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Physiological studies for growth improvement of juvenile red-spotted grouper (*Epinephelus akaara*)

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붉바리 (*Epinephelus akaara*) 치어의 성장 개선을 위한 생리학적 연구

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By
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Physiological studies for growth improvement of juvenile red-spotted grouper (*Epinephelus akaara*)

A dissertation

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Abstract

The red-spotted grouper (*Epinephelus akaara*) is a subtropical species distributed in southern China, Korea, Taiwan, and southern Japan. Currently, this species is being developed as an export item in Korea. The rise of water temperature during the summer season is anticipated to affect the physiological functions of red-spotted grouper. Besides, *E. akaara* is a protogynous hermaphroditic fish that function as a female at a young age and undergo sex changes to become male later in life. The reproductive characteristics has slow down their growth performance and limited the availability of mature male broodstock. Hence, the present situation requires to assess the possible impacts of increased water temperature as well as to find out the effective way to accelerate the growth performance of red-spotted grouper.

In the present research, the potential effects of increased water temperature on the morphology, physiology, heat shock proteins (HSPs), growth performance, and growth-related gene expression in juvenile red-spotted grouper were analyzed. In experiment 1, 200-day-old juveniles (TL: 9.4 ± 0.12 cm; BL: 12.89 ± 0.61 g) were exposed to 24 °C (control), 28 °C, 32 °C, 36 °C for 28 days; in experiment 2, 130-day-old juveniles (TL: 8.28 ± 0.10 cm, BW: 8.53 ± 0.27 g) were exposed to 25 °C (control), 28 °C, 31 °C, 34 °C for 42 days. In experiment 1, histological results showed epithelial necrosis and shortening of secondary gill lamellae in gill; cytoplasmic vacuolization, shrinkage, and coalescence of hepatocytes in liver of the 36 °C group. Biochemical observations revealed a significant elevation of glucose, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and cortisol at 36 °C compared with the other groups (24 °C, 28 °C, and 32 °C). Highest heat shock proteins (Hsp60, 70, 90) mRNA expression was also observed in liver, gill, and muscle at 36 °C ($P < 0.05$). In experiment 2, hematological results showed high red blood cell (RBC) and white blood cell (WBC) count, and erythrocytic abnormalities in shape and size (ECA) at 31 °C and 34 °C water temperature. Histological results presented hyperplasia and curling in secondary gill lamellae, and swelling of primary gill lamellae in gill; the coalescence of hepatocytes, cytoplasmic vacuolization,

and dilation of the sinusoid in liver of the 34 °C group. Biochemical results showed high levels of glucose, cortisol, GPT, GOT, and lactate dehydrogenase (LDH); low levels of triglyceride (TG) and total cholesterol (TCHO) in the 34 °C group compared with the others (25 °C, 28 °C, and 31 °C) ($P < 0.05$). Heat shock proteins (Hsp60, 70, 90 mRNA) were also highly expressed in liver, gill, and muscle at 34 °C. In addition, growth performance data showed high specific growth rate (SGR) and feed efficiency (FER) at 28 °C and 31 °C water temperature. IGF-1 and GHR mRNA expression were also highest in liver at 28 °C and 31 °C compared to 34 °C ($P < 0.05$).

In the second part, the potential effects of 17α-methyltestosterone (MT) treatment on growth performance and sex change process in red-spotted grouper were examined. 90-day-old *E. akaara* juveniles (TL: 5.98 ± 0.14 cm, BW: 3.67 ± 0.24 g) were fed MT incorporated diets at a concentration of 10 and 40 mg/Kg diet for 9 week. Growth performance data showed low weight gain (WG) and down-regulation of IGF-1 and GHR mRNA levels in MT-feeding groups (10mg and 40mg MT/Kg diet) compared to control during the hormonal treatment. After completion of 9 week MT treatment, high specific growth rate (SGR) and up-regulation of IGF-1 and GHR were observed in 40mg MT/Kg diet group compared with the control and 10mg MT/Kg diet group ($P < 0.05$). Gonadal histology showed efferent duct-like structure and transformation of ovaries-to-testis in the MT-fed group (40mg MT/Kg diet) at the end of the experiment (36 week). MT-fed groups showed lower plasma estradiol (E2) levels compared to control during the MT treatment period and higher plasma testosterone (T) levels after completion of MT-feeding ($P < 0.05$).

Collectively, the present research suggests that 36 °C and 34 °C is the lethal and sub-lethal temperature, respectively for *E. akaara*. Prolonged exposure to more than 30 °C can induce physiological stress in red-spotted grouper. Based on the physiological responses, the present study also suggests that glucose, cortisol, GOT, GPT, RBC, and WBC count can be considered as thermal stress biomarkers in red-spotted grouper. The MT treatment results suggest that MT-feeding at 40 mg/Kg diet can promote growth performance and have a high potentiality of inducing sex change from female-to-male in red-spotted grouper compared with the control and 10 mg/Kg diet.

Chapter 1

General Introduction

1.1. Background

Groupers (Pisces: Perciformes, Serranidae) are one of the most commercially important groups of marine fishes that are widely distributed throughout the tropical and subtropical waters of the world (Craig et al., 2011; Sadovy de Mitcheson et al., 2013). Aquaculture of many grouper species is becoming popular throughout Asia because of their high demand. According to FAO (2017), almost 155, 000 tonnes of grouper were produced in 2015 with a total value of USD 630 million. Among the major producer countries, China produced 65% of the total production, followed by Taiwan Province of China (17%), and Indonesia (11%); together, these three countries contribute 92% of reported production. There are 47 grouper species and 15 grouper hybrids that have been trialed or currently aquacultured (Rimmer and Glamuzina, 2019).

Among the groupers, the red-spotted grouper, *Epinephelus akaara* is one of the most important species for aquaculture in southeast asia due to its high consumer demand (Rimmer et al., 2004). It has a relatively small geographic area ranged from southern China, Korea, Taiwan, and southern Japan. The maximum length of *E. akaara* is reported to be 58 cm (Craig et al., 2011). The production of *E. akaara* is partly based upon wild capture, alongside some hatchery-based mariculture. This species is predominantly collected from the wild due to their slow growth rate during the early life stage and poor production in captivity (Sadovy et al., 2018). However, red-spotted grouper has been considered as a potential candidate for aquaculture as it has high value in the live fish food trade in South Korea, China, and Japan (Sadovy de Mitcheneson et al., 2013). Recently, the culture of red-spotted grouper is becoming extensive in South Korea due to its increasing demand (Lee et al., 2019).

Juvenile *E. akaara* prefers to inhabit shallower depths of the coastal area, which is more prone to thermal fluctuations (Sadovy and Cornish, 2000). Moreover, in recent years, the natural

landing of *E. akaara* is reducing to an alarming level due to environmental degradation, indiscriminate fishing, and anthropogenic activities. This species has been enlisted as an endangered species by IUCN (International Union for the Conservation of Nature) (Baillie et al., 2004; Tupper and Sheriff, 2008). In South Korea, the stock of this species declined sharply year by year in the 1990s due to overfishing (Lee et al., 1998). From 1992 to 1994, annual landings reduced from about 2.25 to about 1.3 tonnes and fishing yields in coastal Byonsan Peninsula of Korea declined by more than 10% during the same time period (Lee et al. 1998). Therefore, physiological and growth studies are required to protect this endangered species, especially in consideration of rising water temperature.

1.2. Temperature rise and thermal stress

Climate change has begun to affect the frequency, intensity, and duration of extreme events such as extreme temperatures. Extreme hot weather, especially during the summer season, rises the water temperature (Benitez-Dorta et al., 2017; Gallant et al. 2017; Wong et al., 2018). Anthropogenic sources such as industrial power, paper and pulp mills are also responsible for the increase of water temperature. Discharged wastewater from these powerplants can elevate the temperature of surrounding lakes, rivers, and streams by as much as 8 to 15 °C (Langford, 2001). The increased water temperature presents a significant concern for aquaculturists and fishery biologists. The rising water temperature due to the extreme hot weather is anticipated to affect the productivity of *E. akaara* in wild as well as in aquaculture conditions.

Stress is a common characteristic of living organisms, and fish, like most organisms, have evolved a suite of defence mechanisms to protect themselves from stimuli that pose a challenge to the maintenance of their homeostatic equilibrium. A key component of the stress response is a reallocation of energy away from nonessential physiological functions and toward activities that

contribute to restoration of homeostasis (Fuzzen et al., 2011). A variety of environmental stressors (e.g. Temperature, salinity, photoperiod, crowding) induce stress on the aquatic organisms. Temperature has been considered as one of the most important environmental stressors that has profound effects on aquaculture farming. Temperature beyond the tolerance range of an aquatic organism may produces stress. Thermal stress is a term to describe a temperature change that is severe enough to cause unfavorable and even lethal conditions to aquatic organisms, their populations, community structure, or ecosystem (Portner and Farrell, 2008). Aquatic organisms are able to function most efficiently within an optimal range of water temperature. Removing stressors and minimising stress levels can lead to increase productivity.

Groupers are warm-water fish that grow higher than 24 to 30 °C; most of them prefer a thermal range higher than 15 to 35 °C (Rimmer et al., 2004). The red-spotted grouper is a subtropical species, whose optimal water temperature for growth is reported to be 24-28 °C (Lee and Baek, 2018). Thermal stress resulted from the climate change and anthropogenic activities is expected to affect the distribution and abundances of fish, especially the tropical species (Portner and Farrell, 2008). The effects of increasing temperature are diverse and usually cause multifaceted response including, morphological, physiological and molecular changes (Dent and Lutterschmidt, 2003). The potential effects of increased temperature on fishes have compelled researchers to make continual efforts to understand the physiological stress mechanisms (Kregel et al., 2002). Many studies have been carried out to evaluate the effects of rising water temperatures (Ahmad et al., 2011; Zhang et al., 2017; Shahjahan et al., 2018). However, increased water temperature effects on the physiological functions of red-spotted grouper is not well studied. Hence, the physicochemical responses and growth performances of *E. akaara* to increased water temperature were assessed in chapter 2.

1.2.1. Thermal stress and immune response

Blood is the most efficient indicator that can provide sufficient information about the immune system of fish to environmental changes (Bowden et al., 2007; Simide et al., 2016). Environmental stressors (i.e., temperature, salinity, overcrowding) can easily modify the physiological and biochemical properties of blood (Ruas et al., 2008). The morphology and erythrocyte counts vary with ambient water temperature (Ytrestoyl et al., 2001). Various erythrocytic alterations due to thermal stress are an effective indicator of cytotoxicity (Shahjahan et al., 2018; Islam et al., 2019). Different morphological abnormalities of erythrocytes and differential white blood cell (WBC) count are very important analytical factors to assess the stress caused by any environmental variations (Rowan, 2007; Shahjahan et al., 2018). Therefore, in chapter 2.1, the blood cells have been examined under increased water temperature to evaluate the health status of *E. akaara*.

1.2.2. Thermal stress and morphological response

Temperature fluctuations show a variety of morphological changes in fish. The gill plays the major role in respiratory, osmoregulatory, excretory, and branchial functions of fish. The liver performs several crucial functions including detoxification, metabolism, bile secretion, immune defense, and hormone synthesis in fish and other vertebrates (Wang et al., 2007). The gill and liver are highly sensitive to environmental stressors. Morphological alterations in the gill and liver have been analyzed to assess the effects of stress (Ahmad et al., 2011; Liu et al., 2015; Hernandez-Lopez et al., 2018). Histological modifications in gill (necrosis of gill lamellae) and liver (hepatocyte coalescence, deposition of lipid globule, and cell death) has been reported in thermally stressed Japanese flounder (*Paralichthys olivaceus*) (Liu et al., 2015), bighead carp (*Aristichthys nobilis*) (Aboka et al., 2017), and Pacific sardine, *Sardinops sagax caeruleus* (Hernandez-Lopez

et al., 2018). The morphological responses of different tissue to sudden or prolonged environmental stress has been used as indictors to assess the fish health. In chapter 2.2, the morphological responses of liver and gills in *E. akaara* were investigated to increased water temperature.

1.2.3. Thermal stress and physiological response

Temperature influences the biochemical reactions and therefore, has a significant impact on the physiology and biochemistry of ectothermic organisms. However, temperature beyond the tolerance range can affect the growth and normal physiological processes of fish (Person-Le Ruyet et al., 2004; Fazio et al., 2018). Increased water temperature can also alter the metabolic activities (Lu et al., 2016), weaken the non-specific immune defense system (Qiang et al., 2013), and intensification of disease risk (Karvonen et al., 2010). Thus, temperature change affects virtually all biochemical and physiological activities of an animal. Therefore, thermal stress is one of the most important environmental challenges that fishes may face (Portner and peck, 2010). Hematological and biochemical parameters are a common endpoint measured for assessing the physiological status of fish exposed to different environmental stressors, including temperature (Mora and Maya, 2006; Fu et al., 2018). Cortisol is the primary stress hormone secreted from the hypothalamus-pituitary-interrenal (HPI) axis, when a fish encounters environmental disturbance. Prolonged or acute thermal stress induces the secretion of cortisol, followed by an elevation of plasma glucose levels to support increased energy utilization (Lima et al., 2006). The activities of GPT, GOT and LDH in the blood can be used as stress biomarkers to monitor fish physiological responses to thermal stress (Cheng et al., 2018). Changes in plasma total protein are used as an indicator of liver impairment (Fırat and Kargın, 2010). In addition, blood lipids have also been proposed as an important chronic stress indicator during thermal stress (Ming et al., 2012). These

parameters are considered useful markers for fish health analysis (Cataldi et al., 1998; Fazio et al., 2018; Panase et al., 2018). In chapter 2.2, the hemato-biochemical responses of *E. akaara* were investigated to increased water temperature.

1.2.4. Thermal stress and heat shock protein (HSP) response

Temperature change can significantly affect the integrity of physiological system at various cellular and molecular levels (Cossins and Bowler, 1987). The common well-known feature of heat shock response is the induction of heat shock proteins (HSPs). Among the HSP protein families, Hsp60, Hsp70, and Hsp90 are highly conserved and most extensively studied (Feige et al., 1996; Parsell and Lindquist, 1993). The members of Hsp60, Hsp70, and Hsp90 family play a significant role in cell survival, stress, and thermal tolerance in response to various heat shocks. These proteins also play an important role as molecular chaperones in intracellular organelles that prevent protein aggregation, assist in refolding any misfolded proteins, stabilize unstable proteins, and maintain the integrity of the mitochondrial membrane (Parsell and Lindquist, 1993; Ranford et al., 2000). Understanding the molecular mechanisms is important to gain a clear concept of the adaptation process of fish with the environmental changes (i.e. thermal fluctuations). Therefore, in chapter 2.3, Hsp60, Hsp70 and Hsp90 mRNA expression in different tissues of *E. akaara* were investigated under elevated water temperature to evaluate the heat shock protein (HSPs) responses.

1.2.5. Thermal stress and growth response

The growth and development of ectothermic animals are very temperature sensitive; low temperatures suppress metabolic rates, feeding rates, and growth rates; elevated water temperatures tend to cause an increase in growth up to an optimum point above which thermal stress occurs (Baum et al., 2005). The adequate temperature can improve the food ingestion, digestion, nutrient utilization, and, thus, enhance the growth performance of fish (Bogevik et al.,

2010). Among the growth parameters, the specific growth rate (SGR), feed conversion value (FCR), and feed efficiency value (FER) have been considered as a better growth performance indicators as they are used to do a comparison of growth among different species. These parameters fluctuated significantly under temperature treatments (Otterlei et al., 1999; Person-Le Ruyet et al., 2004). In the molecular level, growth hormone (GH), insulin-like growth factor (IGF-1), and insulin, are three important factors that can contribute to growth and metabolism (Banos et al., 1999). Temperature influences growth via the actions of IGF-1 secreted by the liver following stimulation by GH. The activity of GH initiates after binding to growth hormone receptor (GHR) in the liver (Gabillard et al, 2003a, b; Reinrck et al., 2005). The production and secretion of these hormones are directly or indirectly related to some exogenous factors, including water temperature (Imsland et al., 2007), salinity (Taylor et al., 2008), and photoperiod (Cruz and Brown, 2009). Thus, the GH/GHR/IGF-1 system is an important regulatory network that can control fish growth. Hence, in chapter 2.4, the growth parameters, and GHR and IGF-1 gene expression of *E. akaara* were assessed under different rearing temperatures to make a better understanding of temperature-induced growth performance and the possible endocrine control of this process in red-spotted grouper.

1.2.6. Thermal acclimation and temperature tolerance

Temperature tolerance in fish varies with species, and acclimation time and temperature (Das et al., 2004; Manus et al., 2004). The acclimation temperature has been suggested to be the most important factor that closely related to the critical thermal maxima (CTmax) of fish (Beitinger and Lutterschmidt, 2011). CTmax is the temperature for a given species, above which most individuals respond with unorganized locomotion, subjecting to likely death. The CTmax has been considered as an effective indicator of the thermal tolerance of an organism that allows the

identification of the temperature at which the first signs of stress occur (Beitinger et al., 2000). Assessment of the thermal tolerance limit can help to understand the adaptive biology of aquatic animals to any particular habitat. Determining the thermal tolerance of a species is important for understanding its physiology and ecology, as well as predicting the long-term effects of climate change (Portner and Peck, 2010). This knowledge is required to improve management and conservation efforts for endangered species (Deslauriers et al., 2016). Thus, the thermal tolerance of *E. akaara* was assessed under different acclimation temperature in chapter 2.5.

1.3. 17α-methyltestosterone (MT) treatment and growth enhancement

The red-spotted grouper is a protogynous hermaphroditic fish: function as a female at a young age and later on undergo sex changes to become the male when they have reached a larger size (Bhandari et al., 2003; Liu and Sadovy, 2009; Sao et al., 2012). Thus, a significant amount of absorbed energy has been used for maintaining gonadal development, which ultimately slow down the growth performance. The complicated reproductive characteristics of groupers have made their aquaculture less profitable. The natural or synthetic androgens and estrogens have shown growth-promoting effect in many cultured species (James and Sampath, 2006). Steroids both androgenic and estrogenic can increase growth and food conversion efficiency when administered in food (Jensi et al., 2016). 17α-methyltestosterone (MT) is a synthetic male hormone, which has an androgenic effect. Synthetic androgens are used in fish culture either by immersion or through the diet as sex controlling agents or growth promoters if energy is shut away from developing ovaries towards the growth of somatic tissues (Rizkallah et al., 2004). The endocrinological knowledge, especially about the GH/GH receptor/IGF-1 system, is important to understand the growth patterns induced by MT-treatment. Hence, in chapter 3.1, the growth-inducing potentiality of MT incorporated diet on the red-spotted grouper was investigated.

1.4. 17α-methyltestosterone (MT) treatment and sex change

The sexual maturation of male *E. akaara* occurs at 3 years when the standard length is about 23-24 cm (Tseng and Ho, 1988). The spawning period of the red-spotted grouper in the Jeju coastal area takes place between July and August as because they are summer breeder. Mature males are an important prerequisite in artificial seed production of grouper; however, the shortage of mature male broodstock has limited the success of artificial propagation. The unreliable and limited supply of seed has slow down the culture of red-spotted grouper. Mass production of seed requires quality male brood fish that will help to reduce the generation gap as well as improve the aquaculture of *E. akaara*. Artificial sex change has been successfully performed in many grouper species to overcome the deficiency of mature males for breeding (Yeh et al., 2003; Bhandari et al., 2004). Treatments with exogenous sex steroids can induce permanent sex change in some fish, regardless of their genetic sex. Several studies suggest that treatment with exogenous 17α-methyltestosterone (MT) or aromatase inhibitor (AI) can induce sexually mature females to become functional males of some grouper species by downregulating the endogenous estrogen synthesis (Bhandari et al., 2006; Shi et al., 2012). Therefore, the potentiality of MT-feeding in sex change of red-spotted grouper was examined in chapter 3.2.

1.5. Objectives of the present research

The present study investigated the potential effects of increased temperature on the physiology of red-spotted grouper, especially concerning the rising water temperature. Besides, the probable effects of MT treatment as growth promoter and sex change inducer in *E. akaara* was also examined.

The overall purpose of the present study is to improve the growth performace of red-spotted grouper based on the findings of temperature and hormonal-induced physiological responses. The

findings of our study are also expected to be useful for the better aquaculture management of *E. akaara*, especially in face of rising water temperature.

The specific objectives of the present study include:

- To assess the effects of increased water temperature on morphology, physiology, heat shock proteins (HSPs), growth performance, and growth-related gene expression in juvenile red-spotted grouper

- To evaluate the effects of MT treatment on growth performance, growth-related gene expression, gonad histology, and sex steroids profiles in juvenile red-spotted grouper.

Chapter 2

Physicochemical responses and growth performances of red-spotted grouper (*Epinephelus akaara*) to increased water temperature

Chapter 2.1

Evaluation of blood cell morphology, morphometry, and count in red-spotted grouper

Abstract

The present study carried out to investigate the changes in blood cell morphology and morphometry of red-spotted grouper (*Epinephelus akaara*) under different rearing temperatures. *E. akaara* juveniles were exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days following 2 week of acclimation at 25 °C. Blood cells were examined at three sampling points (2, 7, and 42 days) from a total of 180 fish. Major erythrocytic cellular abnormalities (ECA) observed at 31 °C and 34 °C were echinocytes, teardrop-like cells, swollen cells and vacuolated cells. Both red blood cell (RBC) and white blood cell (WBC) count were significantly ($P < 0.05$) increased at 31 °C and 34 °C group after 42 days of thermal exposure. Differential leucocyte number showed significant increase in neutrophil (N) and decrease in lymphocytes (L) at higher water temperature (34 °C). Erythrocyte major axis (EL) and nucleus major axis (NL) were decreased, whereas erythrocyte minor axis (EW) and nucleus minor axis (NW) was increased at higher water temperature (31 °C and 34 °C). The major-minor axis proportions of erythrocytes and nucleus (EL/EW; NL/NW) were decreased with increasing water temperature (31 °C and 34 °C). The strong relationships were observed among the morphometric indices of erythrocytes and their nucleus, especially in EL *vs*. NL and EW *vs*. NW. Taken together, our results suggest that high water temperature (31 °C and 34 °C) can interfere with the immune system of red-spotted grouper by altering their blood cell morphology and morphometry. This fine information may use to monitor the health status of *E. akaara* and probably for other fish species.

Key words: *Epinephelus akaara*, Temperature, Blood cell morphology, Blood cell morphometry, Blood cell count

1. Introduction

Blood is the most efficient indicator that can provide sufficient information about the immune system of fish to environmental changes (Bowden et al., 2007; Simide et al., 2016). Examining blood cells has been considered a valuable approach for analyzing the health status of fish. Unlike human and most mammals, fish possess an oval-shaped structure of erythrocyte with a condensed nucleus (Jagoe and Welter, 1995). Fish erythrocytes are more responsive to environmental stresses and often vary in morphology (Lowe-Jinde and Niimi, 1983). However, variations in erythrocytes, nuclear size, and structure within the species are smaller than interspecies differences (Najiah et al., 2008; Fang et al., 2014).

The erythrocyte morphology and counts vary with ambient water temperature (Ytrestoyl et al., 2001). Various erythrocytic alterations due to thermal stress are an effective indicator of cytotoxicity (Shahjahan et al., 2018; Islam et al., 2019). Red blood cells (RBC) and white blood cells (WBC) count, different morpho-metrical abnormalities of erythrocytes, and differential white blood cell (WBC) count are very important analytical factors to assess the stress caused by any environmental variations (Rowan, 2007; Shahjahan et al., 2018). Several ECAs (echinocytic, elongated shape, fusion, tear-drop shape, and twin) have been used as temperature stress (bio)markers for fish (Islam et al., 2019; Shahjahan et al., 2019). Therefore, examining blood cells in response to thermal stress has been considered as a valuable approach to get a rapid feedback about the immune response of the stressed fish.

The red-spotted grouper is a subtropical species of serranidae family, which prefers to inhabit the thermal range higher than 24 to 28 °C (Cho et al., 2015; Lee and Baek, 2018). However, temperature can increase to a point that may affect the immune system of fish (Fu et al., 2018). Though a number of studies were conducted to make a better understanding of blood cell

morphology and morphometry of fishes under various environmental toxicants (Rowan, 2007; Osman et al., 2018; Shahjahan et al., 2018; Islam et al., 2019), the high temperature effects are not well studied. Therefore, the present study aimed to investigate the morphology and morphometry of blood cells under different rearing water temperatures. The results of the present study may help to check the health status of *E. akaara* in a quick time.

2. Materials and methods

2.1. Experimental animals and maintenance

Juveniles of *E. akaara* were obtained from the Marine Science Institute, Jeju National University, Korea. Fish were acclimated in aquaria at a water temperature of 25 ± 0.5 °C, salinity of 34 psu, dissolved oxygen levels ≥ 6.6 mg/L, pH of 7.8, and a 12L:12D photoperiod for 2 weeks before the experiment. Fish maintenance, handling, and sampling were conducted according to the guidelines of the Animal Ethics Committee of Pukyong National University (PKNU) (Regulation No. 554).

2.2. Experimental design and thermal exposure

A total of 180 juvenile red-spotted grouper (total length: 8.28 ± 0.10 cm; body weight: 8.53 ± 0.27 g) were randomly distributed into 12 rectangular glass tanks (75 cm × 45 cm× 45 cm) containing 15 fish per tank. Each tank was filled with 120 L seawater and equipped with a recirculating filtration system. The fishes were exposed to four temperature conditions (25 °C as control, 28 °C, 31 °C, and 34 °C), each with three replications for 6 weeks. The temperature of the experimental tank was constantly increased at a rate of 1 °C h^{-1} using a thermostat (OKE-6422H; OKE, Busan, Korea) from the control temperature (25 °C) until the experimental temperature (28 °C, 31 °C, and 34 °C) achieved. Upon reaching the target temperature, the experimental period was started to count. Water temperature, pH, salinity, and dissolved oxygen were checked daily

using a multiparameter water quality meter (HI9829; Hanna Instrumentals, USA) and total ammonium levels were measured every 2 days using an NH_3/NH_4^+ test kit (Tetra GmbH, Melle, Germany). The water quality parameters are shown in Table 2.1.1. Approximately 10% of the water was replaced daily with filtered clean seawater. Fish were fed a commercial diet (Otohime Hirame; Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) twice daily (09:00 and 18:00 h) until 24 h before the experimental trial. Uneaten food was removed after 30 minutes of feeding to control water quality.

Table 2.1.1. Water quality parameters (Mean ± SEM) measured during the experimental period.

Parameters	**Temperature groups**			
	25 °C	**28 °C**	**31 °C**	**34 °C**
Temp.	24.88 ± 0.04	27.77 ± 0.04	30.90 ± 0.05	33.78 ± 0.04
Salinity (psu)	33.80 ± 0.005	33.89 ± 0.06	33.72 ± 0.05	33.77 ± 0.07
pH	8.05 ± 0.02	8.06 ± 0.01	7.99 ± 0.02	7.99 ± 0.02
DO (mg/L)	6.86 ± 0.04	6.72 ± 0.04	5.68 ± 0.05	4.16 ± 0.03
NH4 (mg/L)	<0.25	<0.25	<0.25	<0.25

2.3. Sampling and blood smear preparation

15 fishes were sampled from each temperature group (i.e., 5 fishes from each aquaria; n=15) on each sampling day (i.e., 2, 7, and 42 days of thermal exposure). Fish were randomly captured and lightly anesthetized with 300 ppm 2-phenoxyethanol (Sigma Aldrich, USA). Blood was collected from the caudal vein using heparinized capillary tubes and stored in 1.5 mL centrifuge tubes. The whole blood was withdrawn less than 1 min per fish to avoid handling stress. Blood smears were prepared immediately after collection, air-dried for 10 min, fixed in 95% methanol

for 5 min, stained with Wright-Giemsa solution, rinsed with distilled water, air-dried and mounted with malinol. Three blood-smeared slides were prepared from each fish sample.

2.4. Counting and observation of blood cells

The red blood cell (RBC) and white blood cell (WBC) were counted under a light microscope (BX-50; Olympus, Tokyo, Japan) using an improved Neubauer Hemocytometer (Blaxhall and Daisley, 1973). 100 blood cells were scored from each blood smear and frequencies of differential leucocytes were recorded according to the methodology proposed by Davis et al., (2008).

Erythrocytic cellular abnormalities (ECA) and differential leucocytes, such as monocytes (Mo), neutrophil (N), lymphocytes (L), eosinophil (Eo), and basophil (Ba) were examined from the blood smear of fishes reared in different thermal conditions under a light microscope (BX-50; Olympus, Tokyo, Japan; ×600 magnification). The ECA was dissimilar from the normal erythrocyte cell (oval-shaped with a condensed nucleus).

2.5. Erythrocyte morphometry analysis

Erythrocytes in fish are elliptical, and as such, two different diameters are provided: erythrocyte major axis (EL) and erythrocyte minor axis (EW). Nucleus major axis (NL) and nucleus minor axis (NW) were also obtained. On each slide, EL and EW of randomly selected 100 mature erythrocytes and their nuclei (NL and NW) were measured by an Olympus ocular micrometer at a magnification of ×600 (Olympus BX-50, Japan). Erythrocyte and nuclear sizes (ES and NS) were calculated according to formulas $[(EL \times EW \times \pi)/4]$ and $[(NL \times NW \times \pi)/4]$, respectively (Metin et al., 2008).

