species in Asia, but it is currently classified as an endangered species by the Food and Agriculture Organization (FAO) and the International Union for Conservation of Nature [19,20].

Thus, this study, we induced puberty of red spotted grouper that require a long period of time to each puberty, by controlling the rearing water temperature. During inducing puberty, the study also comparatively analyzed the gonad development and the expression patterns of Kiss, Gpr54, GnRH, and GtHs, which are essential in exploring the expression mechanisms of reproduction-related genes during puberty.

Materials and Methods
Experimental fish and breeding management
Juvenile red spotted grouper (110 days after hatching; 7.25 ± 0.5 cm; 6.45 ± 1.5 g) seed-produced at the Marine Science Institute of Jeju Island in Korea is between July and August (summer breeder). The sex to male [15-18]. The red spotted grouper is commercially important species in Asia, but it is currently classified as an endangered species by the Food and Agriculture Organization (FAO) and the International Union for Conservation of Nature [19,20].

Thus, this study, we induced puberty of red spotted grouper that require a long period of time to each puberty, by controlling the rearing water temperature. During inducing puberty, the study also comparatively analyzed the gonad development and the expression patterns of Kiss, Gpr54, GnRH, and GtHs, which are essential in exploring the expression mechanisms of reproduction-related genes during puberty.

Materials and Methods
Experimental fish and breeding management
Juvenile red spotted grouper (110 days after hatching; 7.25 ± 0.5 cm; 6.45 ± 1.5 g) seed-produced at the Marine Science Institute of Jeju
National University was used as the experimental fish. The juvenile red spotted grouper used in the experiments had been seed-produced in July 2014, under the condition of 14L:10D photoperiod and 21.0 ± 1.0°C rearing WT control, from the adult red spotted grouper reared at the Marine Science Institute. For the induction of puberty in red spotted grouper through the rearing WT control, two experimental plots were devised: the natural condition WT (NC) and the 20 ± 0.5°C, 24 ± 0.5°C, and 28 ± 0.5°C treatment groups, each group containing 600 fish. The water temperature was controlled using a heat pump. Six tone water tanks (3 m × 85 cm) was used for rearing the fish based on running water culture, with the circulating flow rate at each tank being 15-17 L/min. The experimental duration lasted approx. 8 months from October 2014 to July 2015. The photoperiod during this time was treated as equivalent to the period of natural light. Other conditions were dissolved oxygen (DO) 8 ± 1 mg/L; salinity 30 ± 2 ppt; pH 8.0 ± 0.1; feed was provided twice a day.

**Dissection and histological examination of the experimental fish**

To examine the activities of B-P-G axis and the steps of gonad development, the red spotted grouper was randomly selected by 10-15 fish from each tank during their spawning period in July. The length and weight of the fish were measured after they were anesthetized using 0.01% 2-penoxethanol, and the gonads were stored at -80°C before removing the brain including the pituitary gland for analysis. To compare the gonadosomatic index (GSI) and gonad development between the experimental groups, the gonads of the experimental fish were removed, weighed, and fixed using Bouin’s solution for histological analysis. The tissue samples were cut to 5-7 µm thickness using a microtome (Olympus BX53, Japan) was used for inspection.

**Total RNA extraction and cDNA synthesis**

For total RNA extraction, the brain and gonad tissues obtained from each experimental group were placed in a 1.7 ml tube for centrifugation at 12,000x g for 15 min, after which the supernatant was transferred to a fresh tube for the reaction with the added 500 µl iso-propanol at room temperature. The RNA was precipitated by centrifugation at 4°C and at 12,000 x g for 10 min; the supernatant was removed; the precipitated RNA was diluted and washed by H2O treated with diethylpyrocarbonate (DEPC) and the 75% and 95% Ethanol, before the dissolution in DEPC H2O for extracting the total RNA. For the quantitative and qualitative analyses of total RNA, a spectrophotometer (Nanovue) was used for the measurements, and the sample exhibiting the purity of 1.7-2.1 for A260/A280 nm ratio was chosen and confirmed via electrophoresis.

For cDNA synthesis, the total RNA extracted from the brain tissue was used as template. Using the RQ1 RNase-Free DNase Kit (Promega, USA), the RNA was treated with DNase, then cDNA was synthesized using the PrimeScript™ 1 strand cDNA synthesis Kit (Takara, Japan). The DNase-treated RNA was made to 8.0 µl with RNase-free H2O, and after adding 1.0 µl Random 6mers and 1.0 µl dNTP mixture, the reaction was left at 65°C for 5 min. To this, 4.0 µl 5X PrimeCrtpt Buffer, 0.5 µl RNase inhibitor; 10 µl PrimeCrtpt RTase; 4.5 µl RNase free dH2O, were added and the total volume was made to 20 µl. The reaction proceeded at 30°C for 10 min; at 42°C for 60 min; at 95°C for 5 min, for enzymatic activation and completion of synthesis.