2.6. Statistical analysis

All data are presented as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to assess the significant differences among the different temperature groups. Pearson's correlation coefficient test was conducted to estimate the relationships among the morphometric indices of the erythrocyte. In addition, regression analysis and goodness of fit (R^2) was determined when there was a significant correlation between tested variables. The results were considered significant at $P < 0.05$. Data analysis were performed using SPSS statistics software (ver. 21.0; IBM Corp., USA).

3. Results

3.1. Changes in erythrocyte morphology

Thermal stress induced various erythrocytic cellular abnormalities in 31 °C and 34 °C groups on day 2 (Fig. 2.1.1 C, D) compared with those in the 25 °C and 28 °C groups (Fig. 2.1.1 A, B). After 7 days, 34 °C induced more erythrocytic damage showing echinocytes (Ec) and swelled blood cells (Sc) (Fig. 2.1.1 F), and 31 °C group showed normal erythrocyte structure (Er) with oval-shaped (Oc) (Fig. 2.1.1 E). However, 25 °C and 28 °C group did not show any erythrocytic abnormalities after 7 and 42 days of thermal exposure (Figure not shown). After 42 days, massive erythrocytic cellular damge was recorded in 34 °C group (Fig. 2.1.1 H). Sign of erythrocytic cellular damge was also recorded in 31 °C group (Fig. 2.1.1 G). The other major signs of RBCs alterations included teardrop like cells (Tr), eliptocyte cells (El), round-shaped cells (Rc), microcytic RBCs (Mc), bite cell (Bi), hemolyzed RBC cells (Hc), and vacuolated cells (Va).

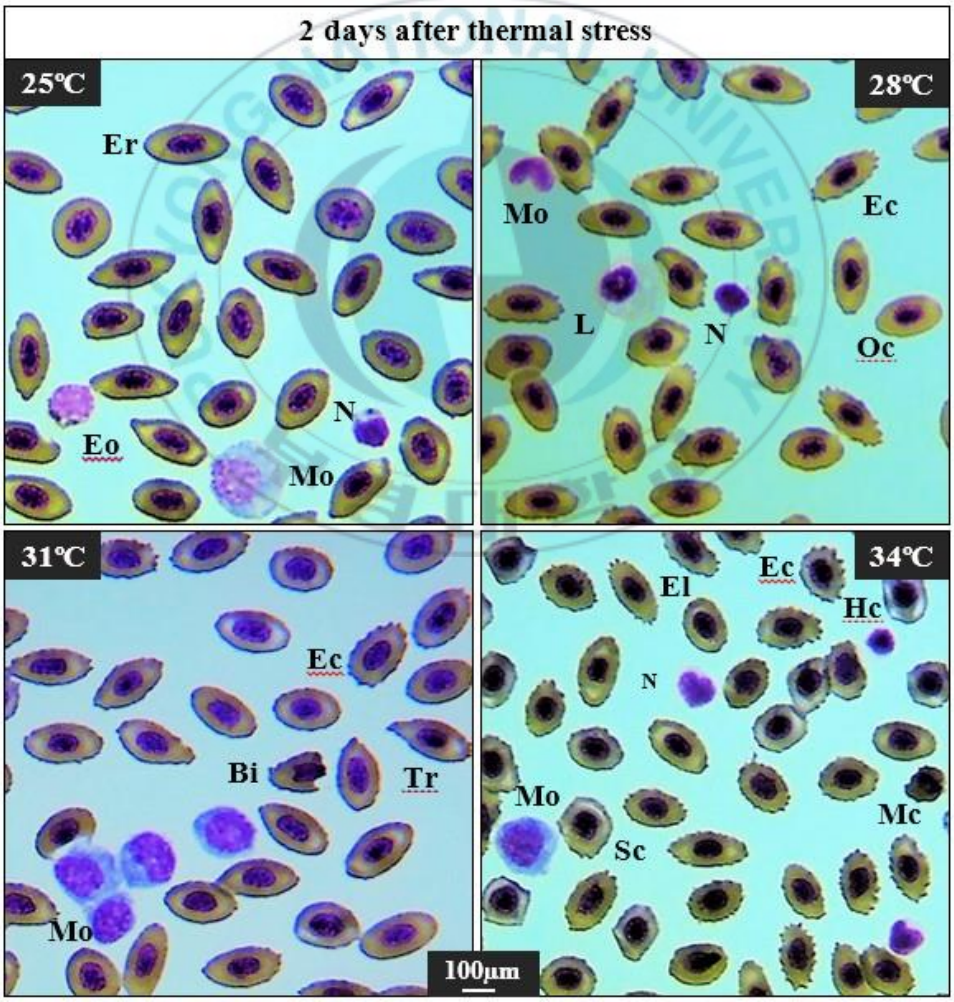
3.2. Changes in RBC and WBC count

Thermal stress significantly elevated the RBCs at 34 °C on day 2, whereas WBCs increased at 31 °C and 34 °C group. After 7 days, both RBC and WBC number were significantly elevated

at 34 °C compared with those in the 25 °C, 28°C and 31 °C groups. However, after 42 days, thermal stress significantly raised both RBC and WBC number in 31 °C and 34 °C group (Table 2.1.2).

3.3. Changes in differential white blood cell (WBC) count

Monocyte and neutrophil number were significantly higher at 31 °C and 34 °C throughout the thermal exposure period (Fig. 2.1.2 A, C), whereas the number of lymphocytes was decreased at 31 °C and 34 °C (Fig. 2.1.2 B). As consequences, the N : L ratio was significantly changed among the thermal treatment groups (Fig. 2.1.2 F). However, no change was recorded in eosinophil and basophil number in any group after 42 days of thermal exposure (Fig. 2.1.2 D, E).



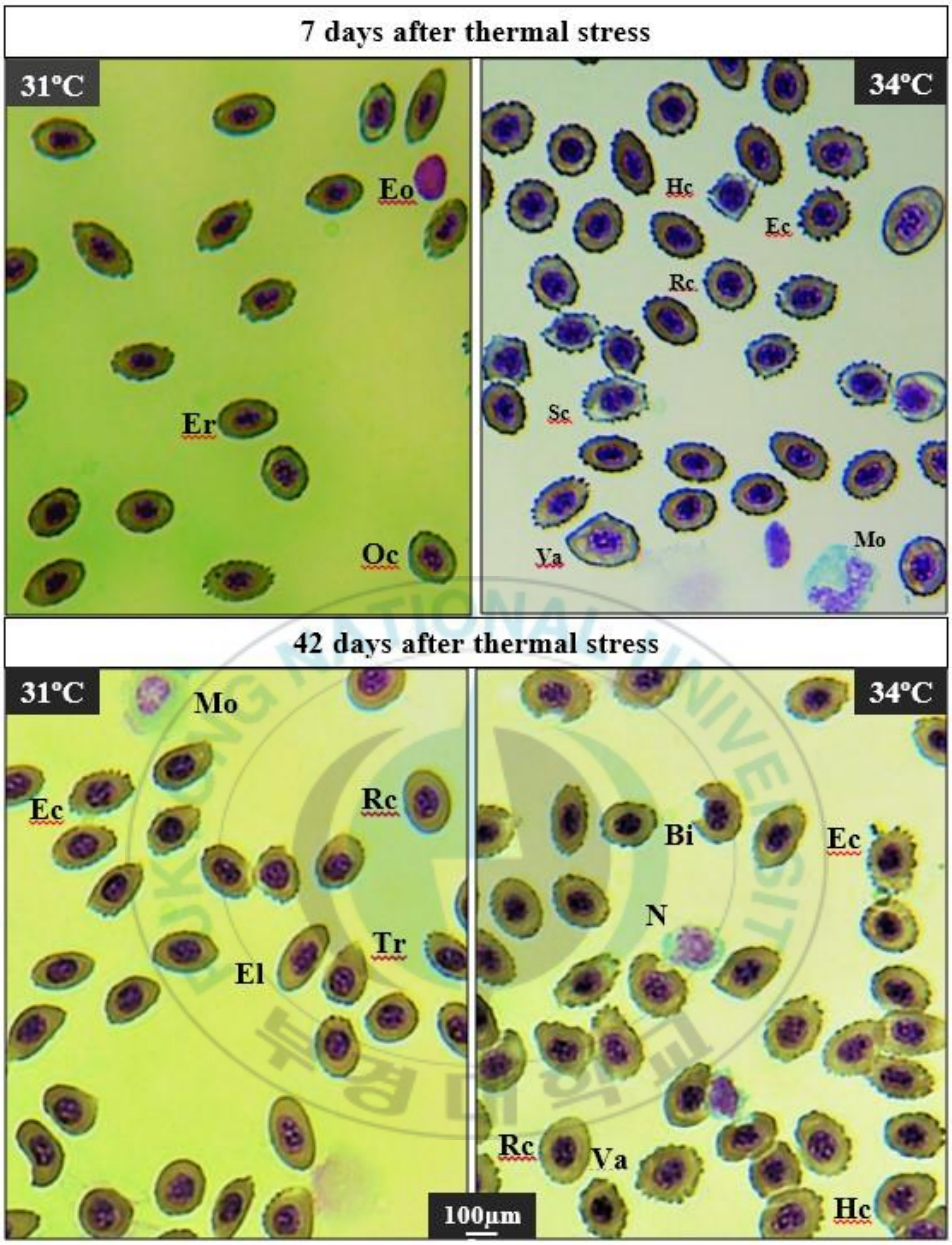
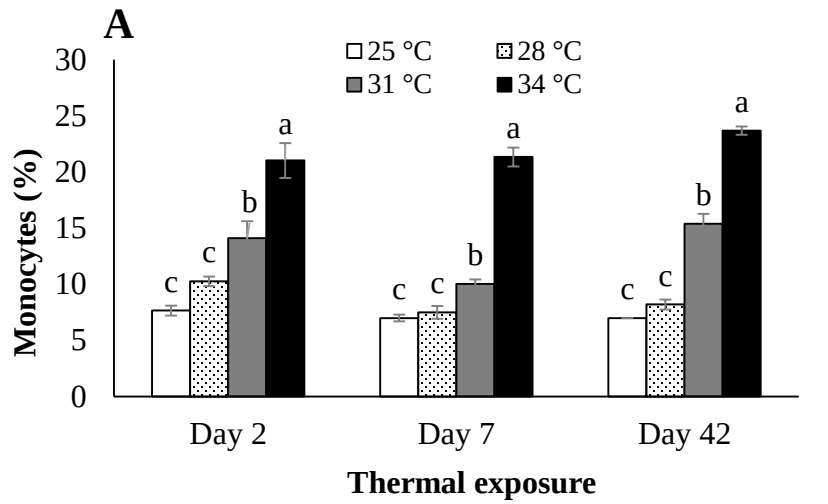


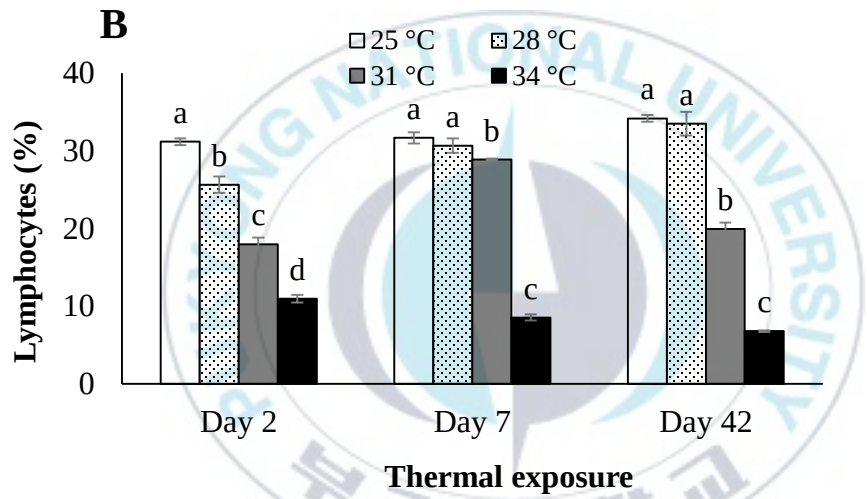
Fig. 2.1.1 (A-H). Various erythrocytic cellular abnormalities in blood smear of red-spotted grouper, *Epinephelus akaara* treated with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. Normal erythrocyte (Er), echinocyte (Ec), teardrop like cells (Tr), bite cell (Bi), eliptocyte (El), swelled cells (Sc), microcytic RBC (Mc), hemolyzed cells (Hc). Wright-Giemsa stain; Scale bars = 100 µm.

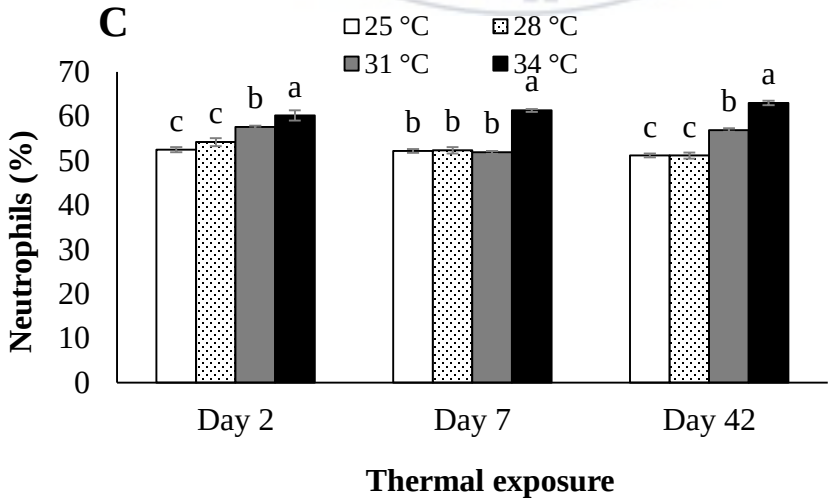
Table 2.1.2: Red blood cell (RBC) and white blood cell (WBC) counts in juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days.

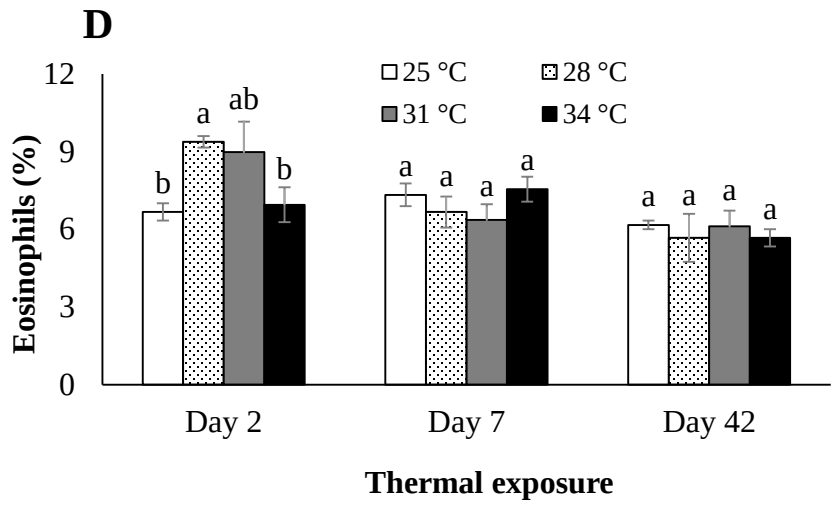
Parameters	Temperature	Exposure time (day)		
		2	**7**	**42**
RBC ($\times 10^6$/mm^3)	25 °C	3.99 ± 0.15^{b}	3.89 ± 0.05^{bc}	3.68 ± 0.04^{c}
	28 °C	3.92 ± 0.09^{b}	3.75 ± 0.16^{c}	3.61 ± 0.07^{c}
	31 °C	4.06 ± 0.19^{b}	4.16 ± 0.09^{b}	4.53 ± 0.19^{b}
	34 °C	5.18 ± 0.24^{a}	5.14 ± 0.04^{a}	5.30 ± 0.09^{a}
WBC ($\times 10^3$/mm^3)	25 °C	9.81 ± 0.49^{c}	9.19 ± 0.43^{c}	8.67 ± 0.30^{b}
	28 °C	9.52 ± 0.14^{c}	8.83 ± 0.18^{c}	8.18 ± 0.63^{b}
	31 °C	12.31 ± 0.02^{b}	11.63 ± 0.27^{b}	13.03 ± 0.36^{a}
	34 °C	13.45 ± 0.21^{a}	14.24 ± 0.08^{a}	13.58 ± 0.25^{a}

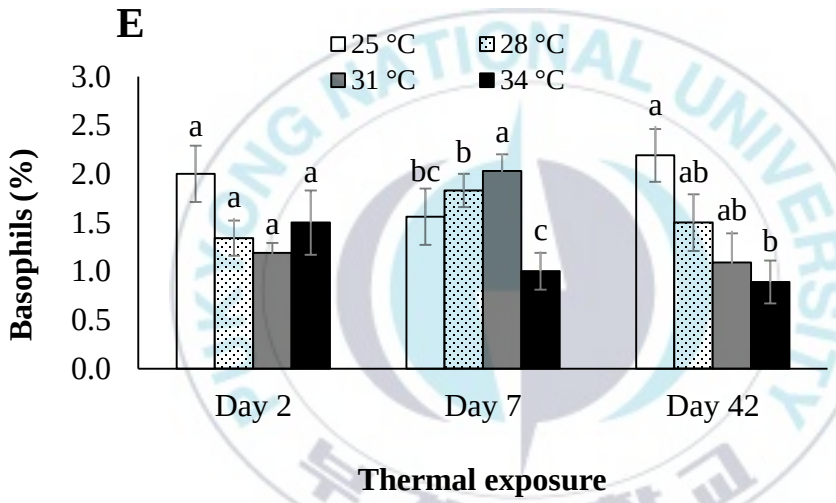
N.B.: Values are mean ± SEM of three replicates (n=15; 5 fish per tank). Different lowercase letters in each column indicate the significant difference between groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).


A
25 °C
28 °C
31 °C
34 °C
Monocytes (%)
30
25
20
15
10
5
0
a
b
c
c
a
b
c
c
a
b
c
c
Day 2
Day 7
Day 42
Thermal exposure


B
25 °C
28 °C
31 °C
34 °C
Lymphocytes (%)
40
30
20
10
0
a
b
c
d
a
a
b
c
a
a
b
c
Day 2
Day 7
Day 42
Thermal exposure


C
25 °C
28 °C
31 °C
34 °C
Neutrophils (%)
70
60
50
40
30
20
10
0
c
c
b
a
b
b
b
a
c
c
b
a
Day 2
Day 7
Day 42
Thermal exposure


D
25 °C
28 °C
31 °C
34 °C
Eosinophils (%)
12
9
6
3
0
b
a
ab
b
a
a
a
a
a
a
a
a
Day 2
Day 7
Day 42
Thermal exposure


E
25 °C
28 °C
31 °C
34 °C
Basophils (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
bc
b
a
c
a
ab
ab
b
Day 2
Day 7
Day 42
Thermal exposure

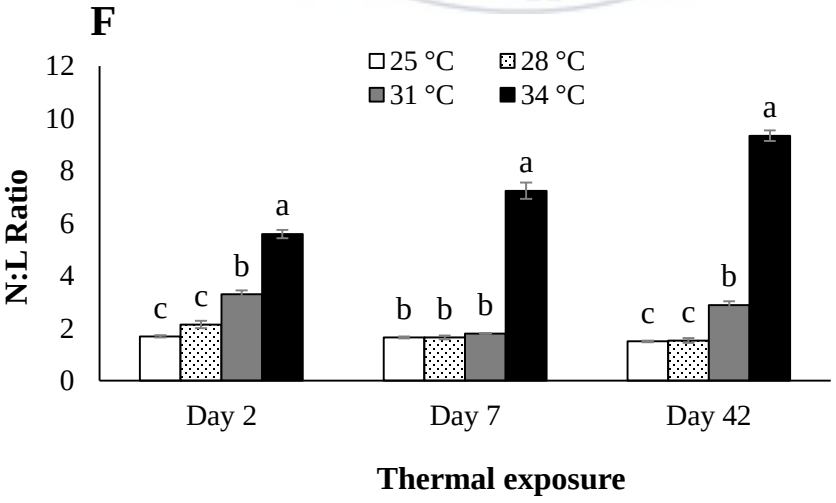

F
25 °C
28 °C
31 °C
34 °C
N:L Ratio
12
10
8
6
4
2
0
c
c
b
a
b
b
b
a
c
c
b
a
Day 2
Day 7
Day 42
Thermal exposure

Fig. 2.1.2 (A-F). Differential white blood cell (WBC) count in juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. Values are mean ± SEM of three replicates (n=15; 5 fish per tank). Different lowercase letters indicate the significant difference between groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.4. Changes in morphometric indices of erythrocytes and their nucleus

Elevated water temperature-induced significant ($P < 0.05$) changes in erythrocytes and nucleus structure of 31 °C and 34 °C groups, compared with those in the 25 °C and 28 °C groups. Fishes of 31 °C and 34 °C groups showed round-shaped erythrocyte structure containing large nucleus, whereas erythrocytes of 25 °C and 28 °C groups showed oval-shaped structure with condensed nucleus (Fig. 2.1.3).

The morphometric indices of erythrocytes and their nucleus under different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) are shown in Table 2.1.3. Major and minor axis morphometry and size of erythrocytes and nucleus differed significantly during the thermal exposure period (42 days). The major and minor axis of the erythrocytes and nucleus were changed reversely. EL and NL were significantly ($P < 0.05$) decreased, whereas EW and NW were increased at higher water temperature (31 °C and 34 °C). In addition, the major-minor axis proportion of erythrocytes and their nucleus (EL/EW; NL/NW) were decreased with increasing water temperature (31 °C and 34 °C). On the other hand, the size of erythrocytes and their nucleus (ES, NS and ES/NS) showed irregular changes under elevated water temperature.

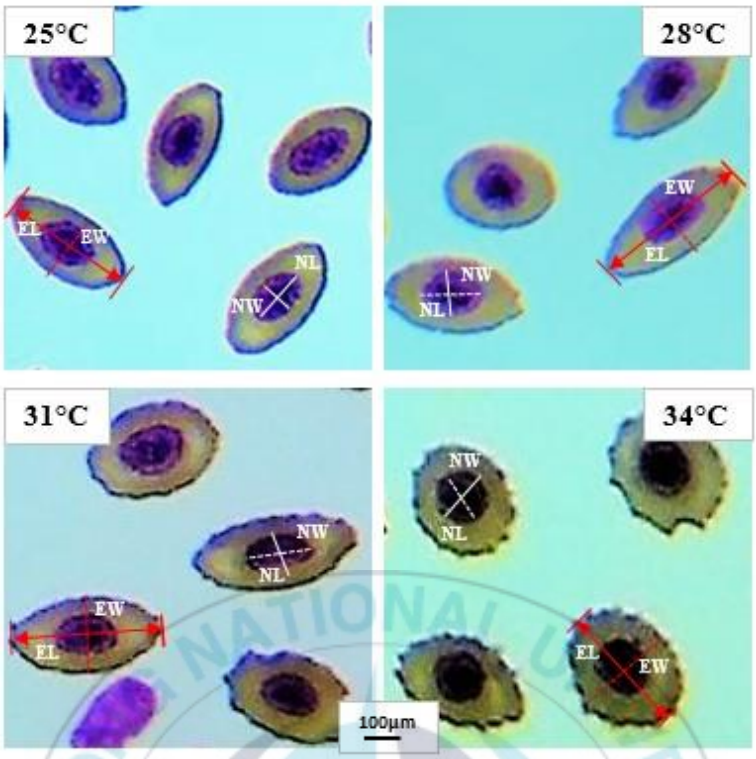


Fig. 2.1.3. Measurement of major and minor axis of erythrocyte and their nucleus in red-spotted grouper, *Epinephelus akaraa*. Red and white line indicates the diameters of erythrocytes and nucleus, respectively. EL, erythrocyte major axis; EW, erythrocyte minor axis; NL, nucleus major axis (NL); NW, nucleus minor axis. Wright-Giemsa stain. Scale bars: 100 μm.

Table 2.1.3. Changes in morphometric indices of erythrocytes and their nucleus in red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days.

Morphometric Indices	Temperature groups	Exposure time (day)		
		2	7	42
EL (μm)	25 °C	11.53 ± 0.09^{a}	11.45 ± 0.07^{a}	11.30 ± 0.08^{a}
	28 °C	11.36 ± 0.02^{a}	11.19 ± 0.07^{a}	10.35 ± 0.22^{b}
	31 °C	9.62 ± 0.17^{b}	9.02 ± 0.14^{b}	8.68 ± 0.18^{c}
	34 °C	8.34 ± 0.04^{c}	8.44 ± 0.12^{c}	8.78 ± 0.19^{c}
EW (μm)	25 °C	5.59 ± 0.10^{b}	5.59 ± 0.08^{c}	5.77 ± 0.22^{c}
	28 °C	5.42 ± 0.03^{b}	5.91 ± 0.07^{b}	5.68 ± 0.14^{c}
	31 °C	6.01 ± 0.02^{a}	6.17 ± 0.09^{b}	6.49 ± 0.07^{b}
	34 °C	6.03 ± 0.09^{a}	7.16 ± 0.08^{a}	7.36 ± 0.11^{a}
EL/EW	25 °C	2.06 ± 0.05^{a}	2.05 ± 0.12^{a}	1.96 ± 0.06^{a}
	28 °C	2.09 ± 0.09^{a}	1.90 ± 0.08^{a}	1.82 ± 0.05^{a}
	31 °C	1.60 ± 0.06^{b}	1.47 ± 0.05^{b}	1.34 ± 0.04^{b}
	34 °C	1.39 ± 0.02^{c}	1.18 ± 0.06^{c}	1.19 ± 0.03^{c}
NL (μm)	25 °C	5.12 ± 0.22^{a}	5.48 ± 0.05^{a}	5.12 ± 0.06^{b}
	28 °C	5.06 ± 0.17^{a}	5.30 ± 0.15^{a}	5.46 ± 0.06^{a}
	31 °C	4.66 ± 0.21^{a}	4.15 ± 0.04^{b}	4.47 ± 0.06^{c}
	34 °C	3.55 ± 0.14^{b}	4.21 ± 0.19^{b}	4.49 ± 0.10^{c}
NW (μm)	25 °C	2.94 ± 0.07^{b}	3.32 ± 0.08^{c}	2.65 ± 0.06^{c}
	28 °C	3.05 ± 0.10^{b}	3.20 ± 0.03^{c}	3.28 ± 0.12^{b}
	31 °C	3.51 ± 0.08^{a}	3.83 ± 0.02^{b}	3.68 ± 0.24^{b}
	34 °C	3.73 ± 0.08^{a}	4.00 ± 0.03^{a}	4.28 ± 0.09^{a}
NL/NW	25 °C	1.74 ± 0.09^{a}	1.65 ± 0.03^{a}	1.94 ± 0.07^{a}
	28 °C	1.67 ± 0.02^{a}	1.66 ± 0.04^{a}	1.71 ± 0.02^{b}
	31 °C	1.33 ± 0.04^{b}	1.09 ± 0.06^{b}	1.20 ± 0.04^{c}
	34 °C	0.95 ± 0.05^{c}	1.05 ± 0.04^{b}	1.05 ± 0.04^{d}
ES (μm^2)	25 °C	50.65 ± 0.65^{a}	50.18 ± 0.62^{b}	51.28 ± 0.88^{a}
	28 °C	48.39 ± 2.46^{b}	51.98 ± 0.28^{a}	46.24 ± 0.88^{b}
	31 °C	45.55 ± 3.88^{ab}	43.98 ± 0.48^{d}	44.25 ± 0.53^{b}
	34 °C	39.44 ± 0.50^{b}	47.44 ± 0.14^{c}	50.78 ± 1.53^{a}
NS (μm^2)	25 °C	11.83 ± 0.54^{a}	14.27 ± 0.12^{a}	10.65 ± 0.13^{c}
	28 °C	12.14 ± 0.23^{a}	13.27 ± 0.04^{b}	13.83 ± 0.33^{b}
	31 °C	12.95 ± 0.39^{a}	12.49 ± 0.57^{b}	13.19 ± 0.26^{b}
	34 °C	10.43 ± 0.12^{b}	13.22 ± 0.15^{b}	15.13 ± 0.24^{a}
ES/NS	25 °C	4.30 ± 0.22^{a}	3.53 ± 0.09^{b}	4.82 ± 0.21^{a}
	28 °C	4.02 ± 0.26^{ab}	3.91 ± 0.08^{a}	3.44 ± 0.28^{b}
	31 °C	3.55 ± 0.20^{b}	3.50 ± 0.04^{b}	3.41 ± 0.06^{b}
	34 °C	3.81 ± 0.14^{ab}	3.59 ± 0.11^{b}	3.41 ± 0.21^{b}

Values are mean ± SEM of three replicates (n=15; 5 fish per tank). 100 erythrocytes and their nucleus were measured from each blood-smeared slide. Different lowercase letters indicate the significant difference between groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$). EL, erythrocyte major axis; EW, erythrocyte minor axis; NL, nucleus major axis; NW, nucleus minor axis; ES, erythrocyte size; NS, nucleus size.

3.5. Correlations among the morphometric indices of erythrocytes and their nucleus

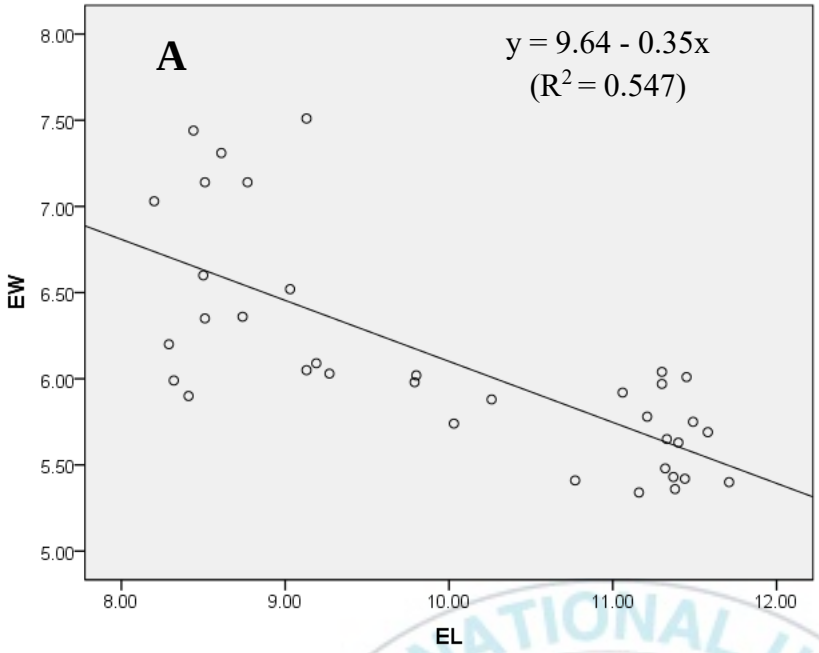
Both positive and negative correlations were observed among the morphometric indices of erythrocytes and nucleus of red-spotted grouper (Table 2.1.4 and Fig. 2.1.4 A-F). A highly significant positive relationship was found in EL *vs*. NL and EW *vs*. NW, whereas significant negative relationship was detected in EL *vs*. EW, NL *vs*. NW, EL *vs*. NW, and EW *vs*. NL.

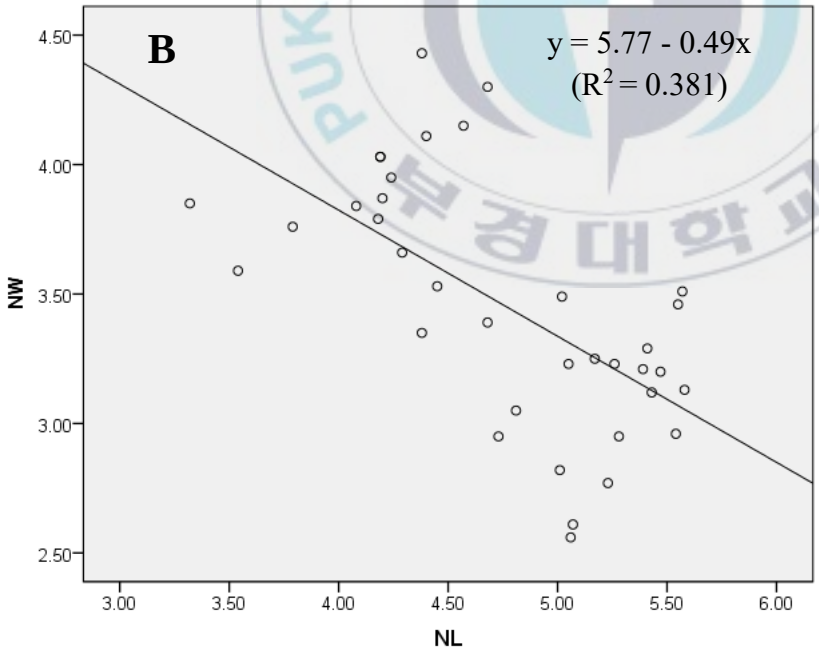
Table 2.1.4. Pearson correlation coefficient and the regression equation for the morphometric indices of erythrocytes and nucleus in red-spotted grouper, *Epinephelus akaara.*

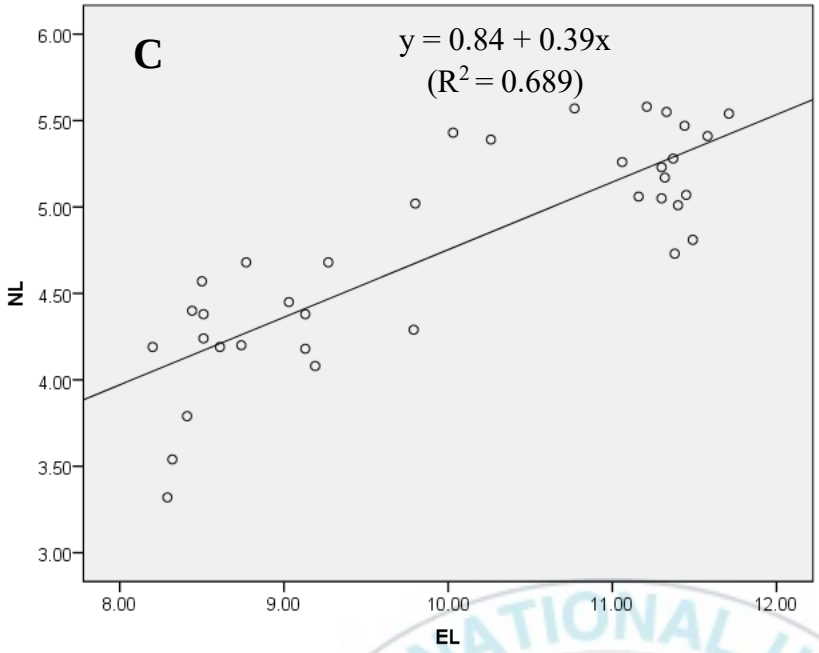
Paired samples	Correlation coefficient (r)
EL (x) vs. EW (y)	- 0.740**
NL (x) vs. NW (y)	-0.617**
EL (x) vs. NL (y)	0.830**
EW (x) vs. NW (y)	0.796**
EL (x) vs. NW (y)	-0.814**
EW (x) vs. NL (y)	-0.536**

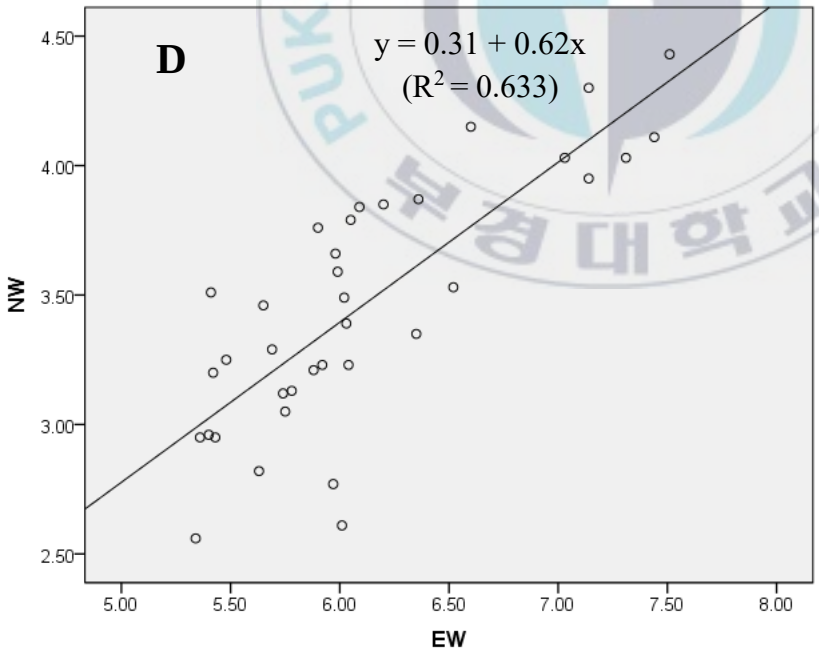
See the regression plot (Fig. 2.1.4A-F) for each paired samples ** $P < 0.01$.

EL, erythrocyte major axis; EW, erythrocyte minor axis; NL, nucleus major axis; NW, nucleus minor axis.









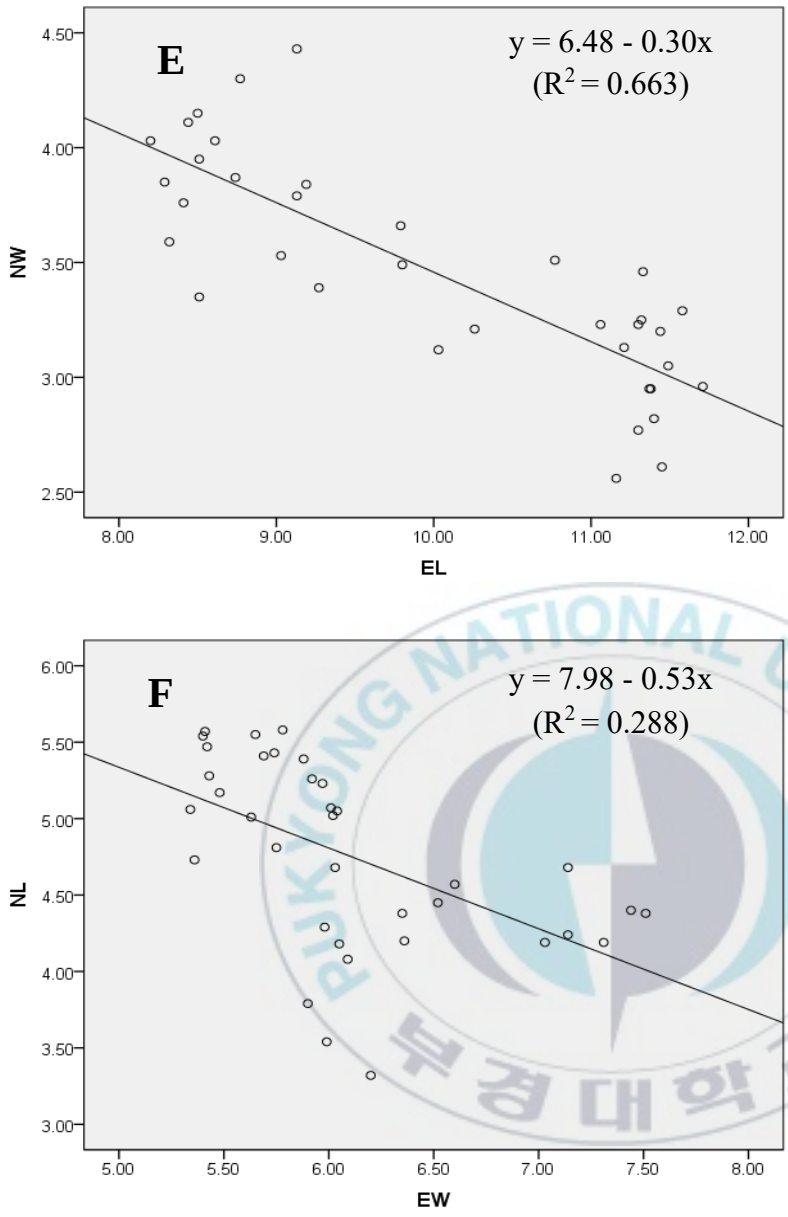


Fig. 2.1.4(A-F). Linear correlations among the morphometric indices of erythrocytes and their nucleus in red-spotted grouper, *Epinephelus akaraa*. (A) erythrocyte major axis (EL) *vs*. erythrocyte minor axis (EW); (B) nucleus major axis (NL) *vs*. nucleus minor axis (NW); (C) erythrocyte major axis (EL) *vs*. nucleus major axis (NL); (D) erythrocyte minor axis (EW) *vs*. nucleus minor axis (NW); (E) erythrocyte major axis (EL) *vs*. nucleus minor axis (NW); (F) erythrocyte minor axis (EW) *vs*. nucleus major axis (NL).

4. Discussion

Since blood cells are influenced by a variety of environmental stressors, they have the potential to be used as stress indicators. Their evaluation in fish has become an important means of understanding the toxicological impacts of exposure hazards (Borges et al., 2007). In this study, changes in morphology and morphometry of blood cells were investigated under different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) to evaluate the thermal stress level in red-spotted grouper. Our results indicated that blood cell morphometry and their count were severely altered at 34 °C water temperature.

In the present study, several ECA like echinocytes, teardrop-like cells, swollen cells and vacuolated cells were observed in blood smears of thermally exposed fish (31 °C and 34 °C) in comparison with other groups (25 °C and 28 °C). These results clearly indicate the sign of stress, which can lead to an anemic condition of experimental animals at higher thermal exposure (34 °C). Similar erythrocytic alterations were reported in striped catfish, *Pangasianodon hypophthalmus* when treated with higher temperature (36 °C) for 7 and 28 days (Islam et al., 2019). The vacuoles in erythrocyte may result from the unequal distribution of hemoglobin (Mekkawy et al., 2011). The swelling of blood cells was recorded as a sign of necrosis (Bushra et al., 2002). Echinocytes is a type of erythrocytic abnormality that formed due to interruption in lipid solubility to the erythrocyte membrane, which ultimately leads to apoptosis (Walia et al., 2013).

In this study, the elevated RBCs at 31 °C and 34 °C after 42 days thermal exposure indicates that the stressed fish tried to cope with adverse conditions by enhancing their respiratory capabilities through evaluated RBC. The higher RBC levels may increase the oxygen carrying capacity of blood, thus supplying oxygen to major organs in response to higher metabolic demand, which is a manifestation of stress (Ruane et al., 1999). Moreover, the observed higher WBC

number at 31 °C and 34 °C after 42 days of thermal exposure can be correlated with additional antibody demand that may help the fish to survive in adverse environmental condition. Similarly, RBC and WBC number were found to be increased with the increased water temperature in striped catfish (24-36 °C) and common carp (20-32 °C) when reared for 30 days (Ahmad et al., 2011; Islam et al., 2018).

In the present study, the increased neutrophil (N) and decreased lymphocytes (L) among the differential leucocyte number in higher temperature (31 °C and 34 °C) indicates the disturbance of the immune system which may lead to infectious disease to the thermally stressed fish. Similar results have been reported in *P. hypophthalmus* where the increase in neutrophil numbers (neutrophilia) and decrease in lymphocyte numbers (lymphocytopenia) resulted due to thermal stress (Shahjahan et al., 2018). Moreover, the N:L ratio can be used as an index of a secondary stress response, since the neutrophils and lymphocytes numbers are affected by stress in opposite directions (Davis et al., 2008). The present study also supports this statement as the highest N:L was recorded in 34 °C group. The higher N: L, like our study, was recorded in stressed dusky grouper *Epinephelus marginatus* (Pereira-Cardona et al., 2016) and pejerrey fingerlings *Odontesthes bonariensis* (Zebral et al., 2015).

In the present study, changes in erythrocytes and their nucleus structure were observed in *E. akaara* under elevated water temperature (31 °C and 34 °C). The erythrocytes of 25 °C and 28 °C were in oval to elliptical shape; however, the erythrocytes were turned into round from elliptical shape after being exposed to 31 °C and 34 °C. These altered structure in erythrocytes and nucleus can induce anemic condition to the thermally stressed fishes. Similar abnormalities in erythrocyte and nuclei were reported in striped catfish, *Pangasianodon hypophthalmus* when treated with higher temperature (36 °C) for 7-28 days (Shahjahan et al., 2018; Islam et al., 2019).

The observed structural changes in erythrocyte and nuclei may be attributed to one or more of the following factors: (i) unequal distribution of hemoglobin (Mekkawy et al., 2011); (ii) necrosis of red blood cells (Ateeq et al., 2002); (iii) interruption in lipid solubility to erythrocyte's membrane (Walia et al., 2013).

Fish erythrocytes and their nucleus have a wide range of shape and sizes among different species (Jagoe and Welter, 1995). The changes in erythrocytes depend on the fish species, age, health and the environment where the fish live (Vazquez and Guerrero, 2007; Zexia et al., 2007; Motlagh et al., 2012). Measurement of morphometric indices of erythrocytes and nucleus (major axis, minor axis, and size) under different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) demonstrated that erythrocytes and their nucleus varied in size at the higher temperature (31 °C and 34 °C). The decline in major axis (EL, NL) and increase in minor axis (EW, NW) of erythrocytes and their nucleus may be related to alteration of erythrocyte's shape. The shape of the erythrocyte was changed from elongate to round which may cause a reduction in major axis and enlargement in the minor axis. The observed variation could also be explained by the following factors: (i) differing the dissolved oxygen levels in experimental groups; (ii) the maturation stage of the erythrocytes; (iii) the sample size of research. These findings are consistent with those of Rowan (2007), who reported that erythrocyte and nucleus morphometry of brown bullheads (*Ameiurus nebulosus*) were affected due to the pollution of river water by agricultural runoffs or industrial effluents. Similar results were also reported in Nile tilapia (*Oreochromis niloticus niloticus*) and African catfish (*Clarias gariepinus*) collected from contaminated sites of the river Nile (Osman et al., 2018). In this study, the strong relationships were observed among the morphometric indices of erythrocytes and their nucleus. Hardie and Hebert (2003) reported that

erythrocytes and nuclear sizes are significantly correlated in ray-finned fishes, cartilaginous fishes and all species combined.

5. Conclusion

In conclusion, the results of the present study revealed that high water temperature (31 °C and 34 °C) significantly altered the blood cell morphology and their count. In addition, the morphometric indices of erythrocytes and their nucleus, especially EL, EW, NL, and NW were also affected at high water temperature. Collectively, our results indicate that the immune system of *E. akaara* may be affected at high water temperature (31 °C and 34 °C) as because of alteration in blood cell morphology and morphometry. This fine information may be helpful to monitor the health status of red-spotted grouper and probably for other fish species.

Chapter 2.2

Physiological and histological responses in red-spotted grouper

Abstract

Rising of water temperature due to climate change is a great concern to fishery biologists. Hence, the present study investigated the physiological and histological responses of red-spotted grouper under different rearing temperatures. The increased temperature effect was examined in two separate experiments (Experiment 1: 24 °C, 28 °C, 32 °C, and 36 °C for 28 days; Experiment 2: 25 °C, 28 °C, 31 °C, and 34 °C for 42 days). Blood and tissue were sampled after 1, 14, 28 days of Exp. 1 and 2, 7, 42 days of Exp. 2. In experiment 1, hematocrit (Ht), hemoglobin (Hb), and red blood cell (RBC) count increased at 36 °C. Biochemical results showed significant changes in glucose, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and cortisol level at 36 °C compared with the other groups (24 °C, 28 °C, and 32 °C). Histological results showed epithelial necrosis and shortening of secondary gill lamellae in gill; cytoplasmic vacuolization, shrinkage, and coalescence of hepatocytes in liver of the 36 °C group. In experiment 2, RBC count and Hb level changed at 31 °C and 34 °C. Biochemical results showed high levels of glucose, cortisol, GPT, GOT, and lactate dehydrogenase (LDH); low levels of triglyceride (TG) and total cholesterol (TCHO) in the 34 °C group compared with the others (25 °C, 28 °C, and 31 °C) ($P < 0.05$). Histological results presented hyperplasia and curling in secondary gill lamellae, and swelling of primary gill lamellae in gill; the coalescence of hepatocytes, cytoplasmic vacuolization, and dilation of the sinusoid in liver of the 34 °C group. In both experiments, high water temperature (34 °C and 36 °C) altered the hemato-biochemical indices and tissue morphology. Collectively, the present study suggests that 34 °C and 36 °C is the sub-lethal and lethal temperature, respectively for *E. akaara*. Besides, 31 °C and 32 °C also provoke physiological disturbances after prolonged exposure.

Keywords: Red-spotted grouper, *Epinephelus akaara*; Temperature; Hematological response; Biochemical response; Histology

1. Introduction

Water temperature is one of the most important environmental factors in aquaculture farming. Increases in water temperature can occur due to natural causes, climate change, or anthropogenic sources. The rise in water temperature in aquatic environment is a great concern of aquaculturists and fishery biologists (Langford, 2001; Somero, 2010). The change in water temperature may significantly affect the normal physiological process, survival, and growth of teleost fish (Person-Le Ruyet et al., 2004; Fazio et al., 2018). In addition, the temperature change can alter the metabolic activities (Lu et al., 2016), weaken the non-specific immune defense system (Qiang et al., 2013), and intensification of disease risk (Karvonen et al., 2010). Temperature affects virtually all biochemical and physiological activities of an animal. Thermal stress is one of the most important environmental challenges that fishes may face (Portner and peck, 2010).

Fish are very susceptible to temperature fluctuations, showing a variety of morphological and physiological changes (Mora and Maya, 2006). Hematological and biochemical parameters are used as reliable indicators of physiological stress responses to endogenous or exogenous changes in fish. These parameters are considered useful indicators for fish health analysis (Cataldi et al., 1998; Lermen et al., 2004; Fazio et al., 2018; Panase et al., 2018). Cortisol is the primary stress hormone secreted from the hypothalamus-pituitary-interrenal (HPI) axis when a fish encounters environmental disturbance. Prolonged or acute thermal stress induces the secretion of cortisol, followed by an elevation of plasma glucose levels to support increased energy utilization (Lima et al., 2006). The activities of GPT, GOT and LDH in the blood can be used as stress biomarkers to monitor fish physiological responses to thermal stress (Cheng et al., 2018). Changes

in plasma total protein are used as an indicator of liver impairment (Fırat and Kargın, 2010). In addition, blood lipids have also been proposed as an important chronic stress indicator during thermal stress (Ming et al., 2012). Besides, morphological changes of gill and liver in response to sudden or prolonged thermal stress have also been observed in fish (Liu et al., 2015; Hernandez-Lopez et al., 2018).

Groupers are warm-water fish that grow higher than 24 to 30 °C; most of them prefer a thermal range higher than 15 to 35 °C (Heemstra and Randall, 1993; Rimmer et al., 2004). The red-spotted grouper (*Epinephelus akaara*) is a subtropical species of serranidae family, which prefers to inhabit shallower depths of the coastal area. It is a promising aquaculture species due to its high market value in southern Japan, Korea, Hong Kong, Taiwan, and southern China (Sadovy and Cornish, 2000). The red-spotted grouper has been enlisted as an endangered species by IUCN (International Union for the Conservation of Nature) as its natural landing is reducing due to overexploitation, habitat degradation, and climate change effects (Baillie et al., 2004; Tupper and Sheriff, 2008). Therefore, in-depth knowledge about the thermal physiology of red-spotted grouper is required for better management and aquaculture.

The effect of water temperature on blood biochemical and hematological indices have been studied in many species (Shajahan et al., 2018; Panase et al. 2018; De et al., 2019; Islam et al., 2019; Mattioli et al., 2019); but, there remains a paucity of information about this in *E. akaara*. Our previous research investigated a narrow range of water temperature to evaluate the physiological and growth responses of red-spotted grouper (Cho et al., 2015; Lee and Baek, 2018). However, physiological and histological responses of *E. akaara* under a wide thermal range needs to evaluate in details, especially, in the face of rising water temperature due to climate chang effect. We, thus, intended to conduct the present study to make a better understanding of physiological

and histological responses in red-spotted grouper under different rearing temperatures. The results obtained from this work could be used to monitor the physiological status of the red-spotted grouper, which will ensure better management approach in grouper aquaculture.

2. Materials and methods

2.1. Experimental fish collection and maintenance

E. akaara juveniles were collected from the Marine Science Institute, Jeju National University, Korea and reared at the laboratory of Marine Biology Department, Pukyong National University (PKNU), Busan, Korea. Experimental fish were immediately dipped in 30 ppm oxytetracycline (Chamshin Pharma Co. Ltd., Seoul, Korea) after being transferred to the laboratory. Fish were acclimated for 2 weeks before starting the experiment. Fish maintenance, handling, and sampling were conducted according to the guidelines of the Animal Ethics Committee of Pukyong National University (PKNU) (Regulation No. 554).

2.2. Experimental design and thermal exposure

In experiment 1, 180 juveniles of red-spotted grouper (total length: 9.4 ± 0.12 cm; body weight: 12.89 ± 0.61 g) were exposed to four temperature conditions (24 °C as control, 28 °C, 32 °C, and 36 °C), each with three replications for 28 days. Upon completion of experiment 1, we carried out the experiment 2 with 180 juveniles of red-spotted grouper (total length: 8.28 ± 0.10 cm, body weight: 8.53 ± 0.27 g). Fish were exposed to different water temperatures such as 25 °C as control, 28 °C, 31 °C, and 34 °C for 42 days.

In both experiments, fish were randomly stocked in 12 glass aquaria (15 fish/aquarium), each with a volume of 120 L. Each aquarium had equal size and height (75cm × 45cm × 45cm), and was equipped with the recirculating filtration system. The temperature of the experimental tank was constantly increased at a rate of 1 °C h^{-1} using a thermostat (OKE-6422H; OKE, Busan,

Korea) from the control temperature until the experimental temperature achieved. Upon reaching the target temperature, the experimental period was started to count. During the experiment, water temperature, pH, salinity, and dissolved oxygen were monitored daily using a multiparameter water-quality meter (HI9829; Hanna Instrumentals, USA) and total ammonium levels were checked every 2 days with NH_3/NH_4^+ test kit (Tetra GmbH, Melle, Germany). The water quality parameters measured during the experimental period are shown in Table 2.2.1. During the acclimation and experimental period, fish were fed with a commercial diet (Otohime Hirame, Marubeni Nisshin Feed Co., Ltd., Japan; 50~52% protein and 7~10% lipids). Fish were fed 2 times daily (09:00 and 18:00 h) at a rate of 2% of their body weight during the experimental period. Uneaten food was removed after 30 minutes of feeding and 10% of the water in each aquarium was replaced daily with filtered clean seawater.

2.3. Sampling protocol

In experiment 1, fish were sampled 1, 14, and 28 days after thermal exposure. The second (14 days) and third sampling (28 days) were conducted only in the 24 °C, 28 °C, and 32 °C groups as because all fish in the 36 °C group died after 1 day of thermal exposure. In experiment 2, fish were sampled 2, 7, and 42 days after thermal exposure. The sampling schedule is shown in Figure 2.2.1a.

All fish were fasted for 24h prior to sampling. 15 fishes were randomly sampled from each temperature group (i.e., 5 fishes from each aquarium; n=15) on each sampling day. All fish were lightly anaesthetized with 300 mg/L 2-phenoxyethanol (Sigma Aldrich, USA) before blood collection. Blood samples were drawn from the caudal vein using heparinized capillary tubes and stored in 1.5 mL centrifuge tubes for further analysis of hematological and biochemical indices. Gill and liver were surgically removed on ice and preserved for histological analysis.

2.4. Hematological analysis

The Ht values were determined by collecting blood samples in glass capillary tubes and centrifuged (5 min, 12,000 rpm) in a microhematocrit centrifuge machine (Hematocrit Centrifuge VS-12000; Vision Scientific Co. Ltd., Seoul, Korea). The Hb levels were measured by placing the blood sample (10 µL) on Hb slides that were then read by an automatic analyzer (FUJI DRI-CHEM 400i; Fuji Film Co., Tokyo, Japan). RBCs were counted under a microscope with a hemocytometer, using Hayem diluting fluid (Ricca Chemical Co., USA). Mean corpuscular volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated from the average values of HB % (Dacie and Lewis, 1984).

2.5. Biochemical analysis

Plasma was immediately separated from blood samples by centrifugation at 4 °C (15 min at 13,000 g) and the supernatant was stored at –70 °C for further analysis. The studied biochemical parameters were determined from the collected plasma samples using an automatic analyzer that had been validated as a suitable method for analyzing fish plasma (Krome, 2014).

2.6. Radioimmunoassay

To measure cortisol levels in plasma, steroids were extracted twice using 2 mL diethyl ether, dried under liquid nitrogen gas and re-suspended in phosphate buffer (pH = 7.5). Plasma cortisol levels were determined by radioimmunoassay (RIA), following the method of Kobayashi and Mikuni (1987). Cortisol antiserum was purchased from Cosmo-Bio Co. Ltd. (Tokyo, Japan). Non-radioactive steroid standards were procured from Steraloids Inc. (Wilton, USA). Radio-labeled steroids ([3H]-cortisol) were purchased from Amersham Life sciences (Piscataway, USA).

2.7. Gill and liver histology

For histological observations, gills and liver were dissected immediately and fixed in 10% neutral formalin, dehydrated through a graded series of ethanol concentrations, and embedded in paraffin to form tissue blocks. Sections with a thickness of 5 μm were stained with Mayer's hematoxylin-eosin and observed under a light microscope (BX-50; Olympus, Tokyo, Japan).

2.8. Statistical analysis

Data analysis were performed using SPSS Statistics software Version 21 (IBM Corp., USA). All data are presented as means ± standard error of the mean (SEM) unless otherwise stated. Normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) were verified prior to statistical evaluation. One-way analysis of variance (ANOVA) was performed to examine the effects of different temperature treatments on physiological parameters at different sampling points. Duncan's multiple range tests was used to identify significant differences among treatment groups ($P < 0.05$).