**Expression patterns of the reproduction-related genes (Quantitative RT-PCR)**

For the primer of reproduction-related genes (Kiss, GnRH, GtHs), the sequences registered at the National Center for Biotechnology Information (NCBI) were used for the design and subsequent use (Table 1). To investigate the expression patterns of reproduction-related genes, Quantitative Real-time (RT-) PCR was used for the quantitative analysis. For this, the BioRad CFX96” Touch” Real Time PCR (BioRad, Hercules, CA) and the EvaGreen 2X qPCR MasterMix-RoxKit (abm, Canada) were used. To 2.0 µl of cDNA template, 5.0 µl EvaGreen 2X qPCR MasterMix; 0.3 µl forward primers; 0.3 µl reverse primers; 2.4 µl RNasefree H2O, were added and the total volume was made to 10 µl. Next, 40 cycles of denaturation (45s, 94°C), annealing (45s, 58°C), and extension (1m, 72°C), were carried out for amplification. The procedure was repeated twice for each sample, and B-actin was used for the relative quantification, from which the average experimental values were obtained and subsequently used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer information</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiss1</td>
<td>Kiss1 F</td>
<td>TGCACGACTCATTGTGTC</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>Kiss1 R</td>
<td>AGCTCAACATCTCGGAGAC</td>
<td>166</td>
</tr>
<tr>
<td>Kiss2</td>
<td>Kiss2 F</td>
<td>GGGCCTGATGTTGGGACCA</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Kiss2 R</td>
<td>CTCCAGTCGGAGAACAC</td>
<td></td>
</tr>
<tr>
<td>GPR54</td>
<td>GPR54 F</td>
<td>TCTCCACGAGGATCTGTG</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>GPR54 R</td>
<td>GACCCAACTCAAAGATG</td>
<td></td>
</tr>
<tr>
<td>sbGnRH</td>
<td>sbGnRH F</td>
<td>ACTGTCTGCGTTGGGAG</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>sbGnRH R</td>
<td>TTGCGAAAAGGTGTTGGC</td>
<td></td>
</tr>
<tr>
<td>FSHβ</td>
<td>FSHβ F</td>
<td>AGCTGAGACCTCGAGAC</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>FSHβ R</td>
<td>AGTTCCTGGCCACAGGAG</td>
<td></td>
</tr>
<tr>
<td>LHβ</td>
<td>LHβ F</td>
<td>TACAGGCTCCAGGAGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LHβ R</td>
<td>CTCGCAAGGCTCCAGGAG</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>β-actin F</td>
<td>GAGCCTGGACTCCTTCAC</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>β-actin R</td>
<td>AGGAAGGAGGCGTGGAGAG</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Primer set used for real time quantitative PCR.
Artificial fertilization

In August 2015, the 1-year-old red spotted grouper fish whose puberty had been induced under 28 ± 0.5°C condition, were selected. For sperm production and egg ovulation, the abdominal compression method was used, and artificial fertilization was performed through wet process using 24 ± 0.5°C filtered sea water. The rates of fertilization and hatching were estimated under the 24 ± 0.5°C rearing WT condition. The fertilized eggs displayed the characteristics of floating eggs, and the fertilization rate of red spotted grouper was determined by the proportion of the eggs that floated following the artificial fertilization. The hatching occurred after 48-hour incubation under the 24 ± 0.5°C rearing WT condition, and the hatching rate was determined by placing 500 washed eggs in a 1000 ml beaker and counting the larvae and spawned eggs.

Statistical analysis

SPSS version 21 was used for statistical analysis, for applying One-Way analysis of variance (ANOVA), and the Duncan’s multiple range test (Duncan, 1955) to validate the significance. The values were expressed in mean ± standard deviation, and the significance of difference was accepted at $P < 0.05$.

Result

Change of growth and gonadosomatic index

After 8 months of water temperature (WT) manipulation, we have measurement of experimental fish total length (TL) and body weight (BW). At the initiation of an experiment (110 days after hatching), juvenile red spotted grouper TL and BW were no different between control and treatment groups. At the end of the experiment, TL and BW of 1-year-old red spotted grouper in the natural condition were 12.2 ± 1.0 cm and 28.5 ± 11.5 g, respectively. The BW were 54.3 ± 15.0 g, 89.0 ± 24 g and 131.4 ± 39 g, respectively (Table 2 and Figure 1). The TL and BW were tended to increase as the rearing water temperature increased ($P < 0.05$).