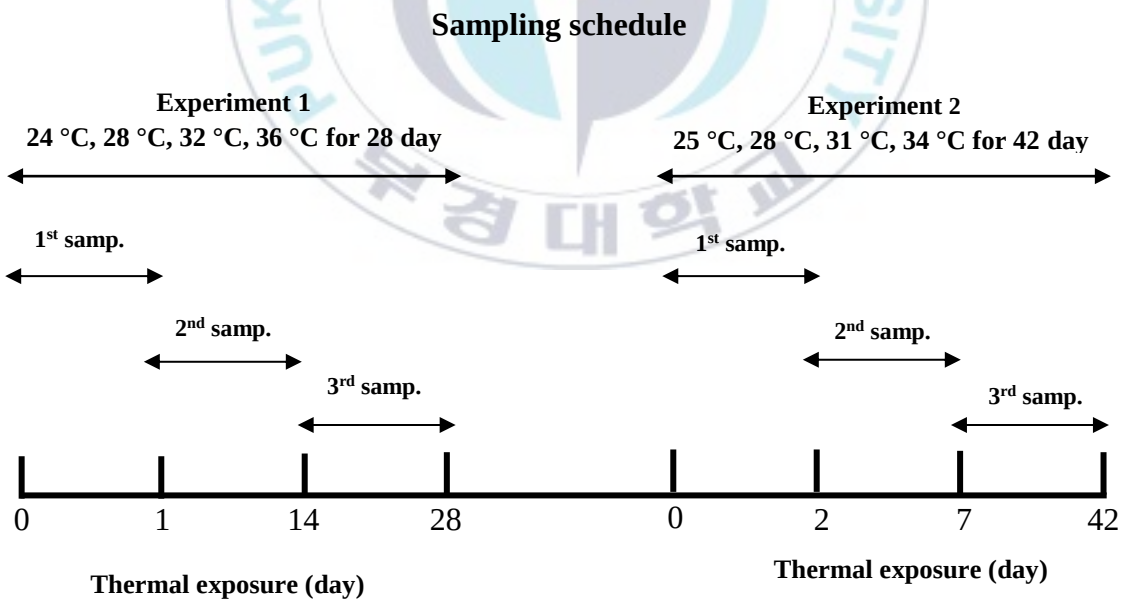


Fig. 2.2.1a. The sampling schedule during the experimental period.

Table 2.2.1. Water quality parameters (Mean ± SEM) measured during the period of experiment.

Parameters	Experiment 1				Experiment 2			
	Temperature groups				Temperature groups			
	24 °C	28 °C	32 °C	36 °C	25 °C	28 °C	31 °C	34 °C
Temp.	23.82 ± 0.02	27.71 ± 0.17	31.88 ± 0.02	35.65 ± 0.31	24.89 ± 0.04	27.77 ± 0.04	30.90 ± 0.05	33.78 ± 0.04
Salinity (psu)	33.67 ± 0.09	33.81 ± 0.04	33.76 ± 0.06	33.80 ± 0.13	33.80 ± 0.05	33.89 ± 0.06	33.72 ± 0.05	33.77 ± 0.07
pH	7.95 ± 0.02	7.89 ± 0.02	7.90 ± 0.02	7.97 ± 0.01	8.05 ± 0.02	8.06 ± 0.01	7.99 ± 0.02	7.99 ± 0.02
DO (mg/L)	6.96 ± 0.06	6.66 ± 0.04	5.62 ± 0.06	4.16 ± 0.14	6.86 ± 0.04	6.72 ± 0.04	5.68 ± 0.05	4.16 ± 0.03
NH4 (mg/L)	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

3.1. Results: Experiment 1

3.1.1 Behavioral changes and mortality

In experiment 1, fish in the 36 °C group exhibited gasping, rapid opercular movements, jerky motions, attempts to escape the testing tank, revolution along their axis, loss of equilibrium, and finally death due to acute thermal stress. All fish in the 36 °C group died following the thermal shock received on day 1 (Table 2.2.2).

Table 2.2.2. Surviaval rate (%) of juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (24 °C, 28 °C, 32 °C, and 36 °C) for 28 days.

	Treatments	Exposure time (day)		
		1	14	28
Survival (%)	24 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	28 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	32 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	36 °C	0.00 ± 0.00[b]	-	-

Data are presented as mean ± SEM of three replicates (n=15). Means with different lowercase letters in the same column indicate the significant difference among treatment groups at equivalent time points (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.1.2 Hematological effects

After 1 day, thermal stress induced a significant increase in Ht level at 28 °C, progressing to a maximum at 36 °C. Significantly higher Ht values were detected in the 32 °C group after 28 days of exposure. RBC counts were significantly higher in the 36 °C group on day 1, whereas no changes were observed in the other thermal groups (24 °C, 28 °C, and 32 °C) after 28 days of exposure. Fish exposed to 36 °C had significantly lower Hb levels after 1 day of exposure, whereas in the other groups no effects were observed after 28 days of exposure (Table 2.2.3).

Table 2.2.3: Changes in hematocrit, red blood cell (RBC) count, and hemoglobin levels of red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (24 °C, 28 °C, 32 °C, and 36 °C) for 28 days.

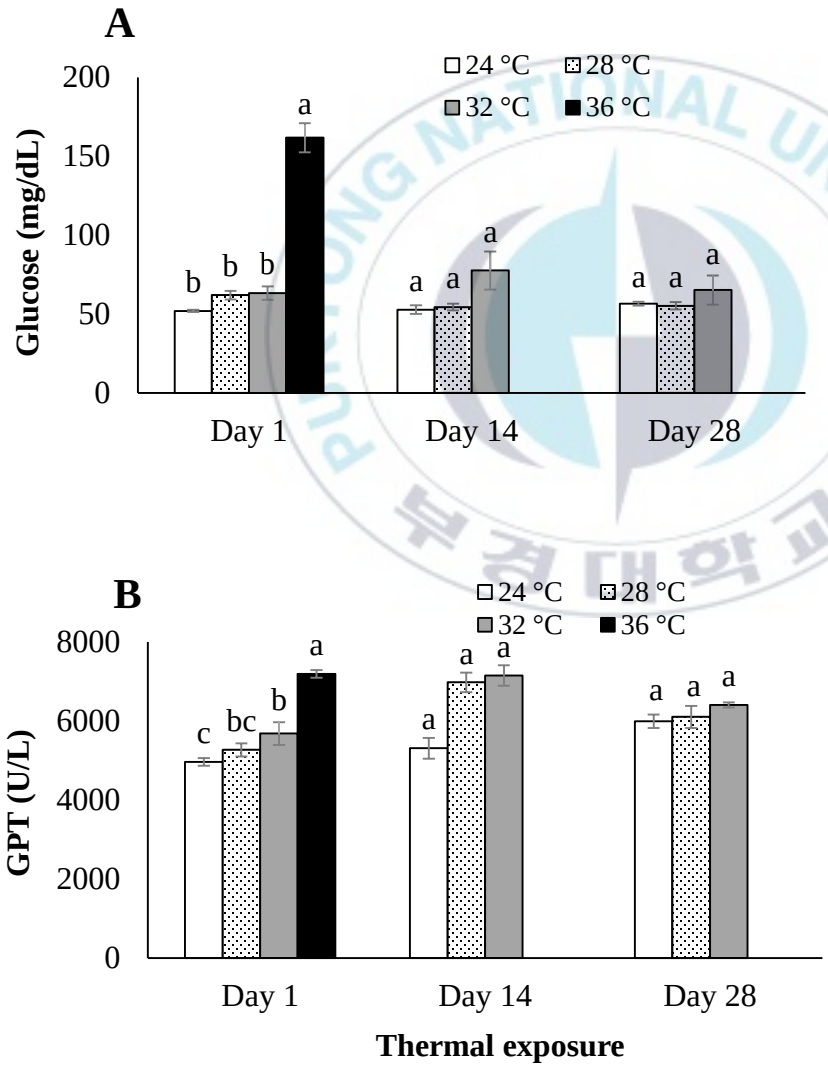
Parameters	Treatments	Thermal exposure (days)		
		1	14	28
Ht (%)	24 °C	31.44 ± 1.11^{c}	37.83 ± 0.25^{a}	33.66 ± 0.28^{b}
	28 °C	34.92 ± 1.12^{b}	38.95 ± 0.69^{a}	33.93 ± 0.34^{b}
	32 °C	36.16 ± 0.25^{b}	39.89 ± 0.95^{a}	38.13 ± 0.07^{a}
	36 °C	41.24 ± 0.12^{a}	-	-
Hb (g/dL)	24 °C	8.10 ± 0.27^{a}	8.75 ± 0.23^{a}	8.54 ± 0.54^{a}
	28 °C	8.13 ± 0.14^{a}	8.45 ± 0.15^{a}	8.53 ± 0.07^{a}
	32 °C	8.18 ± 0.39^{a}	7.67 ± 0.19^{b}	8.02 ± 0.18^{a}
	36 °C	7.42 ± 0.18^{b}	-	-
RBC ($\times 10^{6}$/mm^{3})	24 °C	5.31 ± 0.05^{bc}	5.48 ± 0.07^{a}	5.31 ± 0.28^{a}
	28 °C	5.12 ± 0.07^{c}	4.87 ± 0.05^{b}	5.18 ± 0.14^{a}
	32 °C	5.53 ± 0.17^{b}	5.15 ± 0.13^{ab}	5.53 ± 0.18^{a}
	36 °C	6.01 ± 0.05^{a}	-	-

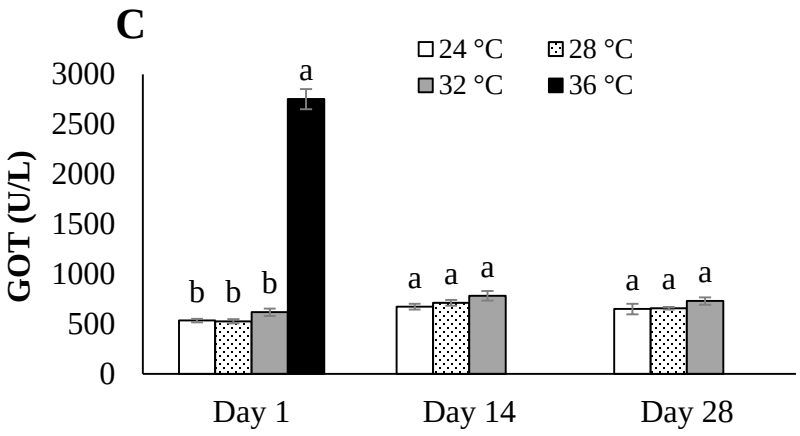
Values are mean ± SEM of three replicates (n=15). Different lowercase letters in the same column indicate the significant difference between groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).

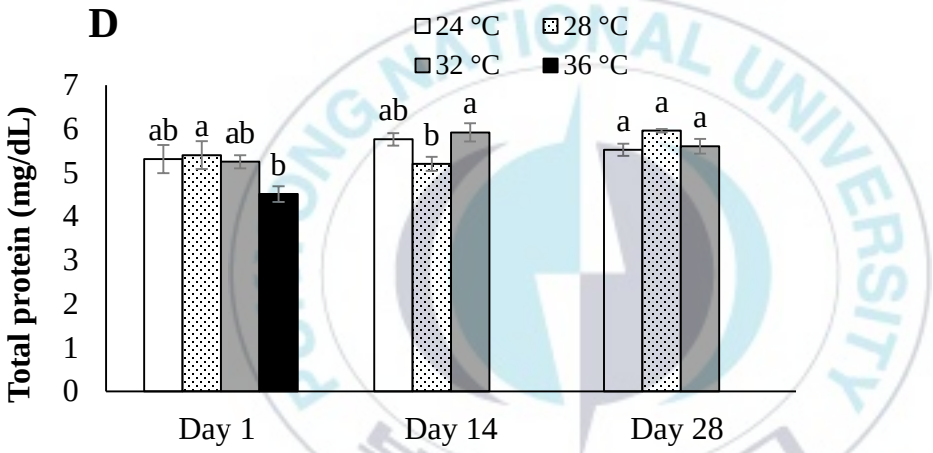
3.1.3 Biochemical effects

Plasma glucose levels in the 36 °C group increased drastically on day 1, but remained unchanged in the other test groups (Fig. 2.2.1A). Plasma GPT in the 36 °C group was significantly increased after 1 day of exposure, whereas no significant changes were recorded in the other groups at the same sampling points (Fig. 2.2.1B). Thermal stress significantly increased plasma GOT

levels in the 36 °C group on day 1 to five times higher than those observed in the other groups; no significant effects were observed in the other groups after 14 and 28 days (Fig. 2.2.1C). After 1 day, plasma total protein levels decreased in the 36 °C group compared with those in the other groups (24 °C, 28 °C, 32 °C) (Fig. 2.2.1D). Plasma cortisol levels were significantly higher in the 32 °C and 36 °C groups than in the 24 °C and 28 °C groups. After 28 days, a significant increase in cortisol levels was detected in the 32 °C group (Fig. 2.2.1E).




C
24 °C
28 °C
32 °C
36 °C
GOT (U/L)
3000
2500
2000
1500
1000
500
0
b b b a
a a a
a a a
Day 1
Day 14
Day 28


D
24 °C
28 °C
32 °C
36 °C
Total protein (mg/dL)
7
6
5
4
3
2
1
0
ab a ab b
ab b a
a a a
Day 1
Day 14
Day 28

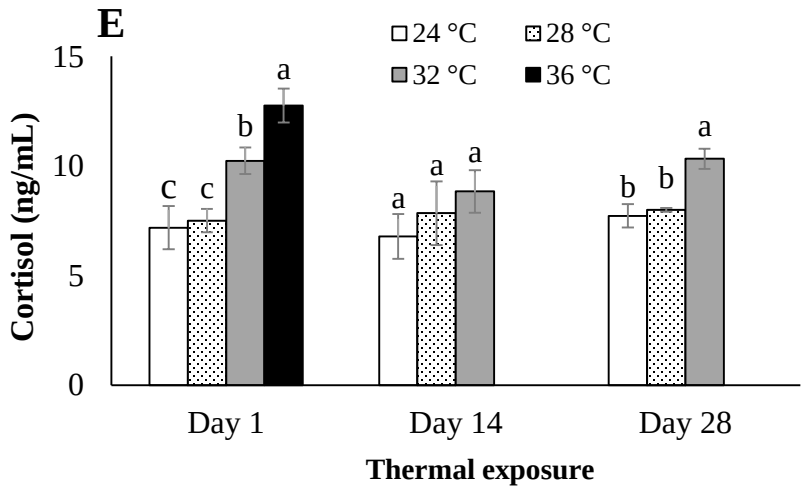
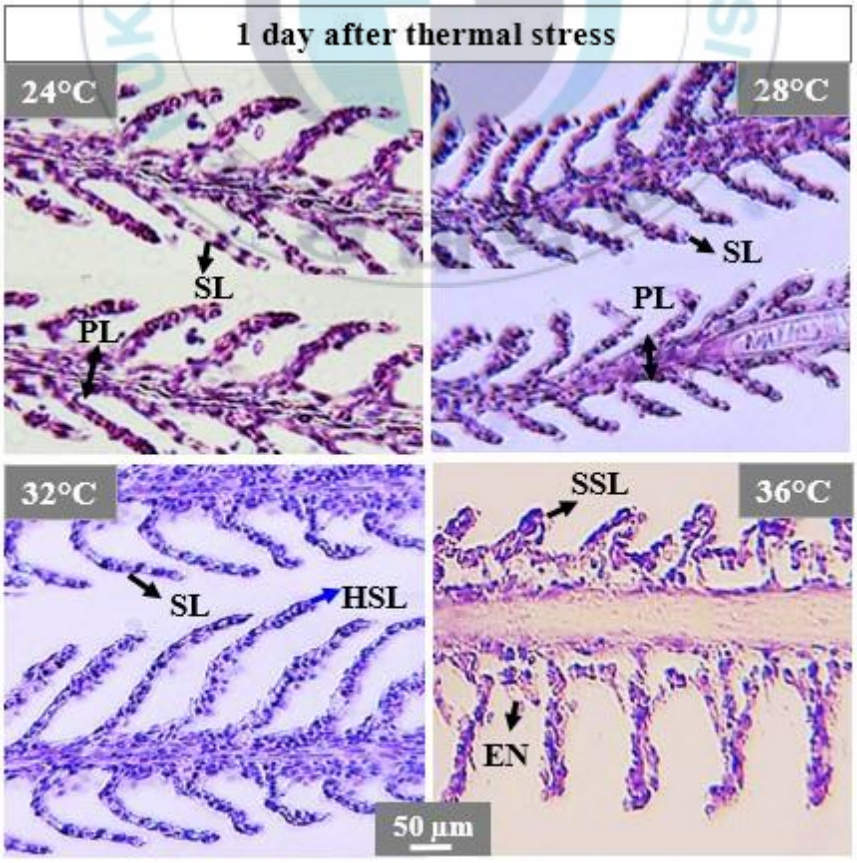

E
24 °C
28 °C
32 °C
36 °C
Cortisol (ng/mL)
15
10
5
0
c c b a
a a a
b b a
Day 1
Day 14
Day 28
Thermal exposure

Fig. 2.2.1(A-E) Changes in plasma glucose, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), total protein and cortisol levels of *Epinephelus akaara* exposed with different water temperatures (24 °C, 28 °C, 32 °C, and 36 °C) for 28 days. Values are mean ± SEM of three replicates (n=15). Different lowercase letters indicate the significant difference between groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.1.4 Histological effects in gills

One day after exposure, gills of the 36 °C group were sharply affected by thermal stress compared with those in the 24 °C, 28 °C, and 32 °C groups (Fig. 2.2.2A–C). The major alterations detected in the 36 °C group were epithelial necrosis (EN), shortening of secondary gill lamellae (SSL), and lamellar disorganization (Fig. 2.2.2D). 14 and 28 days after exposure, no thermal effects were observed (Fig. 2.2.2E-I), except for hyperplasia in the secondary gill lamellae (HSL) in the 32 °C group on day 28 (Fig. 2.2.2J).



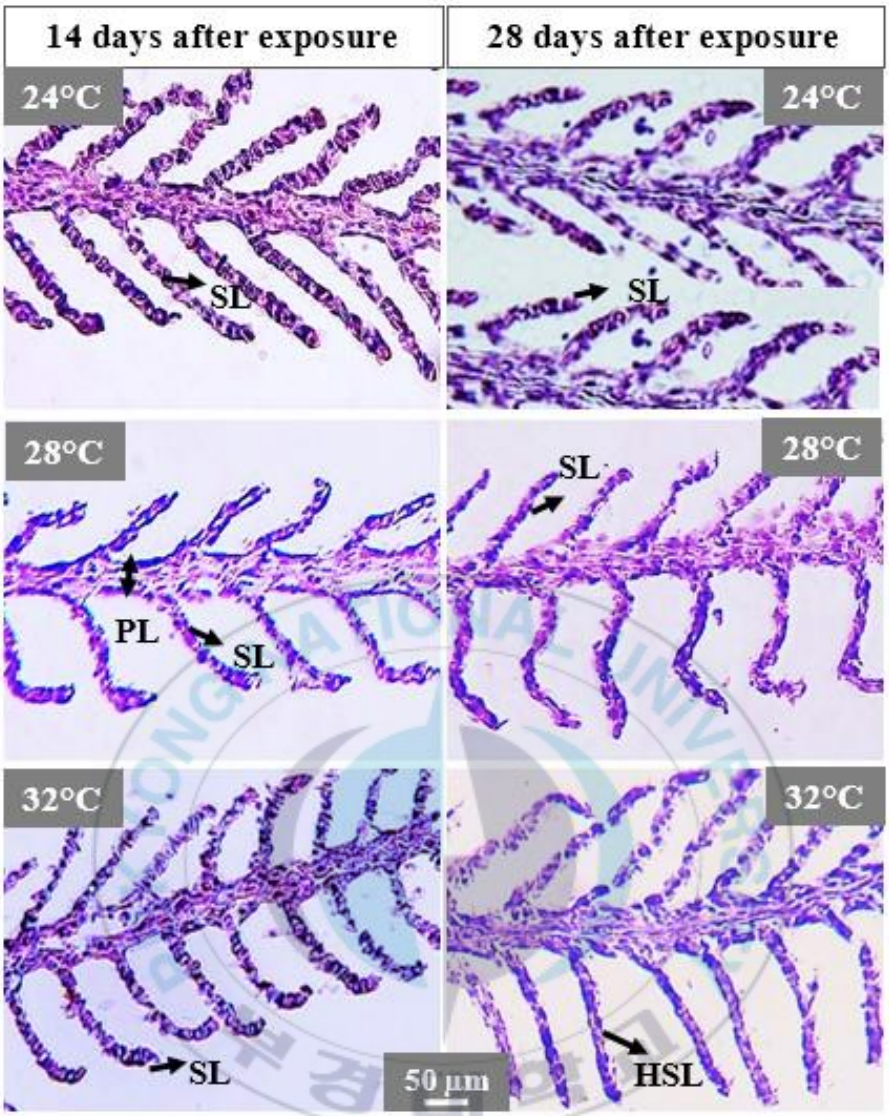
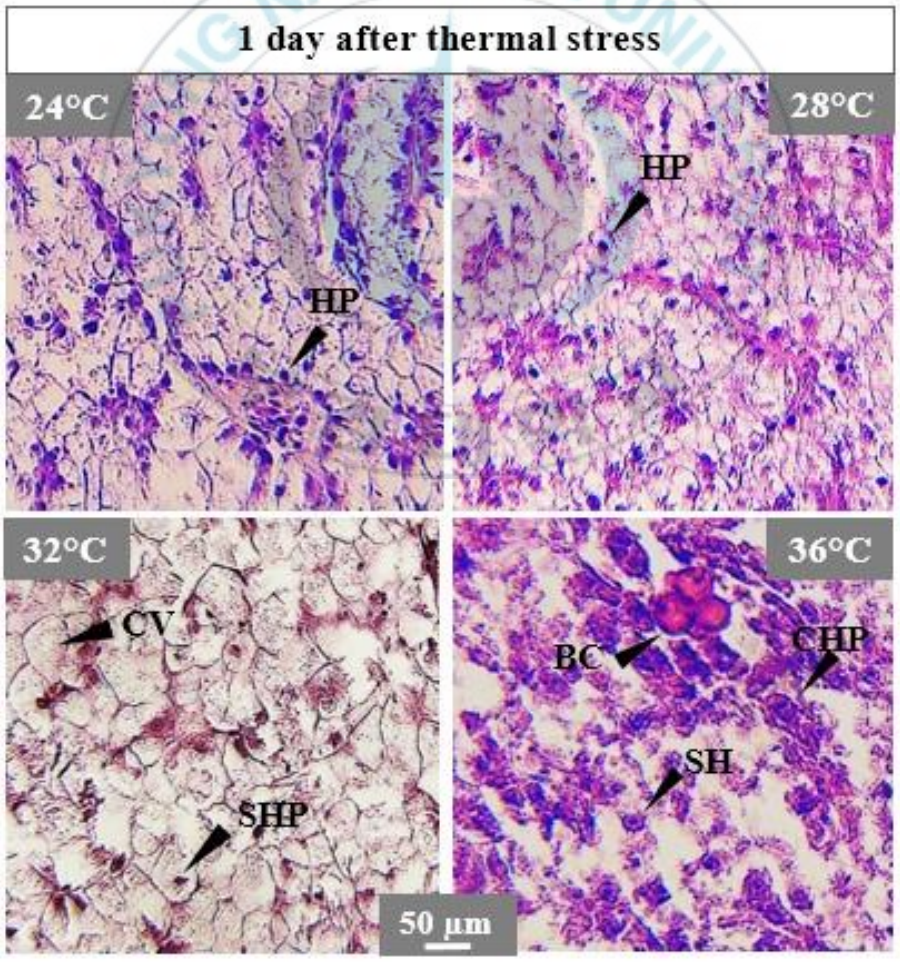


Fig. 2.2.2 (A-I). Gill histology of red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (24 °C, 28 °C, 32 °C, and 36 °C) for 28 days. PL, secondary gill lamellae; SL, secondary gill lamellae; HSL, hyperplasia of secondary gill lamellae; EN, epithelial necrosis; SSL, shortening of secondary gill lamellae. Scale bars = 50 μm.

3.1.5 Histological effects in the liver

One day after exposure, liver exposed to both 32 °C and 36 °C were acutely damaged compared with those in the 24 °C and 28 °C groups (Fig. 2.2.3A-B). Signs of this damage in the 36 °C group included coalescence of hepatocytes (CHP), blood clotting in the sinusoid (BC), and shrinkage of hepatocytes (SH) (Fig. 2.2.3D), whereas swollen hepatocytes (SHP) and cytoplasmic vacuolization (CV) were observed in the 32 °C group (Fig. 2.2.3C). 14 and 28 days after exposure, no visible change was noted in the thermal exposure groups (Fig. 3E-I), except for CV in the 32 °C on day 28 (Fig. 2.2.3J).



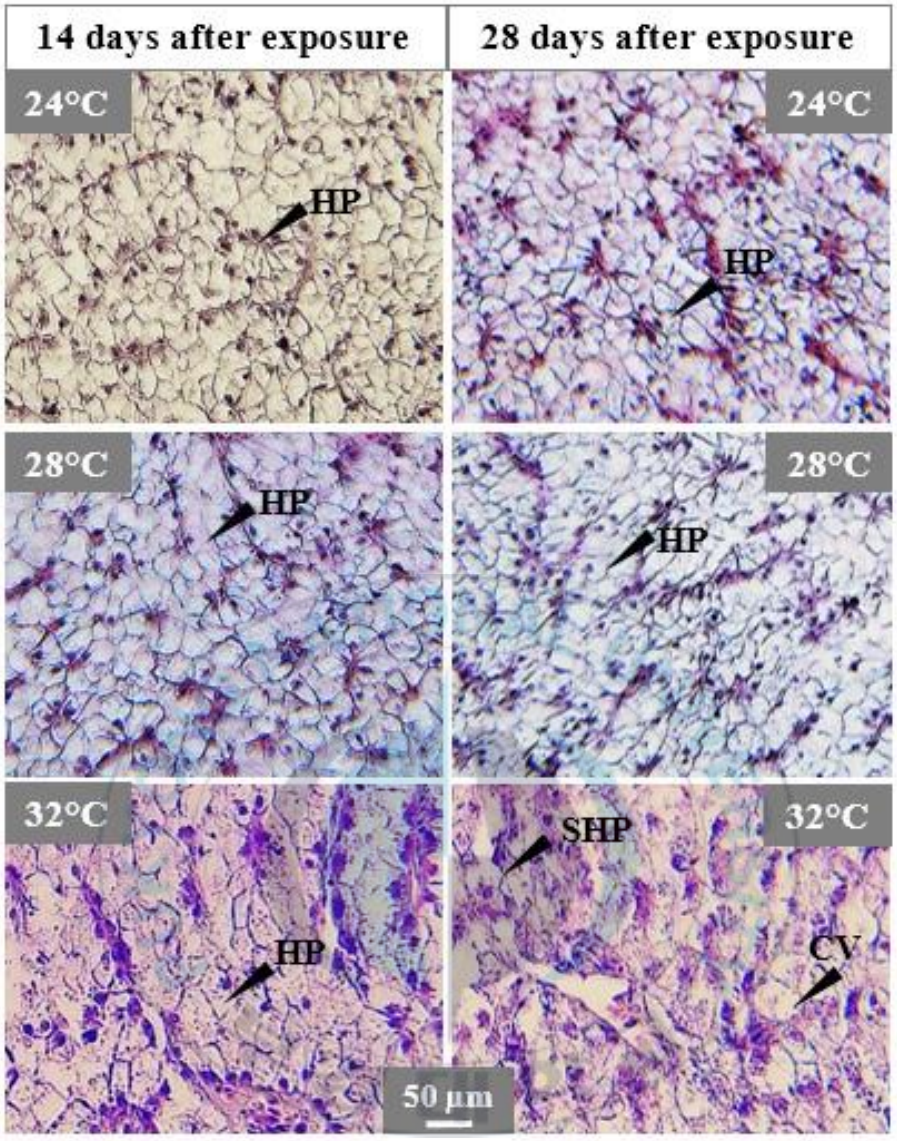


Fig. 2.2.3 (A-J). Liver histology of red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (24 °C, 28 °C, 32 °C, and 36 °C) for 28 days. HP, hepatocytes; SHP, swollen hepatocytes; CV, cytoplasmic vacuolization; SH, shrinkage of hepatocytes; LG, lipid globule, BC, blood clotting in sinusoid; DS, dilation of sinusoid; CHP, coalesce of hepatocytes. Scale bars, 50 µm.

3.2. Results: Experiment 2

3.2.1 Behavioral changes and mortality

In experiment 2, fish in the 34 °C showed fast opercular activities and irregular body movements. 42 days after thermal exposure, mortility was observed in 34 °C group (Table. 2.2.4). On the other hand, fish of the 25 °C, 28 °C, and 31 °C groups exhibited normal body movements without any signs of external distress.

Table 2.2.4. Survival rate (%) of juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days.

	Treatments	Exposure time (day)		
		2	7	42
Survival (%)	25 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	28 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	31 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	34 °C	100.00 ± 0.00[a]	100.00 ± 0.00[a]	80.95 ± 4.76[b]

Data are presented as mean ± SEM of three replicates (n=15). Means with different lowercase letters in the same column indicate the significant difference among treatment groups at equivalent time points (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.2.2 Hematological changes

The results for the hematological analysis are shown in Table. 2.2.3. The thermal shock did not affect ($P > 0.05$) Hb, Ht, and MCHC of juvenile *E. akaara* throughout the experimental period. The erythrocyte count significantly increased in 34 °C group at each sampling points (2, 7, and 42 days), compared with other temperatures (25 °C, 28 °C, and 31 °C), which was associated with a significant change in MCV and MCH values ($P < 0.05$). Additionally, changes in

erythrocyte count, MCV and MCH were also observed in 31 °C group after 42 days of exposure, though the changes were insignificant for MCV and MCH ($P > 0.05$; Table. 2.2.5).

Table. 2.2.5. Hematological values (HB, Ht, RBC, MCV, MCH, and MCHC) of blood samples of juvenile red-spotted grouper, *Epinephelus akaara* submitted to different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days.

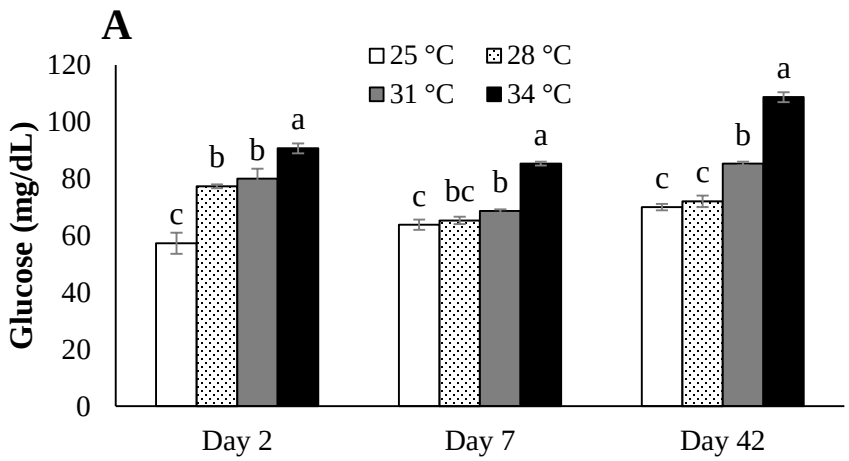
Parameters	Treatments	Exposure time (day)		
		2	7	42
Ht (%)	25 °C	36.92 ± 0.68^{b}	32.55 ± 0.62^{b}	34.78 ± 0.22^{c}
	28 °C	36.83 ± 1.08^{b}	35.78 ± 0.62^{ab}	36.67 ± 0.84^{bc}
	31 °C	38.17 ± 1.02^{ab}	34.11 ± 1.82^{ab}	38.89 ± 0.59^{ab}
	34 °C	40.92 ± 1.16^{a}	36.34 ± 0.67^{a}	40.13 ± 0.99^{a}
Hb (mg/dL)	25 °C	7.84 ± 0.11^{a}	7.80 ± 0.18^{c}	7.62 ± 0.09^{b}
	28 °C	7.83 ± 0.27^{a}	7.84 ± 0.16^{bc}	8.69 ± 0.09^{a}
	31 °C	8.23 ± 0.33^{a}	8.23 ± 0.04^{ab}	8.90 ± 0.15^{a}
	34 °C	8.39 ± 0.12^{a}	8.68 ± 0.13^{a}	9.18 ± 0.27^{a}
RBC ($\times 10^{6}$/mm^{3})	25 °C	3.99 ± 0.15^{b}	3.89 ± 0.05^{bc}	3.68 ± 0.04^{c}
	28 °C	3.92 ± 0.09^{b}	3.75 ± 0.16^{c}	3.61 ± 0.07^{c}
	31 °C	4.06 ± 0.19^{b}	4.16 ± 0.09^{b}	4.53 ± 0.19^{b}
	34 °C	5.18 ± 0.24^{a}	5.14 ± 0.04^{a}	5.30 ± 0.09^{a}
MCV (fl)	25 °C	93.25 ± 5.46^{a}	83.71 ± 1.78^{b}	95.25 ± 1.84^{ab}
	28 °C	94.90 ± 4.62^{a}	95.83 ± 3.87^{a}	101.80 ± 0.74^{a}
	31 °C	94.62 ± 2.27^{a}	78.60 ± 1.95^{bc}	86.53 ± 4.08^{b}
	34 °C	79.60 ± 2.08^{b}	71.97 ± 1.19^{c}	76.01 ± 2.05^{c}
MCH (pg)	25 °C	19.72 ± 0.88^{ab}	20.07 ± 0.37^{a}	20.89 ± 0.48^{b}
	28 °C	20.16 ± 1.03^{ab}	20.97 ± 0.45^{a}	24.11 ± 0.53^{a}
	31 °C	20.54 ± 1.69^{a}	20.03 ± 0.33^{a}	19.85 ± 1.13^{b}
	34 °C	16.31 ± 0.82^{b}	16.87 ± 0.25^{b}	17.34 ± 0.36^{c}
MCHC (g/dL)	25 °C	21.33 ± 0.38^{a}	24.04 ± 0.26^{ab}	21.96 ± 0.19^{a}
	28 °C	21.33 ± 0.19^{a}	22.02 ± 0.49^{c}	23.78 ± 0.37^{a}
	31 °C	21.75 ± 1.56^{a}	25.54 ± 0.39^{a}	22.97 ± 0.61^{a}
	34 °C	20.58 ± 0.83^{a}	23.58 ± 0.79^{bc}	22.97 ± 1.11^{a}

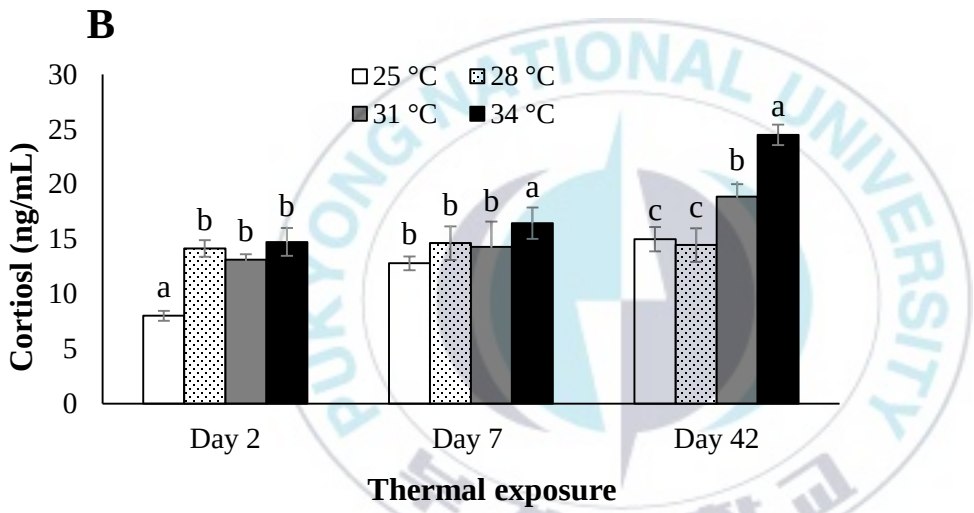
Values are mean ± SEM of three replicates (n=15). Different lowercase letters in the same column indicate the significant differences among the temperature groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).

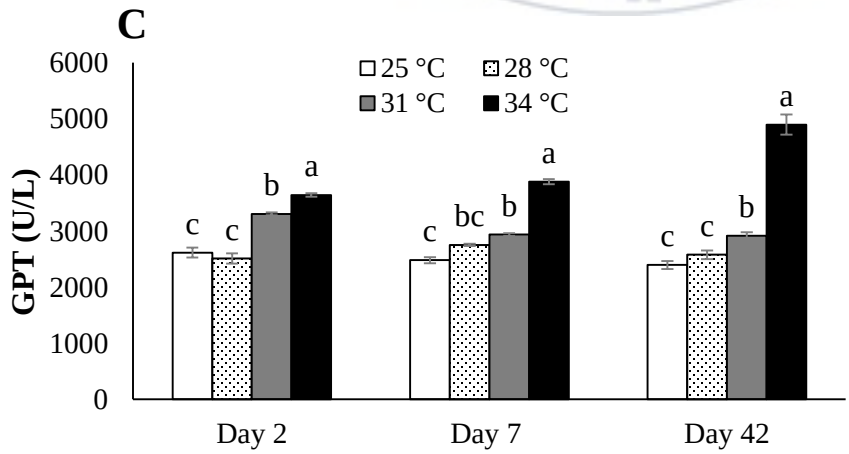
3.2.3 Biochemical changes

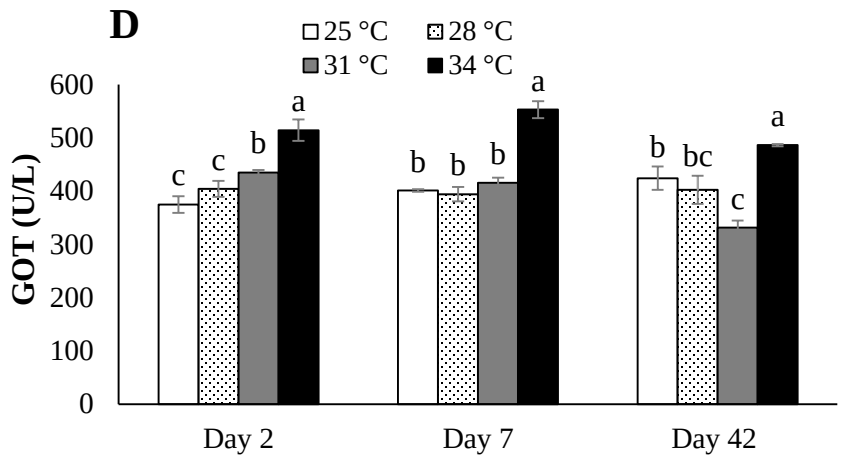
After exposure to thermic shock, plasma glucose, cortisol, GPT, and GOT were significantly increased ($P < 0.05$) in the 34 °C group in comparison to the others (25 °C and 28 °C) and remained unchanged until 42 days of exposure. However, in case of 31 °C, the elevation ($P < 0.05$) were observed after 2 and 42 days of sampling points (Fig. 2.2.4A-D).

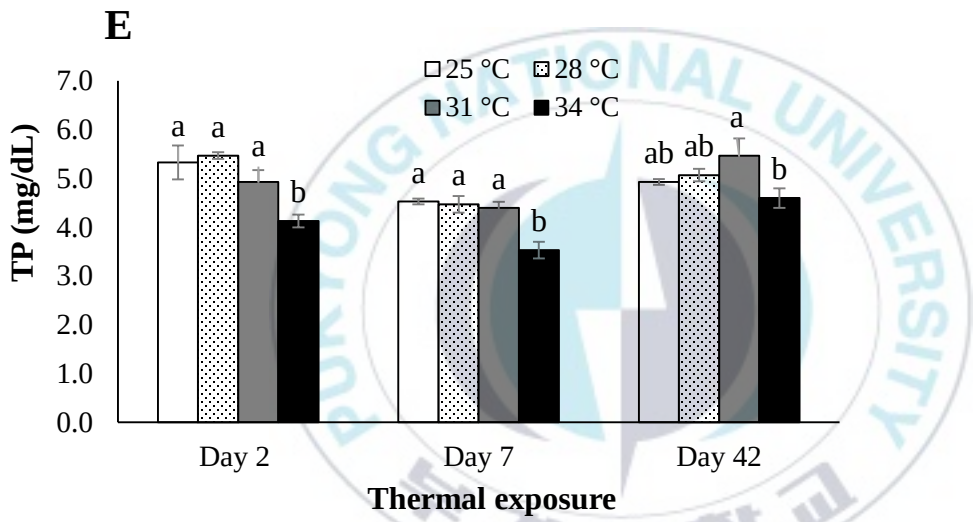
The plasma TP, TG, and TCHO level of 34 °C group were found significantly lower ($P < 0.05$) at each sampling points (2, 7 and 42 days), compared with the other temperatures (25 °C, 28 °C, and 31 °C) (Fig. 2.2.4 E-G). Besides, lower TG and TCHO values ($P < 0.05$) were recorded in 31 °C group after 42 days of exposure (Fig. 4F-G). The plasma enzyme LDH exhibited higher values ($P < 0.05$) in the 31 °C and 34 °C groups than in the 25 °C and 28 °C groups after 2 days of exposure; however, its level became normal ($P > 0.05$) in 31 °C group after 7 and 42 days of exposure, though the level was mounting higher in 34 °C group (Fig. 2.2.4 H). The plasma ALP showed significantly higher level ($P < 0.05$) in 31 °C and 34 °C groups compared with 25 °C and 28 °C groups at each sampling points (Fig. 2.2.4 I).

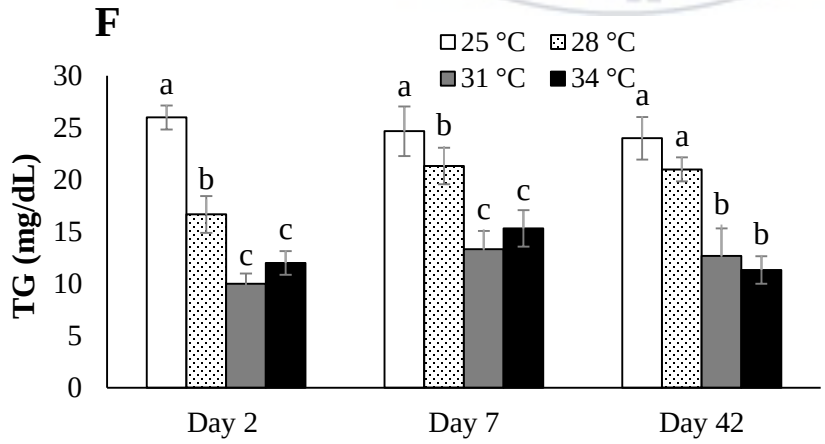

A
25 °C
28 °C
31 °C
34 °C
Glucose (mg/dL)
120
100
80
60
40
20
0
c
b
b
a
c
bc
b
a
c
c
b
a
Day 2
Day 7
Day 42

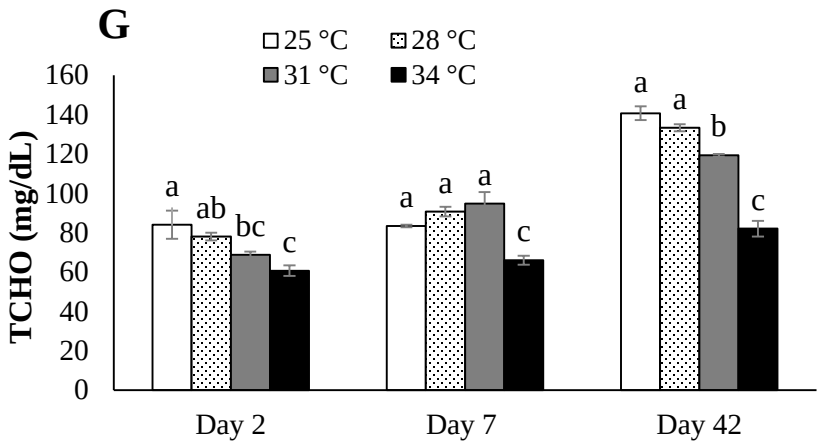

B
25 °C
28 °C
31 °C
34 °C
Cortiosl (ng/mL)
30
25
20
15
10
5
0
a
b
b
b
b
b
b
a
c
c
b
a
Day 2
Day 7
Day 42
Thermal exposure

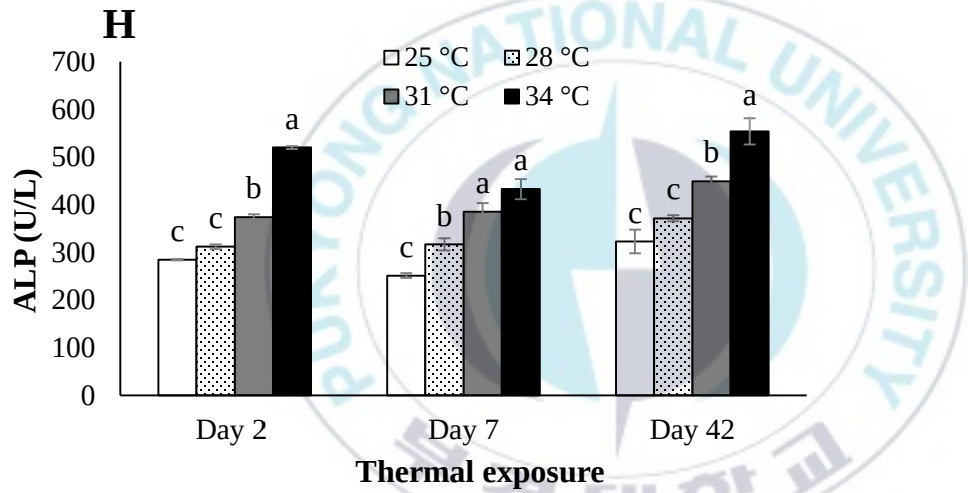

C
25 °C
28 °C
31 °C
34 °C
GPT (U/L)
6000
5000
4000
3000
2000
1000
0
c
c
b
a
c
bc
b
a
c
c
b
a
Day 2
Day 7
Day 42


D
25 °C
28 °C
31 °C
34 °C
GOT (U/L)
600
500
400
300
200
100
0
c c b a
b b b a
b bc c a
Day 2
Day 7
Day 42


E
25 °C
28 °C
31 °C
34 °C
TP (mg/dL)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a a a b
a a a b
ab ab a b
Day 2
Day 7
Day 42
Thermal exposure


F
25 °C
28 °C
31 °C
34 °C
TG (mg/dL)
30
25
20
15
10
5
0
a b c c
a b c c
a a b b
Day 2
Day 7
Day 42


G
25 °C
28 °C
31 °C
34 °C
TCHO (mg/dL)
160
140
120
100
80
60
40
20
0
a
ab
bc
c
a
a
a
c
a
a
b
c
Day 2
Day 7
Day 42


H
25 °C
28 °C
31 °C
34 °C
ALP (U/L)
700
600
500
400
300
200
100
0
c
c
b
a
c
b
a
a
c
c
b
a
Day 2
Day 7
Day 42
Thermal exposure

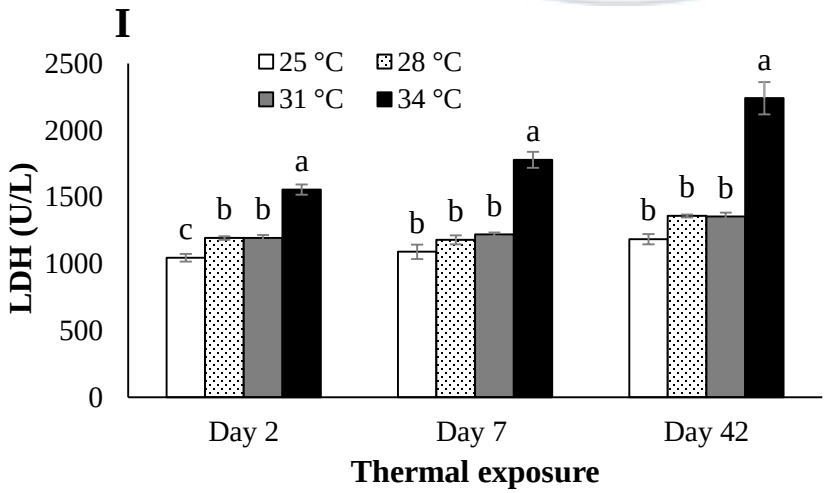
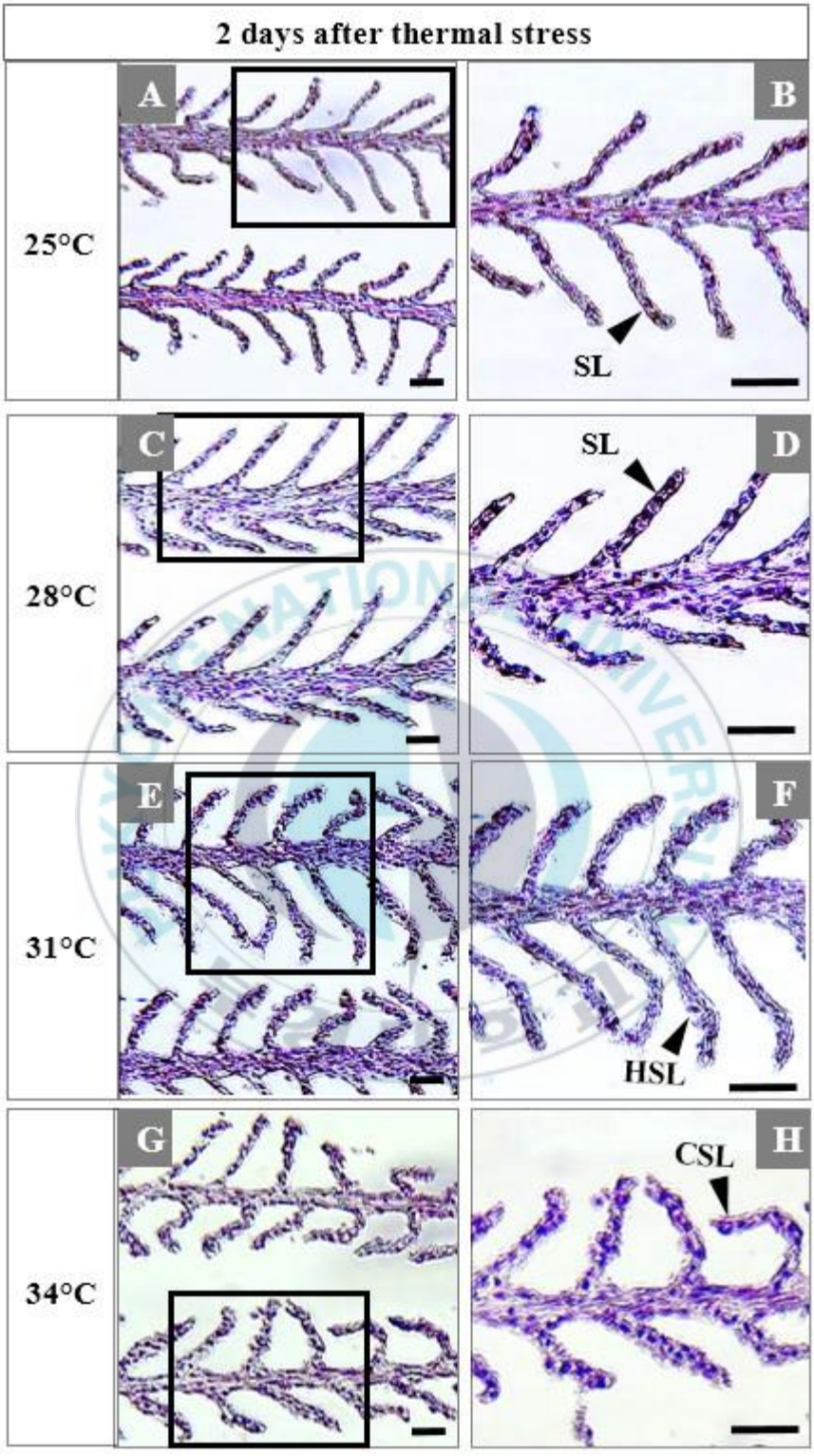

I
25 °C
28 °C
31 °C
34 °C
LDH (U/L)
2500
2000
1500
1000
500
0
c
b
b
a
b
b
b
a
b
b
b
a
Day 2
Day 7
Day 42
Thermal exposure

Fig. 2.2.4. Biochemical values (glucose, cortisol, GPT, GOT, TP, TG, TCHO, LDH, and ALP) of blood samples of juvenile red-spotted grouper, *Epinephelus akaara* submitted to different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. Values are mean ± SEM of three replicates (n=15). Different lowercase letters indicate the significant differences among the temperature groups at equivalent times (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.2.4 Histological changes in gills

The gills of 34 °C group were acutely affected by elevated water temperature compared with those in the 25 °C, 28 °C, and 31 °C groups. After 2 days, well-arranged secondary gill lamellae (SL) were observed in 25 °C and 28 °C groups (Fig. 2.2.5 A-D). However, hyperplasia of secondary gill lamellae (HSL) in 31 °C group (Fig. 2.2.3E–F) and curling of secondary gill lamellae (CSL) in 34 °C group were recorded after 2 days of thermal exposure (Fig. 2.2.5G–H). No noticeable change was recorded among the temperature treated groups after 7 days of exposure (Figure not shown). After 42 days, the gills of the 34 °C group were severely affected by prolonged thermal stress. Thermal stress at 34 °C produced hyperplasia in secondary gill lamellae (HSL), telangiectasia (TL), swelling of primary gill lamellae (SPL), and curling of secondary gill lamellae (CSL) (Fig. 2.2.5 I–N). However, no noticeable change was recorded in 25 °C, 28 °C, and 31 °C temperature groups (Figure not shown).


2 days after thermal stress
25°C
A
B
SL
28°C
C
SL
D
31°C
E
F
HSL
34°C
G
CSL
H

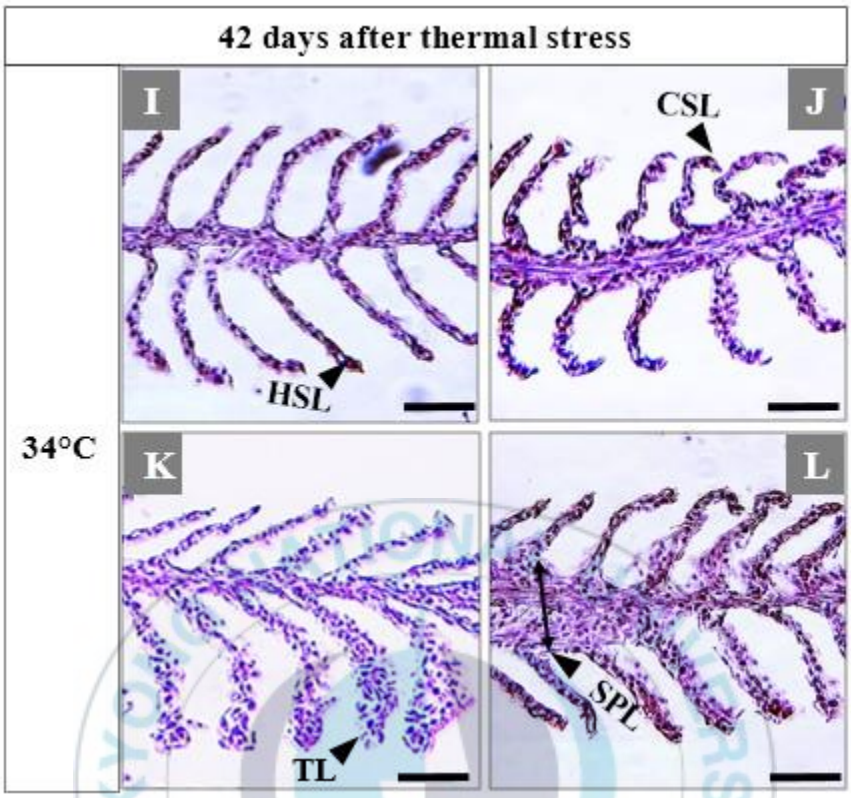
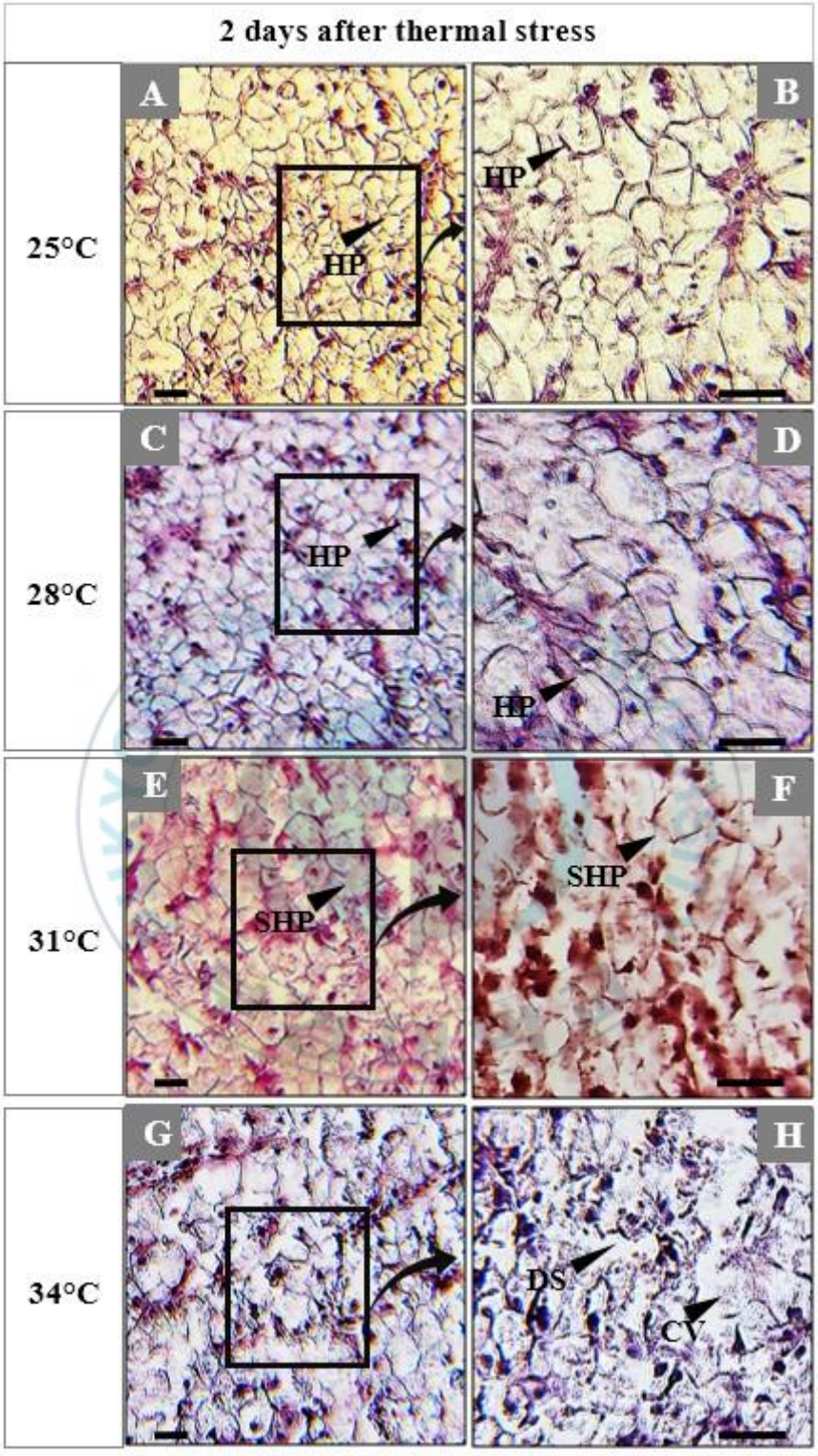


Fig. 2.2.5 (A-N). Gill histology of red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. SL, secondary gill lamellae; HSL, hyperplasia of secondary gill lamellae; CSL, curling of secondary gill lamellae; TL, telangiectasia; SPL, swelling of primary gill lamellae. Scale bars = 25 μm.