At the end of the experiment, both GSI of 1-year-old red spotted grouper in the natural condition and 20 ± 0.5°C treatment group were 0.003 ± 0.001. But GSI of 24 ± 0.5°C and 28 ± 0.5°C treatment groups were 1.34 ± 0.48 and 3.04 ± 1.79, respectively (Figure 2). Their GSI were shown to have developed further compared to the natural condition and 20 ± 0.5°C group.

Gonadal development for each WT treatment groups

After 8 months of WT manipulation, the gonad development in each WT group was comparatively analyzed through histological analysis. The ovaries of 1-year-old red spotted grouper from the natural condition and 20 ± 0.5°C treatment group were immature, mainly contained oogonia stage oocytes of 12 to 17 µm in diameter and perinucleolus stage oocytes of 30 to 50 µm diameter, respectively (Figures 3A and 3B). But, in the 24 ± 0.5°C and 28 ± 0.5°C treatment groups, the ovaries become mature as vitellogenic oocytes of 320 to 350 µm diameter (Figures 3C and 3D), with 33.3% and 53.3% mature individuals in each group (Figure 4).

The mRNA expression patterns of the reproduction-related genes

To explore the activities of B-P-G axis according to the rearing WT manipulation, mRNA expression patterns of the reproduction-related genes from whole brain including the pituitary gland were investigated. The expressions of Kisspeptin1, 2 mRNAs did not show significant difference between each WT treatment groups. In the case of Kisspeptin1, relatively higher expression was observed from the 28 ± 0.5°C treatment group compared to other groups (Figure 5A). In the case of Kisspeptin2, relatively higher expression was observed from 24
Figure 2: Gonadosomatic index of the female red spotted grouper under each other rearing water temperatures. Means represent by different letters are significant (p<0.05). NC: Natural condition.

Figure 3: Development of red spotted grouper gonad by histological analysis in different rearing water temperatures (n= 5; 5; 10; 10). (A) The fish reared in natural condition; (B) An immature fish reared in 20 ± 0.5°C treatment group; (C) A few mature fish reared in 24 ± 0.5°C treatment group; (D) A mature fish reared in 28 ± 0.5°C treatment group. Oo, oogonia; PNS, peri-nucleolus stage; ODS, oil droplet stage; Vo, vitellogenic oocytes. Scale bars indicate 20 μm (A and B) and 100 μm (C and D).

± 0.5°C and 28 ± 0.5°C treatment group than the other groups (Figure 5B). The expression of Gpr54 (kiss1r) mRNA was the highest in the 28 ± 0.5°C treatment group, showing a similar trend to Kisspeptin1 mRNA expression (Figure 5C). The expression of GnRH (seabream type) mRNA did not show significant difference between the natural condition and the 20 ± 0.5°C treatment group, but expressions in the 24 ± 0.5°C and 28 ± 0.5°C groups were significantly higher than the natural condition (Figure 5D, P<0.05). The expression of GtHs (FSHβ, LHβ) mRNA was higher in the 24 ± 0.5°C and 28 ± 0.5°C treatment group than the other groups (Figures 5E and 5F, P<0.05).

Fertilized egg production of 1-year-old red spotted grouper fish in puberty

In August 2015, a male (19.7 ± 1.8 cm TL, 110.8 ± 40.4 g BW) and a female (18.6 ± 0.3 cm TL, 131.0 ± 40.2 g BW) red spotted grouper reared in 28 ± 0.5°C group until they became a year old, were selected. Using the abdominal compression method, their sperms and eggs were collected. The amount of eggs was 6 – 13 ml, which corresponded to
approx. 10% of the body weight of fish. For the artificial fertilization, the fertilization rate was 95% and the hatching rate was 97% (Table 3).

**Discussion**

The puberty of fish has been shown to bear correlation with age, growth, and the activities of B-P-G axis which is the reproductive endocrine axis. The activities of B-P-G axis are in turn affected by light, WT, weight, and feed [3]. The puberty of sea bass and Nile tilapia could be induced through the increased expressions of the reproduction-related genes by photoperiod control [2,21], and the puberty of pikeperch could be induced under the conditions of natural photoperiod and 14°C rearing WT [22]. The puberty of longtooth grouper and dusky grouper were shown to be correlated with weight [12,23], and that of various other farmed fish were shown to be correlated with factors such as age and growth [3]. As such, puberty in fish is induced when the reproductive endocrine axis of fish receives the signals from environmental and physiological factors, which initiates the expressions of the reproduction-related genes (Kiss, Gpr54, GnRH, GtH) and the maturation of gonad.