3.2.5 Histological changes in liver

Liver of the 34 °C group were severely affected by elevated water temperature compared with the other groups (25 °C, 28 °C and 31 °C) after 42 days of thermal exposure. After 2 days, nucleated hepatocytes (HP) were observed in 25 °C and 28 °C group (Fig. 2.2.6 A-D). In 31 °C group, swollen hepatocytes (SHP) was observed, whereas dilation of the sinusoid (DS), cytoplasmic vacuolization (CV), and SHP were detected in 34 °C group (Fig. 2.2.6E-H). After 7 days of exposure, no noticeable changes were recorded among the temperature treated groups (Figure not shown).

After 42 days of thermal exposure, no change was recorded in 25 °C and 28 °C group (Figure not shown). However, lipid globules (LG) were observed around the hepatocytes of 31 °C group (Fig. 2.2.6I-J). The liver of 34 °C group showed the severe damage including coalescence of hepatocytes (CHP), blood clotting in the sinusoid (BC), cell death (CD), and DS (Fig. 2.2.6K-N).


2 days after thermal stress
25°C
28°C
31°C
34°C
A
B
C
D
E
F
G
H
HP
HP
HP
HP
SHP
SHP
DS
CV

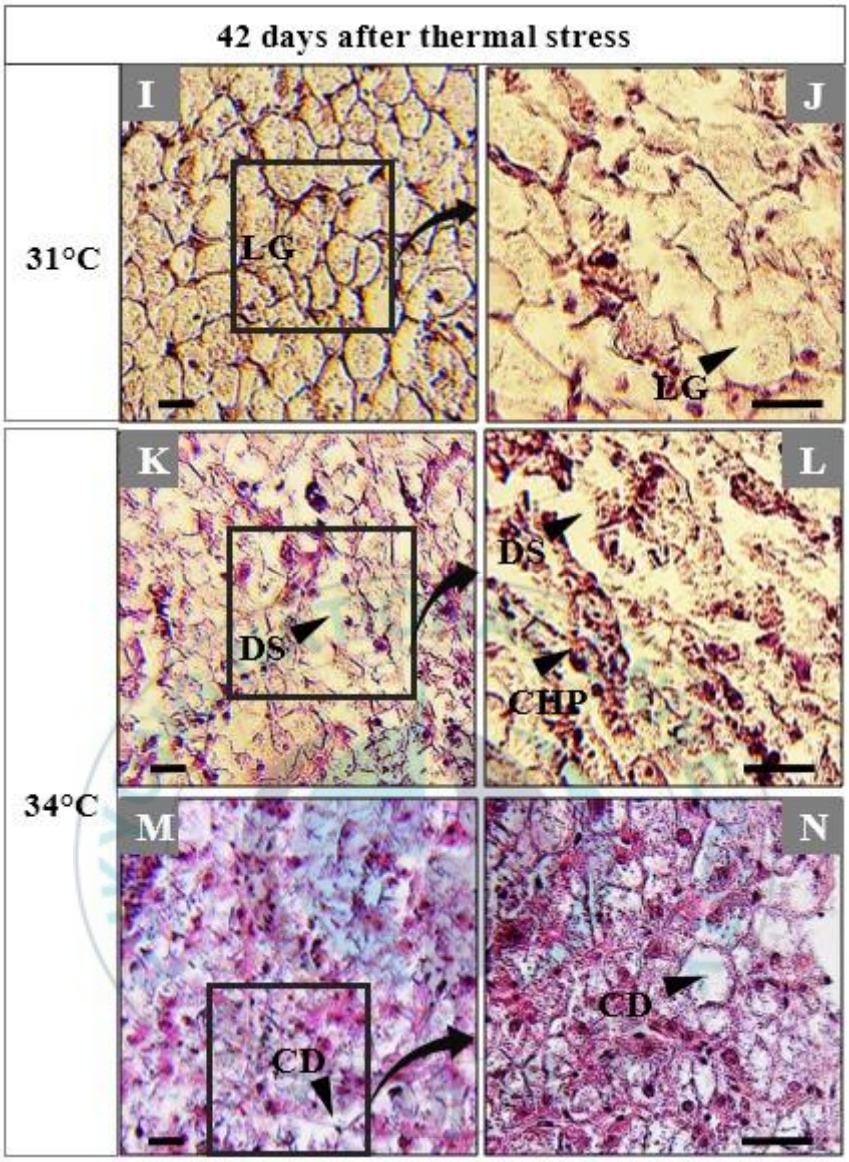


Fig. 2.2.6 (A-N). Liver histology of red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. HP, hepatocytes; SHP, swollen hepatocytes; CV, cytoplasmic vacuolization; LG, lipid globule, BC, blood clotting in sinusoid; DS, dilation of sinusoid; CHP, coalesce of hepatocytes; CD, cell death. Scale bars, 25 µm.

4. Discussion

Temperature is one of the important environmental factors, which influences the biochemical reactions and, therefore, has a significant impact on the health of aquatic animals (Person-Le Ruyet et al., 2004). The results of the present study demonstrated that increase in water temperature lead to the alterations in the hemato-biochemical parameters, and tissue morphology of *E. akaara*. Our results indicated that the highest stressful conditions observed at 36 °C and 34 °C in experiment 1 and experiment 2, respectively.

Thermal fluctuations beyond the tolerance level reduce appetite, irregular behavior of swimming, gulp for air, and often cause fish mortality (Cheng et al., 2013). In experiment 1, red-spotted grouper juveniles were affected by thermal stress and mortality occurred at 36 °C after 1 day, indicating that the upper thermal tolerance limit of *E. akaara* is around 36 °C. Our results are consistent with those of Cheng et al. (2013), who reported that the critical thermal limit of brown-marbled grouper (*E. fuscoguttatus*) was 35.9–38.3 °C, at which 50% of fish died. Orange spotted grouper (*E. coioides*) also showed sensitiveness to temperature change and mortality occurred after being transferred from 27 °C to 35 °C (Cheng et al. 2009). In experiment 2, *E. akaara* showed poor appetite for feed, rapid opercular movements, and irregular body movements. Similar results were reported by Islam et al. (2019) in Thai pangas (*Pangasianodon hypophthalmus*) after being exposed to 36 °C for 28 days.

Hematological parameters have been used to assess the functional status of oxygen-carrying capacity in the bloodstream (Shah and Altindag, 2004). In our study, Ht and Hb values, and RBC counts were changed with the increase in water temperature. Elevated Ht and RBC levels increase the oxygen-carrying capacity of the blood, thus supplying oxygen to major organs in response to higher metabolic demand, which is a manifestation of stress (Ruane et al., 1999). The Hb levels were reported to decrease as Ht increased when Nile tilapia (*Oreochromis niloticus*) were exposed

to 37 °C for 4 hours (Panase et al., 2018). Hematological responses to increased water temperatures differ among aquatic species and by the duration of thermal exposure (Radoslav et al., 2013). The other hematological parameters affected by water temperature fluctuations include MCV and MCH. The decrease in MCV and MCH values at the higher temperature (34 °C) is associated with the increase of RBC and Hb values. Similar result was reported by Ahmad et al. (2011) in common carp, *Cyprinus carpio communis* after being exposed to a water temperature of 32 °C for 30 days.

In this study, plasma glucose levels in *E. akaara* juveniles increased dramatically at the higher temperature (34 °C and 36 °C) compared with the other treatment groups, indicating increased energy demand to overcome this unstable physiological condition. Following the introduction of thermal stress, increases in blood glucose levels occur due to glycogenolysis (Naour et al., 2017), to meet additional energy demands (Hsieh et al., 2003). Our result was consistent with those of studies on Nile tilapia, which was subjected to thermal stress at 37 °C (Panase et al., 2018). Changes in blood GPT and GOT levels have been used as indicators of hepatic dysfunction and damage, which result in elevated transaminase activity (Gholami-Seyedkolaei et al., 2013). In this study, increased blood GPT and GOT levels at high temperature (34 °C and 36 °C) indicated liver stress, demonstrating hepatic metabolism hyperactivity. Our results are consistent with those of Cheng et al. (2018), who observed sharp increases in GPT and GOT in pufferfish (*Takifugu obscurus*) at 37 °C. The total protein is commonly used to diagnose fish immunity and nutritional and metabolic status (Ortuno et al. 2001). In our study, the decline in total protein observed at high temperature (34 °C and 36 °C) may have been related to impaired protein synthesis due to liver damage. Our results are consistent with those for puffer fish, *T. obscurus* (Cheng et al. 2018) and common carp (Ahmad et al., 2011).

In this study, cortisol levels were significantly increased ($P < 0.05$) at high water temperature (34 °C and 36 °C). The increase in cortisol levels observed at high water temperature may have been due to stress-induced by prolonged thermal exposure and long-term confinement. Similar results were reported in Black Sea trout (Balta et al., 2017), Nile tilapia (Panase et al., 2018), and red-spotted grouper (Lee and Baek, 2018). Corticosteroid levels typically surge the following stress and return to basal levels after recovery (Iwama et al. 2006). Changes in plasma cortisol levels have been reported to induce elevated glucose levels (Hur et al., 2008), as observed in this study.

In this study, serum TCHO and TG levels prominently declined with the rise in water temperature. The decreasing trend of serum TCHO and TG suggests that higher temperature may disturb lipid mobilization and circulation between liver and tissue. TCHO and TG may be the energy source of anti-stress in fish to meet the additional energy demand under thermal stress (Ming et al., 2012). LDH is an important glycolytic enzyme in the biochemical system that catalyzes the conversion of lactate to pyruvic acid. LDH is also considered as a potential marker for assessing the toxicity of environmental toxicity. Our results indicate that LDH activity in serum significantly increased at 34 °C. Similarly, previous findings also confirmed a sharp increase in LDH activity in pufferfish at 34 °C and 37 °C (Cheng et al., 2018). ALP is an important enzyme, which directly involves in the transfer of phosphate groups and metabolism of calcium and phosphate. In this study, the ALP levels increased with the rise of water temperature. Similar alternations in ALP activity was observed in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) under different thermal conditions (22 °C, 26 °C, 30 °C, and 34 °C) (De et al., 2019). The changes in ALP levels at higher temperature might result from the disturbances in both physiological and functional mechanisms under thermal stress.

Morphological alterations in the gill and liver have been analyzed to assess the effects of stress; these effects also correlate well with hemato-biochemical observations. Our results demonstrated severe histological modifications to the gill including hyperplasia, curling, and shortening of secondary gill lamellae, swelling of primary gill lamellae, epithelial necrosis and telangiectasia. These findings are consistent with previous reports of gill morphology in thermally stressed Japanese flounder (*Paralichthys olivaceus*) (Liu et al., 2015) and bighead carp (*Aristichthys nobilis*) (Aboka et al., 2017), which were exposed to 32 °C and 38 °C, respectively. Our histological results of the liver showed hepatocyte coalescence, sinusoid blood clotting, and hepatocyte shrinkage, hepatocyte swelling, hepatocyte vacuolization, deposition of lipid globule, dilation of sinusoid, and cell death at higher water temperature. This morphological disruption of liver indicates an adaptive and prolong thermal stress response to compensate for the effects of temperature. Similar effects have been recorded in thermally stressed fish of other species, including Japanese flounder (Liu et al., 2015), Pacific sardine, *Sardinops sagax caeruleus* (Hernandez-Lopez et al., 2018), and common carp (Ahmad et al., 2011).

5. Conclusion

In conclusion, the present study demonstrated that high water temperature-induced severe physiological and morphological modifications in red-spotted grouper. Based on the hemato-biochemical and histological observations, the present study suggests that 34 °C and 36 °C is the sub-lethal and lethal temperature, respectively for *E. akaara*. Besides, the red-spotted grouper began to experience physiological disturbances after prolonged exposure to 31 °C and 32 °C, suggesting that these temperatures are also stressful for this species. The baseline information of plasma profiles and tissue morphology under increased water temperatures may help to monitor the health status of *E. akaara* for better welfare and production.

Chapter 2.3

Heat shock protein (HSPs) gene expression in red-spotted grouper

Abstract

Heat shock proteins (HSPs) prevent cellular damage and therefore, play a significant role in adaptation to temperature. In the present study, HSP gene expression under different water temperature were investigated in red-spotted grouper (*Epinephelus akaara*). The increased water temperature effects on Hsp60, Hsp70 and Hsp90 mRNA expression in various tissues were examined in two separate experiments (Experiment 1: 24 °C, 28 °C, 32 °C, 36 °C for 28 days; Experiment 2: 25 °C, 28 °C, 31 °C, 34 °C for 42 days). Liver, gill, brain, muscle, and heart were collected after 1, 14, 28 day of Exp. 1 and 2, 7, 42 days of Exp. 2. In experiment 1, Hsp60, 70, 90 was highly expressed in liver, gill, brain, muscle and heart of 36 °C group ($P < 0.05$). Hsp60, 70, 90 were also up-regulated in gill, muscle and heart at 32 °C after 28 days of thermal exposure. In experiment 2, Hsp60, 70, 90 mRNA were highly expressed in liver, gill, brain, and muscle at 34 °C temperature. Higher Hsp60, 70, 90 transcript level was also observed in liver and gill of 31 °C group after 42 days. The Hsp60, Hsp70, and Hsp90 expression chronology in different tissues were liver > gill > muscle > brain > heart, liver > muscle > brain > gill > heart, liver > gill > muscle > heart > brain, respectively. The high expression of Hsp60, Hsp70 and Hsp90 at 34 °C and 36 °C, suggesting that these HSPs are required for immediate survival of *E. akaara* at high water temperature. The results obtained from this study can be used as valuable basic data for understanding the defense mechanisms employed by fish to combat thermal stress.

Keywords: Red-spotted grouper, *Epinephelus akaara*; Temperature; Heat shock proteins, mRNA expression

1. Introduction

Temperature is one of the most important abiotic factors that influence the biological process of fishes including growth, reproduction and disease resistance, but a subacute or acute change of water temperature beyond the optimal range can induce a thermal stress response to modulate these processes smoothly (Cossins et al., 1995). Increase in water temperature due to climate change and anthropogenic activities is expected to affect the physiological processes of fish, especially in tropical regions (Portner and Farrell, 2008). Understanding the molecular mechanisms is important to gain a clear concept of the adaptation process of fish with the environmental changes (i.e. thermal fluctuations). Therefore, the potential effect of increased water temperature on fishes has forced researchers to make continual efforts to define cellular stress mechanisms that may help the fish to survive environmental changes.

Water temperature change can significantly affect the integrity of the physiological system at various cellular and molecular levels (Cossins and Bowler, 1987). When the water temperature rises past the tolerable range, cells perceive oxidative stress and individuals show a highly conserved set of immune pathways to withstand such thermal changes. The common well-known feature of heat shock response is the induction of heat shock proteins (HSPs). HSPs are a highly conserved set of proteins that present in all types of cell of living organisms (Parsell and Lindquist, 1993). Generally, HSPs are differentiated by molecular weight and found in the range between 27 and 110 kDa. HSPs are broadly classified into six major groups namely Hsp60, Hsp70, Hsp90, Hsp100, Hsp110, and small HSPs. Among the HSP protein families, Hsp60, Hsp70, and Hsp90 are highly conserved and most extensively studied (Feige et al., 1996; Parsell and Lindquist, 1993).

The Hsp60, Hsp70, and Hsp90 genes are stress-inducible, multigenic, and present in all organisms studied till date (Lindquist and Craig, 1988). The members of Hsp60, Hsp70, and Hsp90

family play a significant role in cell survival, stress, and thermal tolerance in response to various heat shocks. These proteins also play an important role as molecular chaperones in intracellular organelles that prevent protein aggregation, assist in refolding any misfolded proteins, stabilize unstable proteins, and maintain the integrity of the mitochondrial membrane (Parsell and Lindquist, 1993; Ranford et al., 2000). The members of Hsp60, 70, and 90 are also involved in many cellular processes including signal transduction, DNA replication, protein synthesis, and protein trafficking (Otaka et al., 1994). The increased level of these genes has been observed in fish subjected to various stressors such as crowding, transport, bacterial and viral infections, and nutrient deprivation (Stefan et al., 1991). Thus, the increased transcription of these genes can be considered as an early indicator of stress, which is of the utmost importance when dealing with various kinds of stressors.

Although a significant number of studies about the influence of environmental stressors on the physiological parameters in fish have been reported (Gornati et al., 2004; Gornati et al., 2005; Poltroneiri et al., 2007), few studies investigate the effects of increased water temperature on cellular level change such as heat shock protein (HSPs) response in fish. The red-spotted grouper (*Epinephelus akaara*) is a subtropical species, whose optimal temperature for growth is reported to be 24-28 °C (Lee and Baek, 2018). However, the rise of water temperature as a consequence of climate change is anticipated to affect the physiological activities of red-spotted grouper (Baillie et al. 2004; Tupper and Sheriff 2008). Therefore, it is important to find out how red-spotted grouper show the cellular level responses with the changes in water temperature. In the present study, the mRNA expression of Hsp60, Hsp70 and Hsp90 in different tissues of *E. akaara* were investigated to evaluate the heat shock protein (HSPs) responses under increased water temperature.

2. Materials and methods

2.1. Ethics statement and fish collection

The experiments were conducted following the fish maintenance, handling, and sampling guidelines of the Animal Ethics Committee of Pukyong National University (PKNU) (Regulation No. 554). *E. akaara* juveniles were collected from the Marine Science Institute, Jeju National University, Korea and reared in the laboratory of Marine Biology Department, PKNU, Korea. The animals were acclimated for 2 week before starting the experiment.

2.2. Experimental design and thermal exposure

In experiment 1, 180 juveniles of red-spotted grouper (total length: 9.4 ± 0.12 cm; body weight: 12.89 ± 0.61 g) were exposed to four temperature conditions (24 °C as control, 28 °C, 32 °C, and 36 °C), each with three replications for 28 days. Upon completion of experiment 1, we carried out the second experiment using 180 red-spotted grouper juveniles (total length: 8.28 ± 0.10 cm, body weight: 8.53 ± 0.27 g). Fish were exposed to four different water temperatures such as 25 °C as control, 28 °C, 31 °C, and 34 °C for 42 days.

In both experiments, fish were randomly stocked in 12 glass aquaria (15 fish/aquarium), each with a volume of 120 L. Each aquarium had equal size and height (75cm × 45cm × 45cm), and was equipped with the recirculating filtration system. The temperature was gradually increased (Δ1 °C/h) from the normal temperature to the target temperature to avoid the sudden thermal shock. The required temperature was maintained by using thermostat (OKE-6422H; OKE, Busan, Korea). During the acclimation and experimental period, fish were fed with a commercial diet (Otohime Hirame, Marubeni Nisshin Feed Co., Ltd., Japan). Fish were fed two times daily (09:00 and 18:00 h) during the experimental period. Uneaten food was removed after 30 minutes of feeding and 10% of the water in each aquarium was replaced daily with filtered clean seawater. Water temperature, pH, salinity, and dissolved oxygen were monitored daily using a multiparameter water-quality

meter (HI9829; Hanna Instrumentals, USA). Salinity, pH, DO, and NH_4 were 33.67–33.81 g/L, 7.89–7.97, 4.16–6.96 mg/L, and < 0.25 mg/L, respectively, during the experimental trial.

2.3. Sampling protocol

In experiment 1, fish were sampled at 1 day, 14 days, and 28 days of thermal exposure. The second and third sampling was conducted only in the 24 °C, 28 °C, and 32 °C groups as because all fish in the 36 °C group died after 1 day of thermal stress. In experiment 2, fish were sampled three times over the investigations, after 2, 7, and 42 days of thermal exposure. All fish were fasted for 24h before sampling. 15 fishes were randomly sampled from each temperature group (i.e., 5 fishes from each aquarium; n=15) on each sampling day. All fish were anaesthetized with 300 mg/L 2-phenoxyethanol (Sigma Aldrich, USA) before tissue collection. The liver, gill, brain, muscle, and heart were surgically removed and immediately kept in RNA later (Qiagen, Germany) at -80 °C for further analysis.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNAiso plus (Takara, Japan) from the preserved tissues according to the manufacturer's protocol. Total RNA concentration was measured using a spectrophotometer (NanoDrop 1000, Thermo Scientific). The cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The synthesized cDNA was stored at -20 °C for further analysis.

2.5. Quantitative real-time PCR analysis

To evaluate the mRNA levels of heat shock protein 60 (Hsp60), heat shock protein 70 (Hsp70), and heat shock protein 90 (Hsp90), primers were designed using the sequence information available in National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.gov). Real-time PCR was carried out with Chromo4 Real-Time PCR System (Bio-

Rad, USA). The real-time PCR reaction mixture contained 2µl of the diluted cDNA sample, specific primer, and TB Green™ Premix Ex Taq™ (Takara, Japan). The PCR reaction was performed with an initial denaturation at 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s (Hsp70 and Hsp90) or 67 °C for 30 s (Hsp60), 72 °C for 30 s, plate read, and final step at 94 °C for 3 min. The 18S rRNA was used as a reference gene to normalize the expression of the target gene. The primers used for the PCR and qRT-PCR are shown in Table 2.3.1.

2.6. Statistical analysis

Values are presented as the mean ± standard error of the mean (SEM). Data were normalized before statistical evaluation. The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to identify the significant differences among the different temperature conditions. Statistical significance was set at $P < 0.05$. All statistical analysis was done using SPSS program Version 21 (IBM Corp., USA).

Table 2.3.1. Primers used for quantitative real-time PCR of Hsp60, Hsp70 and Hsp90 genes

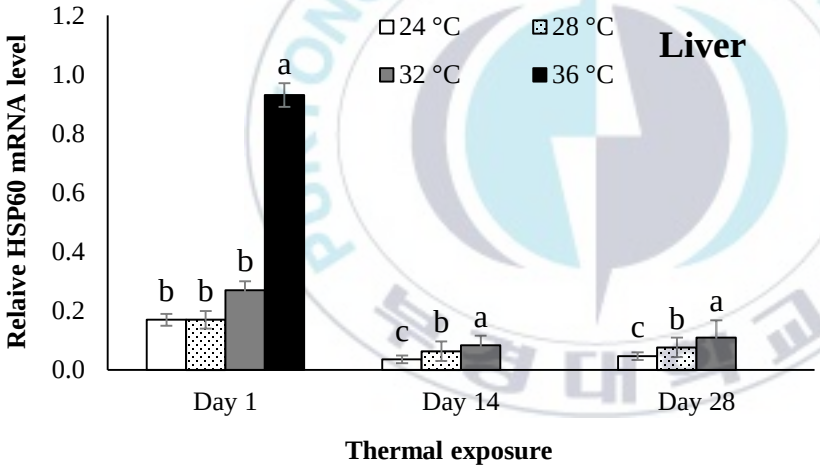
Gene	Direction	Sequence (5' - 3')
Heat shock protein 60	Forward	AGACTCTGCACGATGAGCTGGAG
	Reverse	GCCATGTCCCTCAGCTGATTCTTC
Heat shock protein 70	Forward	TGGCAAGATCAGTGAAGACG
	Reverse	TTCATCCTTCTCGGCAGTCT
Heat shock protein 90	Forward	TGTCCAACAGACTGGTTTCC
	Reverse	ATTCTGTAGATGCGGTTGGA
18S rRNA	Forward	CGGTAATTCCAGCTCCAATAGCG
	Reverse	CTTAATCATGGCCCCAGTTCAGAG

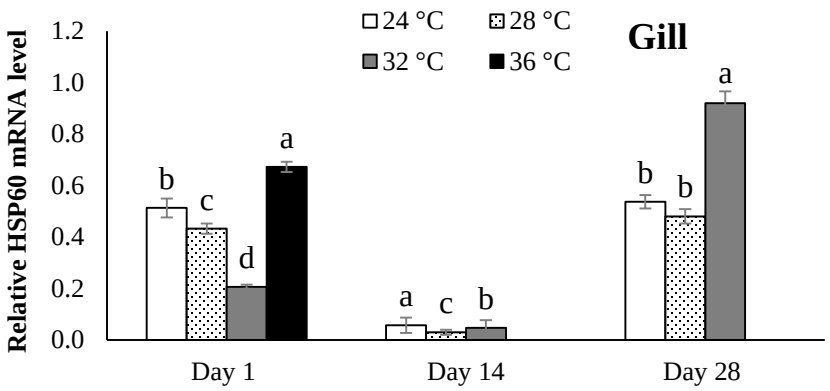
3.1. Results: Experiment 1

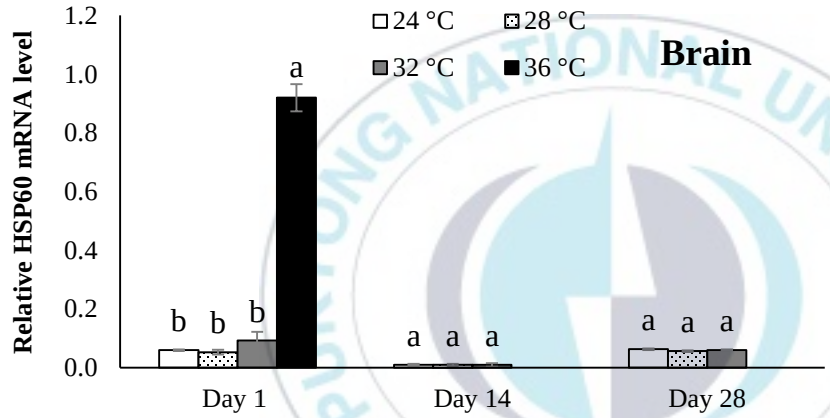
3.1.1. HSP60 mRNA expression

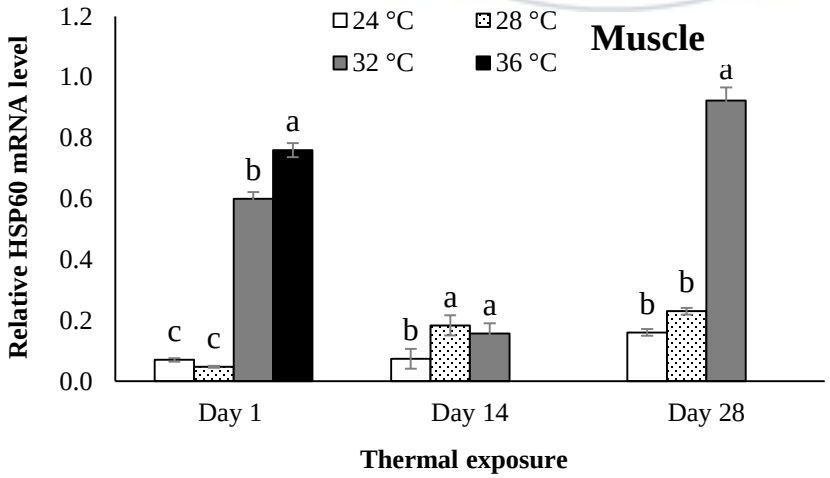
HSP60 mRNA expression levels in each tissue are shown in Figure 2.3.1. After 1 day of exposure, the mRNA level of HSP60 was significantly up-regulated ($P < 0.05$) in liver, gill, brain, muscle, and heart of 36 °C group; however, in case of 32 °C, higher transcript level was recorded in muscle and heart (Fig. 2.3.1 D-E).

14 days after exposure, no significant change was observed in expression; however, higher expression ($P < 0.05$) was detected in gill, muscle, and heart of 32 °C group after 28 days of thermal exposure (Fig. 2.3.1 B, D-E).




Gill
24 °C
28 °C
32 °C
36 °C
Relative HSP60 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b c d a
a c b
b b a
Day 1
Day 14
Day 28


Brain
24 °C
28 °C
32 °C
36 °C
Relative HSP60 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b b b a
a a a
a a a
Day 1
Day 14
Day 28


Muscle
24 °C
28 °C
32 °C
36 °C
Relative HSP60 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
c c b a
b a a
b b a
Day 1
Day 14
Day 28
Thermal exposure

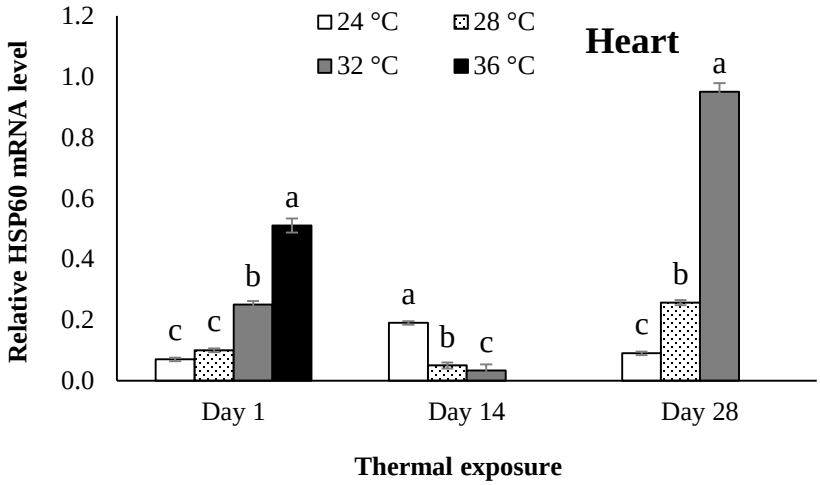
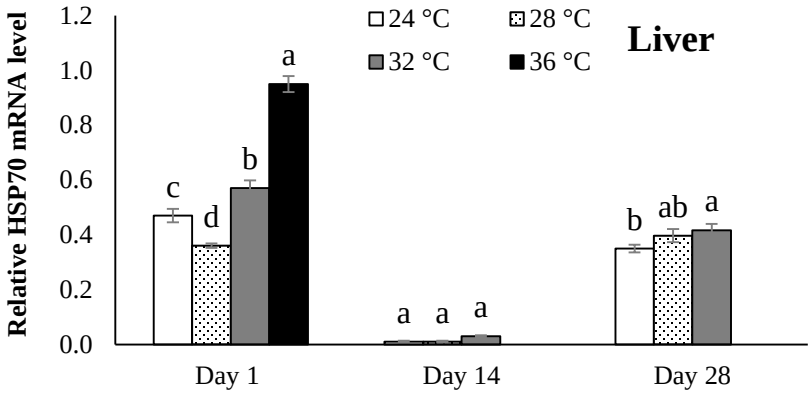


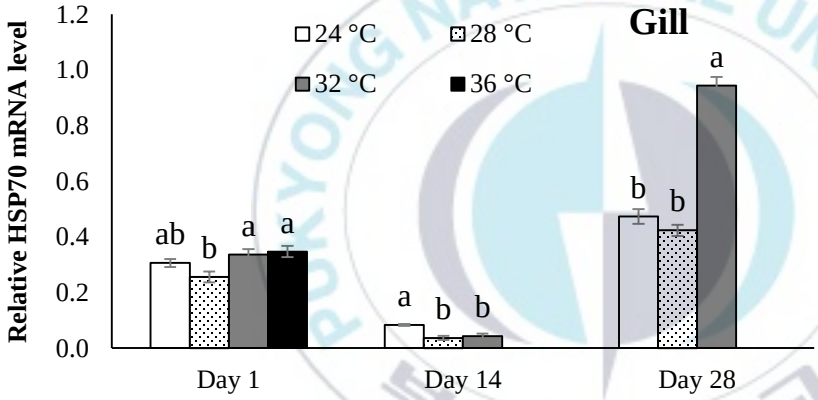
Fig. 2.3.1. The relative mRNA expression of HSP60 in the liver (A), gill (B), brain (C), muscle (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (24 °C, 28 °C, 32 °C and 36 °C) for 28 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

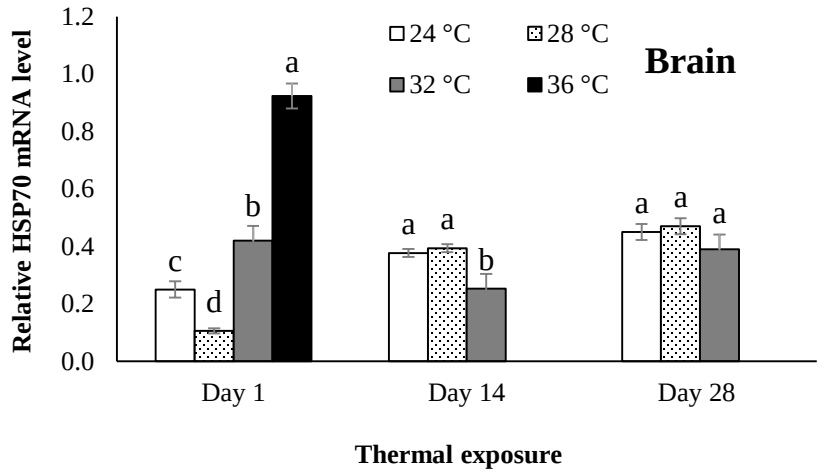
3.1.2. HSP70 mRNA expression

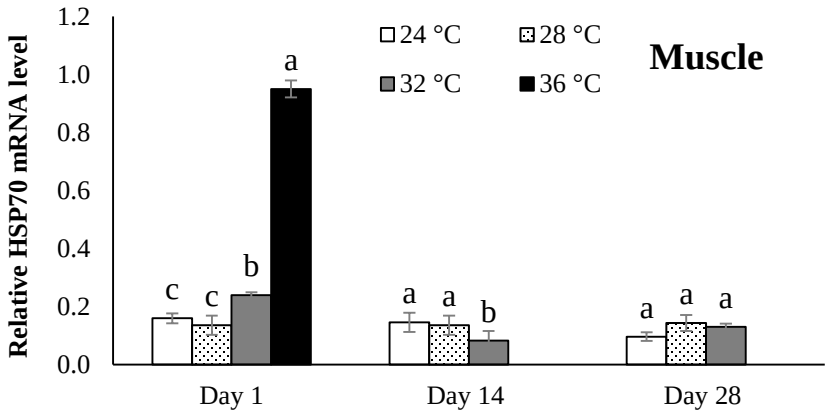
The mRNA level of HSP70 in each tissue is shown in Figure 2.3.2. After 1 day, HSP70 gene expression was significantly increased ($P < 0.05$) in the liver, brain, muscle, and heart of 36 °C group compared with the others (24 °C and 28 °C) (Fig. 2.3.2 A, C-E). In the case of 32 °C, elevation in HSP70 expression was found in liver, brain and muscle (Fig. 2.3.2 A, C-D).

After 14 days, no difference was observed in transcript level; however, upregulation ($P < 0.05$) in HSP70 expression was noted in the liver and heart of 32 °C group after 28 days of exposure (Fig. 2.3.2 A, C, E).









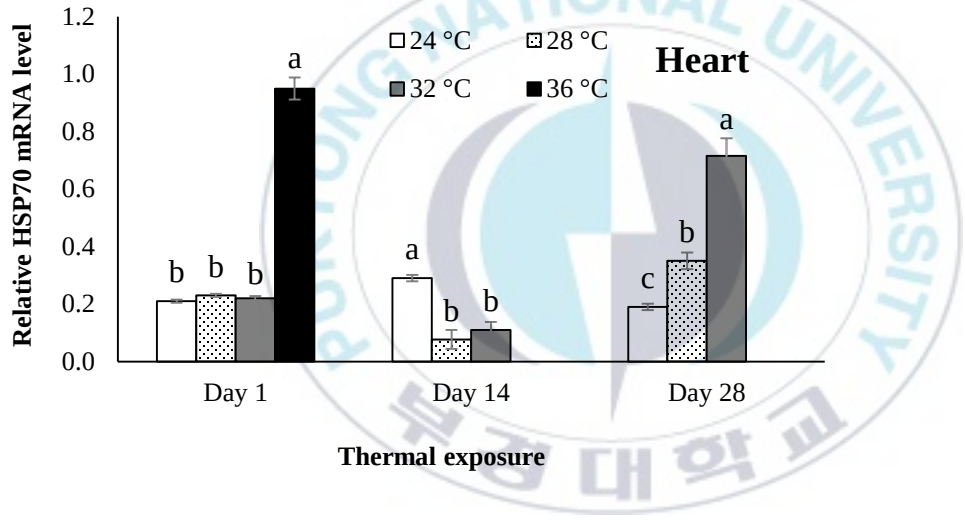
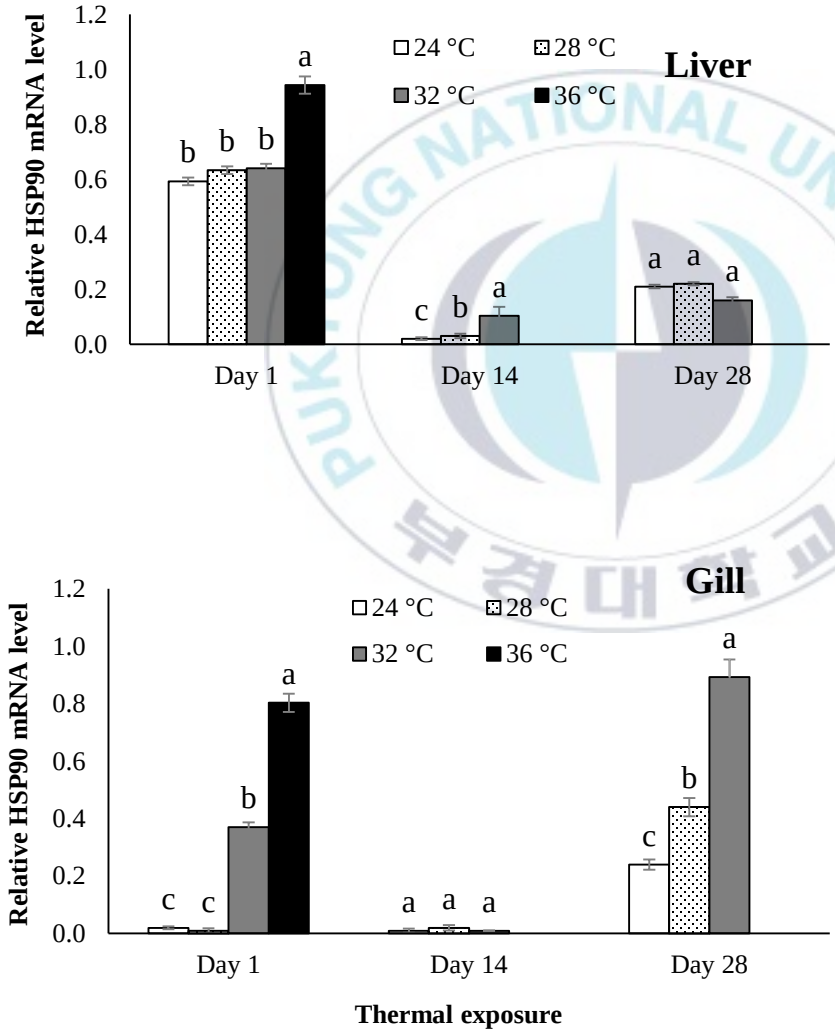


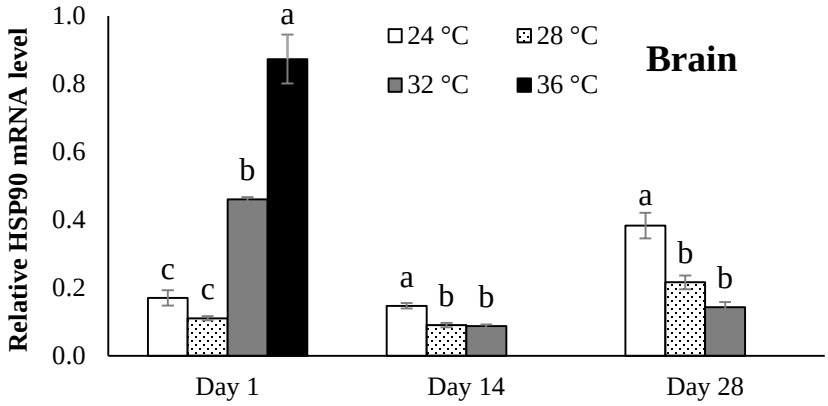
Fig. 2.3.2. The relative mRNA expression of HSP70 in the liver (A), gill (B), brain (C), muscle (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (24 °C, 28 °C, 32 °C and 36 °C) for 28 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

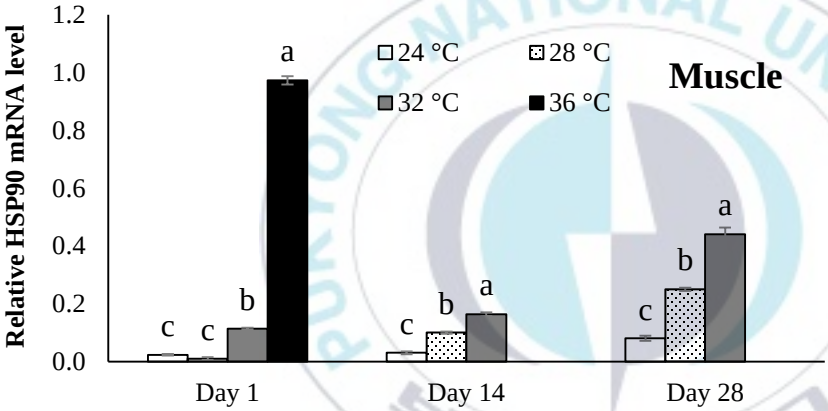
3.1.3. HSP90 mRNA expression

The transcript level of HSP90 in each tissue is shown in Figure 2.3.3. The mRNA level of HSP90 was over-expressed ($P < 0.05$) in liver, gill, brain, muscle, and heart of 32 °C and 36 °C group compared with the others (24 °C and 28 °C) after 1 day of exposure (Fig. 2.3.3 A-E).

14 days after exposure, no differences were observed in mRNA expression; however, elevated HSP90 expression ($P < 0.05$) was observed in gill, muscle and heart of 32 °C group after 28 days of exposure (Fig. 2.3.3 B, D-E).




Brain
24 °C
28 °C
32 °C
36 °C
Relative HSP90 mRNA level
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
c
a
b
b
a
b
b
Day 1
Day 14
Day 28


Muscle
24 °C
28 °C
32 °C
36 °C
Relative HSP90 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
c
a
b
c
a
b
c
Day 1
Day 14
Day 28

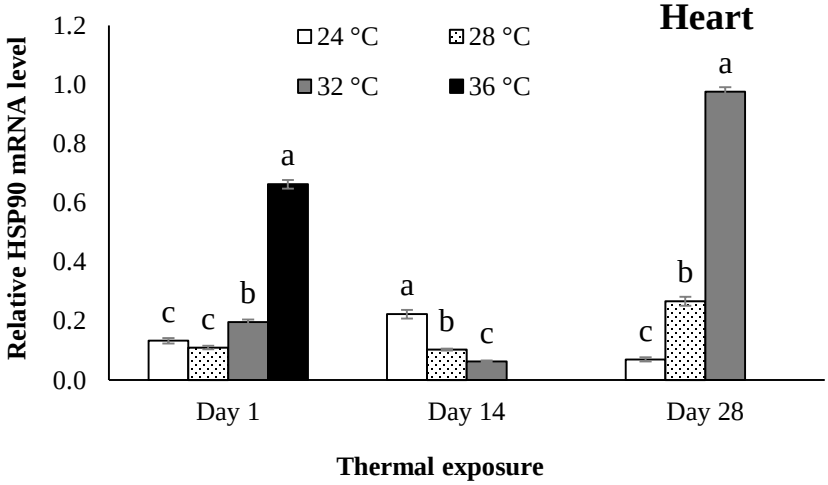
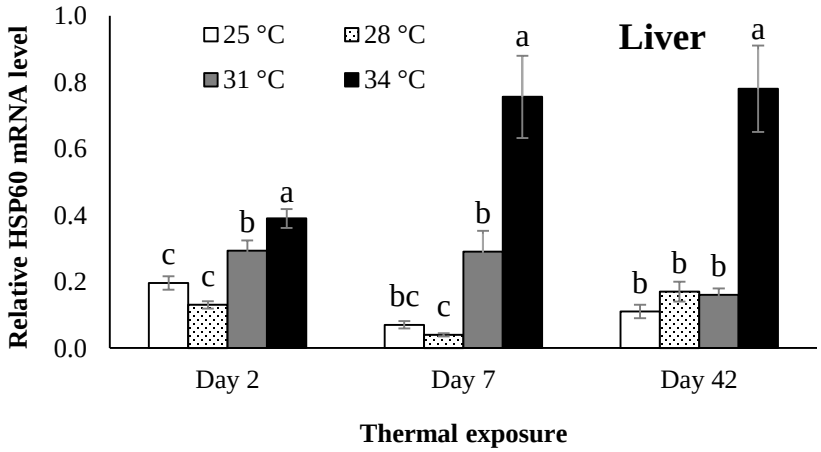

Heart
24 °C
28 °C
32 °C
36 °C
Relative HSP90 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
c
a
b
c
a
b
c
Day 1
Day 14
Day 28
Thermal exposure

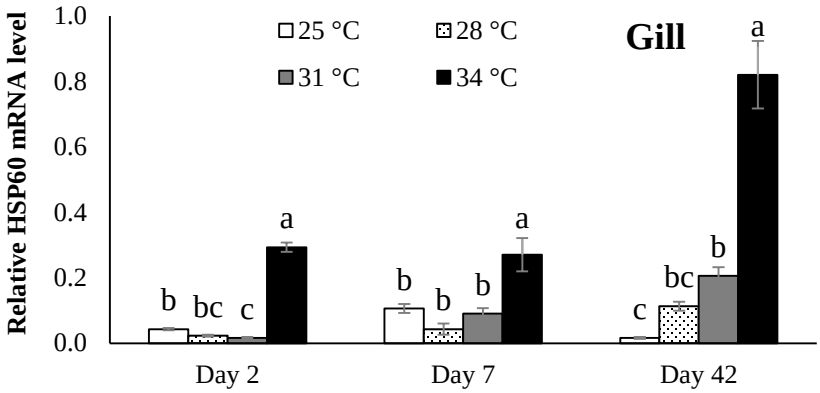
Fig. 2.3.3. The relative mRNA expression of HSP90 in the liver (A), gill (B), brain (C), muscle (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (24 °C, 28 °C, 32 °C and 36 °C) for 28 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

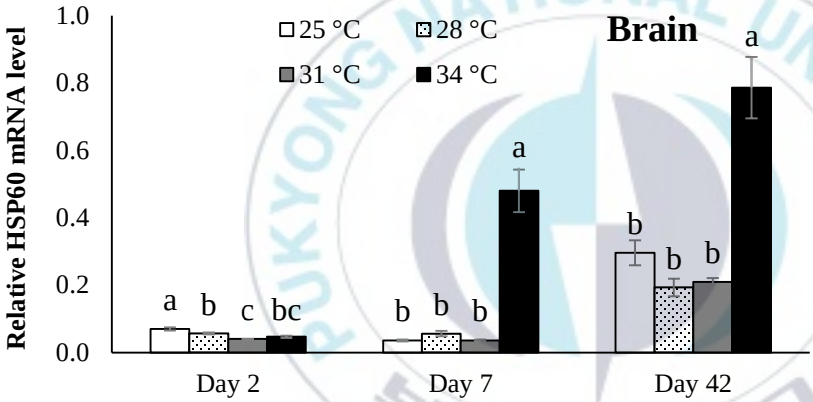
3.2. Results: Experiment 2

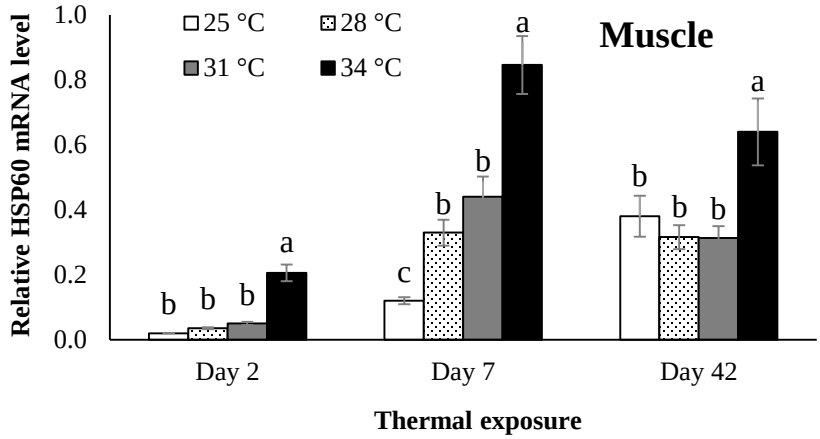
3.2.1. HSP60 mRNA expression

HSP60 mRNA levels in each tissue are shown in Figure 2.3.4. The mRNA level of HSP60 was significantly ($P < 0.05$) up-regulated in liver, muscle, gill, brain, and heart of 34 °C group compared with the others (25 °C, 28 °C, and 31 °C) when fish were reared for 42 days (Fig. 2.3.4 A-E). However, in case of 31 °C, higher HSP70 mRNA expression was found only in the liver after 2 days of exposure and baseline level expression were found in other tissues throughout the trial except in muscle after 7 days of exposure (Fig. 2.3.4 A, D). Tissues of 25 °C and 28 °C groups were found unaffected in response to thermal exposure.









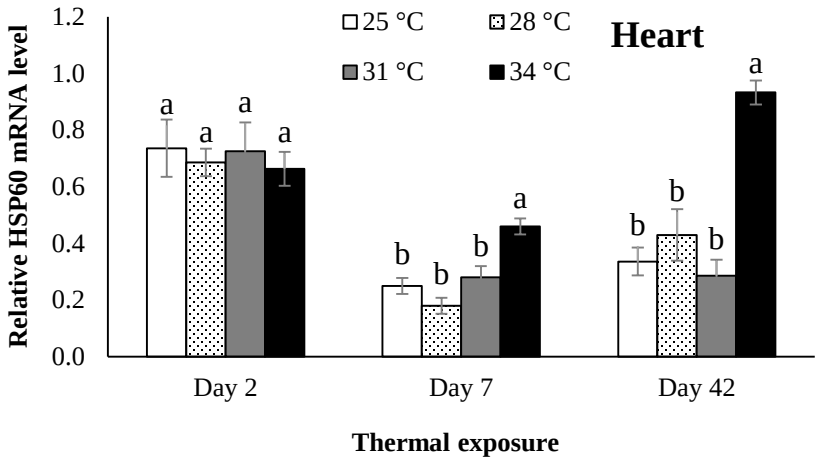
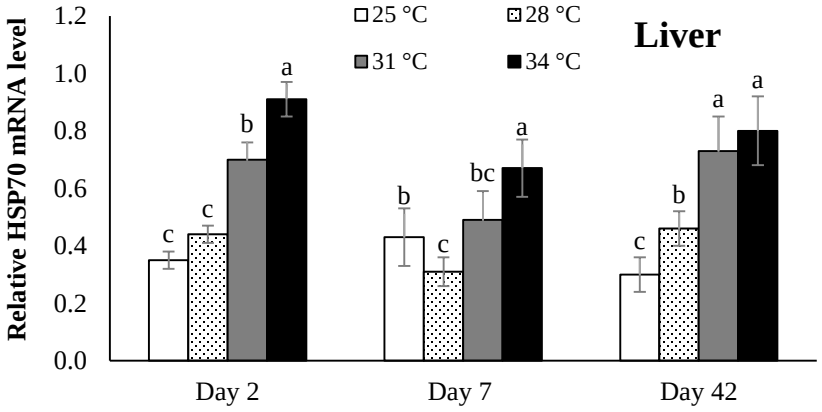
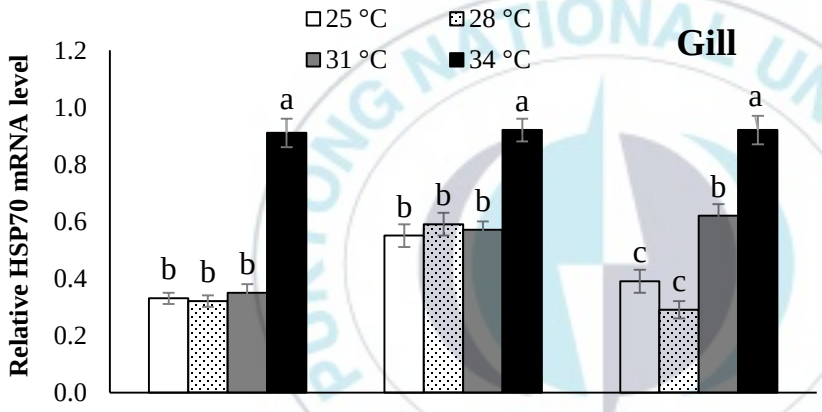


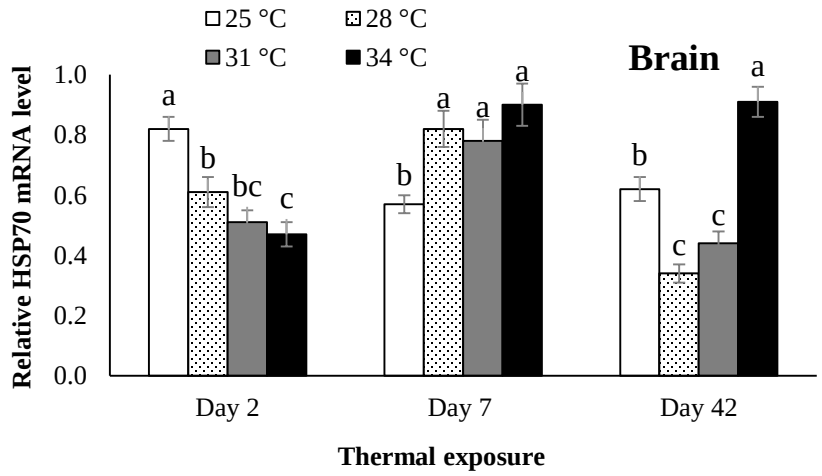
Fig. 2.3.4. Expression patterns of HSP60 mRNA in the liver (A), muscle (B), gill (C), brain (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (25 °C, 28 °C, 31 °C and 34 °C) for 42 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

3.2.2. HSP70 mRNA expression

HSP70 mRNA levels in each tissue are shown in Figure 2.3.5. After 2 days of exposure, the HSP70 gene expression was significantly ($P < 0.05$) increased in liver, muscle, and gill of 34 °C group in comparison to the others (25 °C, 28 °C, and 31 °C); however, higher transcript level was noted in all tissues except for heart when exposed for 42 days (Fig. 2.3.5 A-E). In contrast, the liver, muscle and gill of 31 °C group showed higher expression after 42 days of thermal exposure (Fig. 2.3.5 A-B, D).