Among the reproduction-related genes of fish, Kisspeptin and Gpr54 are known to play a crucial role in puberty [21,24]. Increased Gpr54 expression was observed at the onset of puberty in Nile tilapia [21], and mammals (rat, mouse, sheep) were also shown to induce GnRH secretion through Kisspeptine expression at the onset of puberty [25]. GnRH induced the GtHs secretion and it was influenced by the increase in Gpr54 expression [26,27], while the GnRH mRNA production increased with the onset of puberty [21,28]. Several different types of GnRH exist, and the gonad development in sea basses or basses are often influenced by the sea bream type GnRH expression [29,30].

GtHs is another key factor in puberty [1,2]. In Chinook salmon fish, the expressions of FSHβ and LHβ increased during puberty [31], while in African catfish and European sea bass, the level of LHβ expression also increased during puberty [32]. The present study, likewise, where the puberty of red spotted grouper was induced, showed a gradually increasing trend in the mRNA expressions of Kiss1, 2 with the increase in rearing WT, although no significant between-group differences were found. Also, the mRNA expressions of Gpr54, GnRH (sb), and GtHs, were relatively high in high WT groups than in low WT groups with statistical significance. When the gonad development

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total length (cm)</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td>N.C.</td>
<td>7.5 ± 0.7a</td>
<td>6.80 ± 1.6a</td>
</tr>
<tr>
<td>20 ± 0.5°C</td>
<td>7.1 ± 0.0b</td>
<td>6.05 ± 1.5b</td>
</tr>
<tr>
<td>24 ± 0.5°C</td>
<td>7.2 ± 0.0c</td>
<td>6.42 ± 1.6c</td>
</tr>
<tr>
<td>28 ± 0.5°C</td>
<td>7.2 ± 0.5d</td>
<td>6.16 ± 1.1d</td>
</tr>
</tbody>
</table>

Means represented by different letters are significant (P<0.05).

**Table 2**: Change of red spotted grouper growth in different rearing water temperature.

<table>
<thead>
<tr>
<th>Number of fish</th>
<th>Total length (cm)</th>
<th>Body weight (g)</th>
<th>Ovulated egg (mL)</th>
<th>Floated egg (mL)</th>
<th>Fertilization rate (%)</th>
<th>Hatching rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>18.6 ± 0.3</td>
<td>131.0 ± 40.2</td>
<td>11.8 ± 3.5</td>
<td>11.2 ± 3.3</td>
<td>95</td>
<td>97</td>
</tr>
</tbody>
</table>

**Table 3**: Induced ovulation and fertilization of 1-year-old red spotted grouper.

**Figure 4**: Matured fish rate under each other rearing water temperatures by histological analysis (n=10; 10; 15; 15). NC: Natural condition.
of red spotted grouper was observed through histological analysis, the gonad was not shown to have developed in the natural condition and 20 ± 0.5°C groups, whereas in the 24 ± 0.5°C and 28 ± 0.5°C treatment groups, mature individuals were observed mostly with mature oocytes. Growth hormone (GH) induces the synthesis of Igf1, 2 in the liver [33]. In rainbow trout, WT affects promote growth through Igf1 secretion by the liver following GH stimulation [34], in Atlantic salmon, these hormones were shown to be associated with growth and maturation [35].

Igf1 of Japanese eel and tilapia is known to stimulate the gonad of fish and induce the secretion of 11-Ketosteroid to influence sperm production [36,37]. Igfs expression was also found in gilthead seabream and during the early mature yolk stage of tilapia [38,39], while in goldfish, it was shown to regulate yolk formation [40]. Although the expressions of growth-related genes Igfs and GH were not investigated in this study, faster growth was exhibited by 24 ± 0.5°C and 28 ± 0.5°C WT groups compared to natural condition and 20 ± 0.5°C WT groups.

In fish, the onset of puberty begins with the activity of B-P-G axis. Water temperature and photoperiod are very important environmental factor and it can modulate the activity of B-P-G axis. The manipulation of water temperature can be used to advance or prevent the onset of puberty in some fish. For example, in the initiation puberty of pikeperch, the most efficient effect is observed at 12°C, while high (23°C) or low (6°C) temperature prevented gonadal maturation [41].

### Conclusion

In this study, the puberty of subtropical red spotted grouper may have been induced by direct and indirect influences of water temperature, with close correlation to the rearing WT. The water temperature induces changes of activity of the B-P-G axis and is considered as the most important environmental cue determining the timing of puberty in red spotted grouper. With recent reports on the receptors sensitive to light and WT, metabolism, physiology and behavior of diverse fish including zebrafish and rainbow trout [42-44] the mechanism correlating the WT-sensitive receptor and B-P-G axis should be investigated in future.

### Acknowledgement

This research was supported by Golden Seed Project, Ministry of Agriculture, Food and Rural Affairs(MAFRA), Ministry of Oceans and Fisheries(MOF), Rural Development Administration(RDA) and Korea Forest Service(KFS).

### References