Liver
25 °C
28 °C
31 °C
34 °C
Relative HSP70 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
c
c
b
a
b
c
bc
a
c
b
a
a
Day 2
Day 7
Day 42


Gill
25 °C
28 °C
31 °C
34 °C
Relative HSP70 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
b
b
a
b
b
b
a
c
c
b
a
Day 2
Day 7
Day 42


Brain
25 °C
28 °C
31 °C
34 °C
Relative HSP70 mRNA level
1.0
0.8
0.6
0.4
0.2
0.0
a
b
bc
c
b
a
a
a
b
c
c
a
Day 2
Day 7
Day 42
Thermal exposure

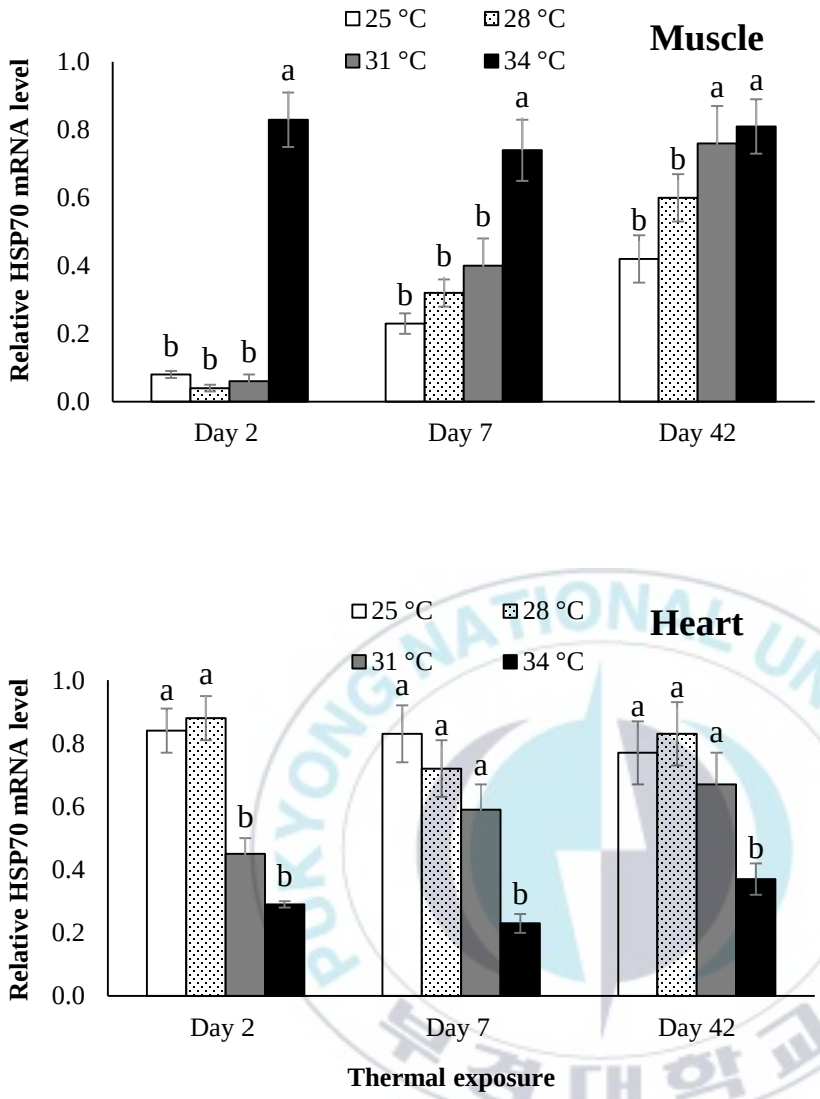
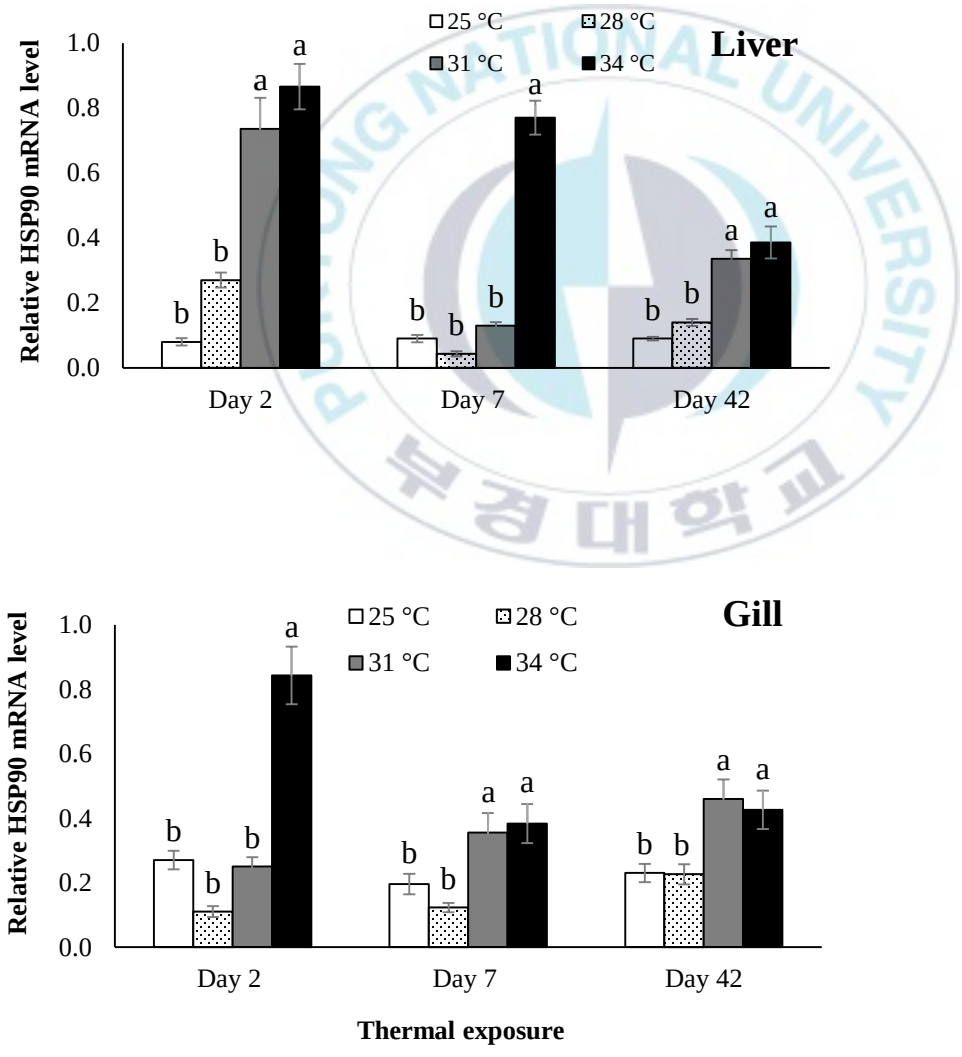
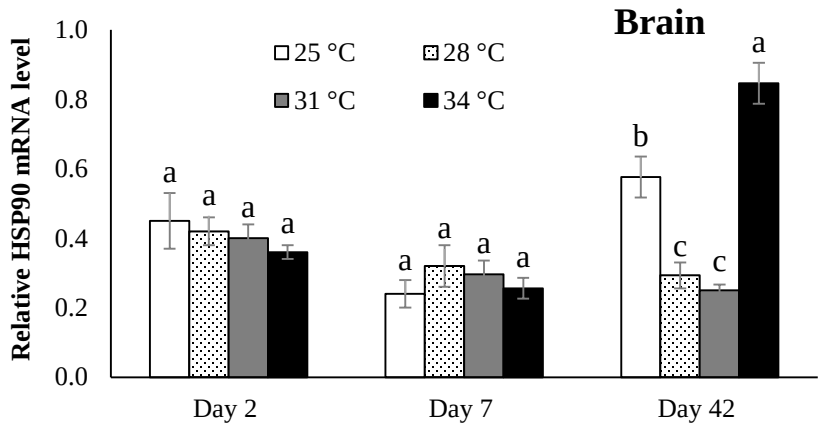


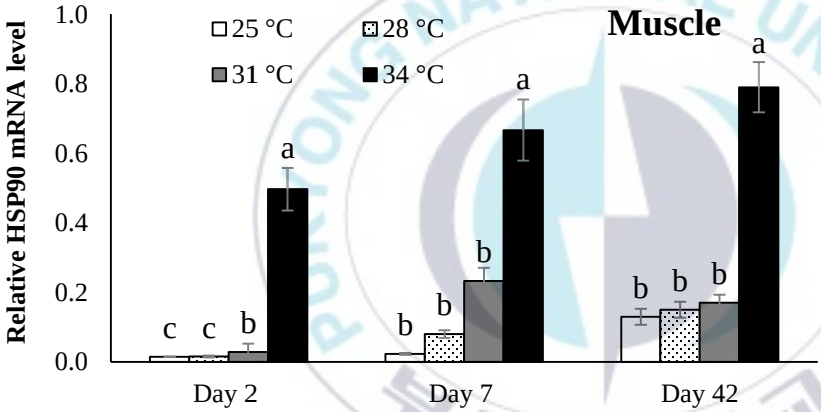
Fig. 2.3.5. Expression patterns of HSP70 mRNA in the liver (A), muscle (B), gill (C), brain (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (25 °C, 28 °C, 31 °C and 34 °C) for 42 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

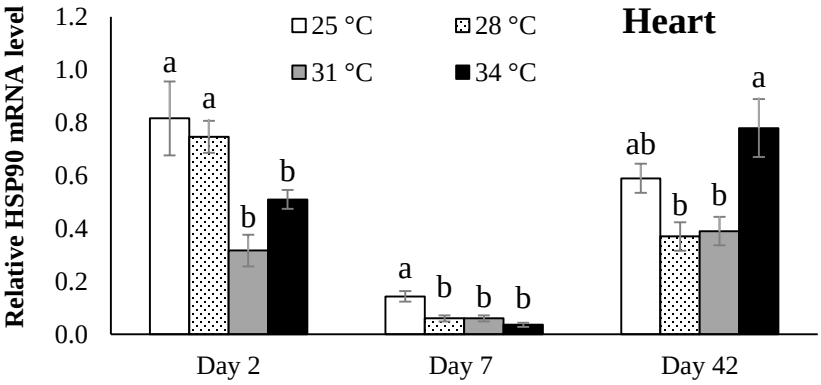
3.2.3. HSP90 mRNA expression

HSP90 mRNA expression levels in each tissue are shown in Figure 2.3.6. In 34 °C group, the HSP90 transcript level increased ($P < 0.05$) in the liver, muscle and gill after 2 days of exposure as compared with 25 °C and 28 °C; however, it showed overexpression in liver, muscle, gill, brain, and heart after 42 days of exposure (Fig. 2.3.6 A-E). In 31 °C group, liver exhibited higher HSP90 expression compared with 25 °C and 28 °C after 2 days of exposure; dropped towards the baseline level after 7 days of exposure, and found significantly higher level in liver and gill after 42 days of exposure (Fig. 2.3.6 A, B).









Thermal exposure

Fig. 2.3.6. Expression patterns of HSP90 mRNA in the liver (A), muscle (B), gill (C), brain (D), and heart (E) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperatures (25 °C, 28 °C, 31 °C and 34 °C) for 42 days. The expression of the target gene was normalized by 18s rRNA expression. Data represent the mean ± SEM of three replicates (n=15). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

4. Discussion

When fish undergo stress because of diverse environmental changes including temperature, cellular damage occurs from the stress that leads to protein denaturation (Kultz, 2005). In response to the stress, protective proteins are synthesized within the body to guard cells against secondary shock caused by stress (Iwama et al., 1999; Hightower, 1991). Consequently, elevated expression of heat shock genes are releasing to produce heat shock proteins (HSPs), which interact with the stress-denatured proteins to maintain or restore their native structures and prevent aggregation and degradation. HSPs transiently reprogram cellular metabolic activity, repair damaged proteins, and thereby maintaining protein homeostasis (Sanders, 1993; Kultz, 2005). HSPs have been considered as suitable biomarkers for assessing the organism's response to environmental stressors because their accumulations in tissues indicate the intensity of the stressors (Iwama et al., 1998; Feder and Hofmann, 1999; Kregel, 2002). In this study, we examined the heat shock proteins responses in various tissues of *E. akaara* under different water temperatures. Our results demonstrated that high water temperature (36 °C and 34 °C) significantly increase the transcript levels of Hsp60, HSP70, and HSP90 in liver, gill, muscle, brain and heart. In the present study, the consistent upregulation of Hsp60, Hsp70, and Hsp90 transcript in liver, gill, muscle of 36 °C and 34 °C groups relative to control, suggesting that tissue damage resulted from thermal stress is not readily restored.

Hsp60 is a stress-inducible, well-characterized chaperone that can show a potent inflammatory response in cells and therefore, their elevated levels have been considered as a "danger signal" of stressed or damaged cells (Ohashi et al., 2000). Hsp60 plays an extremely important role in cell protection (Eggert-Kruse et al., 2015). The Hsp60 transcript is up regulated in response to elevated water temperatures in different organisms including fish (Xie et al., 2015; Shi et al., 2016; Wang et al., 2017). In the present study, we observed the highest upregulation of Hsp60 transcript at 36 °C and 34 °C water temperature in all the studied tissues. The increased levels of Hsp60 were also observed in gill, muscle and heart after prolong exposure to 32 °C which indicates the sensitiveness to temperature. Our results are in accordance with the earlier report of Zhou et al. (2018), who showed that Hsp60 mRNA levels are significantly elevated in various tissues of albino northern snakehead (*Channa argus*) after exposure to 37 °C water temperature. Similar results were also observed in rainbow trout (*Oncorhynchus mykiss*) (Shi et al., 2015), green sturgeon (*Acipenser medirostris*) (Werner et al., 2007), and murrel (*Channa striatus*) (Purohit et al., 2014). In this study, the significant increase in Hsp60 at high water temperature suggesting that Hsp60 might have a greater role to play in the adaptation of *E. akaara* at higher temperatures and therefore it is continuously synthesized depending upon the need thereby aiding survival at a higher temperature. Thus, Hsp60 appears to be an important player in acquired thermo-tolerance and adaptation.

Hsp70 plays an important role in the maintenance of intracellular homeostasis via protein transport across the cellular membrane and protein synthesis (Moseley, 1998). It is the primary family of heat shock proteins that show sensitive to environmental stressors, is induced by thermal stress, and therefore protects cells from stress-induced apoptosis (Mosser et al., 1997; Mallouk et al., 1999). Several studies have been shown the correlation of the Hsp70 gene expression with

changing environments (Dalvi et al., 2012; Purohit et al., 2014; Liu et al., 2015; Shin et al., 2018). In the present study, Hsp70 expression in different tissues increased concomitantly with increased water temperatures (36 °C and 34 °C) and exposure duration. In case of 32 °C and 31 °C, Hsp70 transcript level was up-regulated initially, then returned to the basal level, and again up-regulated in liver, gill and muscle after prolonged thermal exposure. This is indicative of the protective role played by Hsp70 in response to both short and long term thermal exposure. This is likely a result of the apoptotic defense mechanism triggered by increased water temperature. This appears to be an adaptive mechanism as similar results have been reported in *Channa striatus* also, where the Hsp70 is constitutively expressed (Purohit et al., 2014). Similar observations were also reported in Japanese flounder (*Paralichthys olivaceus*) (Liu et al., 2015) and spotted sea bass (*Lateolabrax maculatus*) (Shin et al., 2018).

Hsp90 is one of the most prominent HSPs, which can be induced by various stressors (Feder and Hofmann, 1999). The elevated Hsp90 levels in organism play a vital role in cell survival under stress (Mocanu et al., 1993). Increased synthesis of Hsp90 has been reported in fish following thermal stress (Oksala et al., 2014; Purohit et al., 2014; Cheng et al., 2018). In this study, the up-regulated Hsp90 mRNA levels in different tissues of 36 °C and 34 °C groups may reflect a protective response against cellular damage induced by temperature stress. Our results are consistent with those of Cheng et al. (2018), who observed sharp increases in Hsp90 transcript levels in pufferfish (*Takifugu obscurus*) at 37 °C. In the present study, the expression patterns of Hsp90 in different tissues were more or less similar to Hsp70. Shin et al. (2018) observed the related results in spotted sea bass, where Hsp90 and Hsp70 exhibited the similar expression pattern after exposure to different water temperatures (20 °C, 22 °C, 24 °C and 28 °C). The Hsp90 and Hsp70 bind together and regulates the activation of hormone receptors in the nucleus, protein

translocation and degradation. The Hsp70 and Hsp90 proteins have been shown to prevent apoptotic cell death (Kregel, 2002; Young et al., 2004; Wegele et al., 2004).

Tissue-specific variations in HSP levels are reported in various fish species (Wang et al., 2007; Shi et al., 2015; Dalvi et al., 2017; Shin et al., 2018). In this study, variation in the mRNA expression of Hsp60, Hsp70, and Hsp90 in the studied tissues indicate that the synthesis of Hsp60, Hsp70 and Hsp90 in response to thermal stress is tissue-dependent. In this study, the expression chronology of Hsp60, Hsp70, and Hsp90 in different tissues after exposure at high temperature (34 °C and 36 °C) were, respectively as follow: liver > gill > muscle > brain > heart, liver > muscle > brain > gill > heart, liver > gill > muscle > heart > brain. Dalvi et al. (2017) observed the tissue-specific variations in Hsp70 levels (liver > brain > muscle > gill) in catfish (*Horabagrus brachysoma*) acclimated at 26 °C and 31 °C. The Hsp60 showed the tissue-dependent mRNA expression in *O. mykiss* (Shi et al., 2015) and *C. argus* (Zhou et al. (2018) after exposure to different water temperatures. In this study, the highest induction of Hsp60, 70 and 90 synthesis was clearly apparent in the liver, followed by gill and muscle. The liver is a metabolically active tissue in terms of protein synthesis and turnover. The liver performs several crucial functions including detoxification, metabolism, bile secretion, immune defense, and hormone synthesis in fish and other vertebrates. The gill is a crucial organ for respiratory, osmoregulatory, excretory, and branchial functions, and is highly sensitive to environmental stressors (Wang et al., 2007). The stress responses differ between tissues supports the hypothesis that certain tissues govern the thermal limits of an organism more than the others (Dyer et al., 1991). Such differences in the accumulation of stress proteins have been suggested to be useful in identifying the tissues that are particularly vulnerable to damage by a specific stressor and in identifying the extent of the damage (Sanders, 1993).

5. Conclusion

In summary, increased water temperature significantly affects the Hsp60, Hsp70 and Hsp90 mRNA level in different tissues of *E. akaara*. The highest expression of Hsp60, Hsp70 and Hsp90 at 36 °C and 34 °C suggesting that these HSPs are required for immediate survival of *E. akaara* at high water temperature. 31 °C and 32 °C also induce stress as because transcript levels of Hsp60, 70 and 90 were upregulated after prolong exposure to these temperatures. There were tissue-specific differences in Hsp60, 70 and 90 expressions, indicating that the thermal defense mechanism of red-spotted grouper differs between tissue responses. The results obtained from this study can be used as primary data to understand the molecular mechanisms employed by fish to combat thermal stress.

Chapter 2.4

Growth performance and growth-related gene expression in red-spotted grouper

Abstract

The GH (growth hormone)/GHR (growth hormone recptor)/IGF (insuline-like growth factor) axis play a major role in controlling the fish growth. This study was conducted to investigate the effect of different rearing temperatures on growth and growth-related genes, IGF-1, GHR (growth hormone receptor) in red-spotted grouper (*Epinephelus akaara*). Juveniles of *E. akaara* (total length: 8.28 ± 0.10 cm, body weight: 8.53 ± 0.27 g) were exposed to four water temperature regimes (25 °C as control, 28 °C, 31 °C, and 34 °C) for 42 days, following 2 weeks of acclimation at 25 °C. Results showed that growth performance, in terms of body weight gain was maximum at 31 °C water temperature. Depressed growth performance was observed at 34 °C. Besides, the specific growth rate (SGR), feed conversion ratio (FCR), and feed efficiency ratio (FER) was significantly higher ($P < 0.05$) at 28 °C and 31 °C water temperature compared to 34 °C. IGF-1 and GHR mRNA levels were highly expressed at 28 °C and 31 °C in liver. Taken together, our results suggest that *E. akaara* showed better growth responses to temperature regimes at 28 °C and 31 °C. Besides, our findings also indicated that the IGF-1 and GHR in liver involved in the modulation of temperature-induced growth of red-spotted grouper.

Keywords: Water temperature, Growth performance, Insulin-like growth factor, Growth hormone receptor, Red-spotted grouper (*Epinephelus akaara*)

1. Introduction

The change in the culture water temperature may significantly affect the normal physiological processes, survival, and, mainly, growth (Hansen and Falk-Peterson, 2002; Luckenbach et al., 2007; Islam et al., 2019). Particularly, the growth and development of ectothermic animals are very temperature sensitive; low temperatures suppress metabolic rates, feeding rates, and growth rates; elevated water temperatures tend to cause an increase in growth up to an optimum point above which thermal stress occurs (Baum et al., 2005). The adequate temperature can improve the food ingestion, digestion, nutrient utilization, and, thus, enhance the growth performance of fish (Bogevik et al., 2010). The normal range of water temperature in the tropics to which fish are well adapted is 25–35 °C (Howerton, 2001). However, the rise of water temperature in the consequences of climate change is alarming for aquaculture. Though it varies from species to species, the increasing water temperature may become harmful for fish growth (Portner and Peck, 2010).

Growth hormone (GH), insulin-like growth factor (IGF-1), and insulin, are three important factors that can contribute to growth and metabolism (Banos et al., 1999). The GH/GH receptor/IGF-1 axis has a pivotal role in controlling both fish growth and development. Temperature influences growth via the actions of IGF-1 secreted by the liver following stimulation by GH. The activity of GH is initiated after binding to growth hormone receptor (GHR) in the liver (Kopchick and Andry, 2000; Gabillard et al, 2003a, b; Reinrck et al., 2005). The production and secretion of these hormones are directly or indirectly related to some exogenous factors, including water temperature (Imsland et al., 2007), salinity (Taylor et al., 2008), and photoperiod (Cruz and Brown, 2009). Increased water temperature elevated the hepatic IGF-1 mRNA level in Nile tilapia, *Oreochromis niloticus* (VeraCruz et al., 2006). Luckenbach et al. (2007) reported that muscle IGF-

1 mRNA was significantly affected in response to temperature changes in southern flounder (*Paralichthys lethostigma*). GHR genes were differentially expressed in muscle and liver of rainbow trout (*Oncorhynchus mykiss*) when reared under different water temperature (Gabillard et al., 2006). Thus, the GH/GHR/IGF-1 system is an important regulatory network that can control fish growth.

The red-spotted grouper, *Epinephelus akaara* is one of the important marine fish due to its high commercial value. It is a potential candidate for aquaculture as it has a high value in the live fish food trade in South Korea, China, and Japan (Sadovy de Mitcheneson et al., 2013). Recently, the culture of red-spotted grouper is becoming extensive in South Korea due to its increasing demand. A number of studies have been conducted to evaluate the growth performance and growth-related gene expression in response to water temperature changes in many species (Person-Le Ruyet et al., 2004; Lin et al., 2008; Li and Leatherland; 2008; Qiang et al., 2012; He et al., 2014); but, little is known about this in *E. akaara*. Our previous research investigated the growth performance of red-spotted grouper at a temperature range of 24 °C to 28 °C (Lee and Baek, 2018). However, the increase of water temperature due to climate change is anticipated to affect the growth performance of *E. akaara*. Therefore, in this study, the growth and growth-related genes were investigated under different rearing temperatures to make a better understanding of temperature influences on growth and their possible endocrine control in red-spotted grouper.

2. Materials and Methods

2.1. Experimental fishes

The experiment was conducted according to the fish maintenance, handling, and sampling guidelines of the Animal Ethics Committee of Pukyong National University (PKNU) (Regulation No. 554Experimental fish, *E. akaara* (total length: 8.28 ± 0.10 cm, body weight: 8.53 ± 0.27 g)

were obtained from the Marine Science Institute, Jeju National University and reared in the Marine Biology Department, PKNU, Busan, Korea. Fish were acclimated for 2 weeks before starting the experiment. The animals were maintained at 25 ± 0.5 °C temperature, 34 psu salinity, ≥ 6.6 mg/L dissolved oxygen level, 7.8 pH, and 12L:12D photoperiod during the acclimation period.

2.2. Experimental design

At the end of the acclimation period, 180 juveniles of red-spotted grouper were randomly stocked in 12 glass aquaria (15 fish/aquarium), each with a volume of 120 L. Each aquarium had equal size and height (75cm × 45cm × 45cm), and was equipped with the recirculating filtration system. The fish were exposed to four temperature conditions (25 °C as control, 28 °C, 31 °C, and 34 °C), each with three replications for 42 days. The temperature was gradually increased (Δ1 °C/h) from the control temperature (25 °C) until the experimental temperature (28 °C, 31 °C, and 34 °C) achieved. The required temperature was maintained by using a thermostat (OKE-6422H; OKE, Busan, Korea). During the experiment, water temperature, pH, salinity, and dissolved oxygen were monitored daily using a multiparameter water-quality meter (HI9829; Hanna Instrumentals, USA) and total ammonium levels were checked every 2 days with NH_3/NH_4^+ test kit (Tetra GmbH, Melle, Germany). The water quality parameters measured during the period of the experiment are shown in Table 1. During the acclimation and experimental period, fish were fed with a commercial diet (Otohime Hirame, Marubeni Nisshin Feed Co., Ltd., Japan; 50~52% protein and 7~10% lipids). Fish were fed 2 times daily (09:00 and 18:00 h) at a rate of 2% of their body weight during the experimental period. Uneaten food was removed after 30 minutes of feeding and 10% of the water in each aquarium was replaced daily with filtered clean seawater.

2.3. Sampling

Fish were sampled for growth measurements after 2, 7, and 42 days of thermal exposure. All animals were fasted for 24h prior to any handling and measurements. 15 fishes were randomly sampled from each temperature group (i.e., 5 fishes from each aquarium; n=15) on each sampling day. All fish were anaesthetized with 300 mg/L 2-phenoxyethanol (Sigma Aldrich, USA) to measure the length and weight individually. The liver, muscle, and brain were collected (n = 7) after the measurement and immediately kept in RNA later (Qiagen, Germany) at -80 °C for further analysis.

2.4. Growth performance

At each sampling points, the biometric parameters such as body weight (BW) and body length (BL) were measured individually (n = 15). Growth performance and feed efficiency were evaluated by determining growth and nutrient utilization indices. The following parameters were estimated according to below formulas:

Weight gain (WG; %) = 100 × [final body weight (g) – initial body weight (g)]

Feed efficiency ratio (FER) = weight gain (g) / feed intake (g)

Feed conversion ratio (FCR) = feed intake (g) / body weight gain (g)

Protein efficiency ratio (PER) = weight gain (g) / protein intake (g)

Specific growth rate (SGR; %WG/day) = 100 × [In final weight (g) – In initial weight (g)] / days

Condition factor (K; g/cm^3) = 100 × [fish weight (g) /fish $length^3$ $(cm)^3$]

Mortality (%) = 100 [number of fish died at the end of experiment / initial number of fish stocked]

2.5. Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNAiso plus (Takara, Japan) from the preserved liver, muscle, and brain according to the manufacturer's protocol. Total RNA concentration was

measured using a spectrophotometer (NanoDrop 1000, Thermo Scientific). The cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The synthesized cDNA was stored at -20 °C for further analysis.

2.6. Quantitative real-time PCR analysis

To evaluate the mRNA levels of insulin-like growth factor 1 (IGF-1) and growth hormone receptor (GHR), primers were designed using the sequence information available in National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.gov). Real-time PCR was carried out with Chromo4 Real-Time PCR System (Bio-Rad, USA). The real-time PCR reaction mixture contained 2µl of the diluted cDNA sample, specific primer, and TB Green^{TM} Premix Ex Taq^{TM} (Takara, Japan). The PCR reaction was performed with an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The 18S rRNA gene was used as a reference gene. The list of used primers is shown in Table 2.

2.7. Statistical analysis

Values are presented as the mean ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to identify the significant differences among the different temperature conditions. Statistical significance was set at $P < 0.05$. All statistical analysis was done using SPSS program Version 21 (IBM Corp., USA).

Table 2.4.1. Water quality parameters (Mean ± SEM) measured during the experimental period.

Parameters	Temperature groups			
	25 °C	**28 °C**	**31 °C**	**34 °C**
Temp.	24.88 ± 0.04	27.77 ± 0.04	30.90 ± 0.05	33.78 ± 0.04
Salinity (psu)	33.80 ± 0.005	33.89 ± 0.06	33.72 ± 0.05	33.77 ± 0.07
pH	8.05 ± 0.02	8.06 ± 0.01	7.99 ± 0.02	7.99 ± 0.02
DO (mg/L)	6.86 ± 0.04	6.72 ± 0.04	5.68 ± 0.05	4.16 ± 0.03
NH4 (mg/L)	<0.25	<0.25	<0.25	<0.25

Table 2.4.2. Primers used for quantitative real-time PCR of GHR and IGF-1 genes.

Gene	Sequence (5' - 3')	Direction
Growth hormone receptor	TCAAACCATACACCCTCAGC	Forward
	CTGTACCACTGTGTAGTCTGC	Reverse
Insulin-like growth factor 1	ATGTAGGGAAGGTGCGAATG	Forward
	CCTTTGTCAGCATCCTCTTTG	Reverse
18S rRNA	CGGTAATTCCAGCTCCAATAGCG	Forward
	CTTAATCATGGCCCCAGTTCAGAG	Reverse

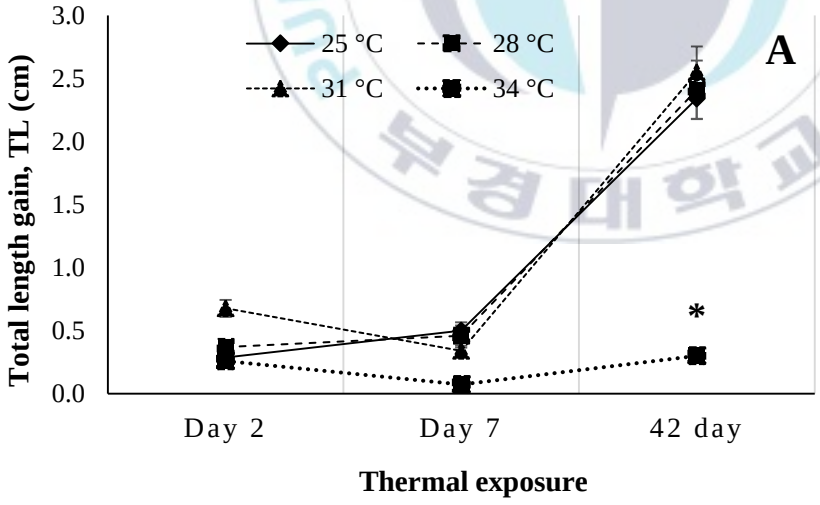
3. Results

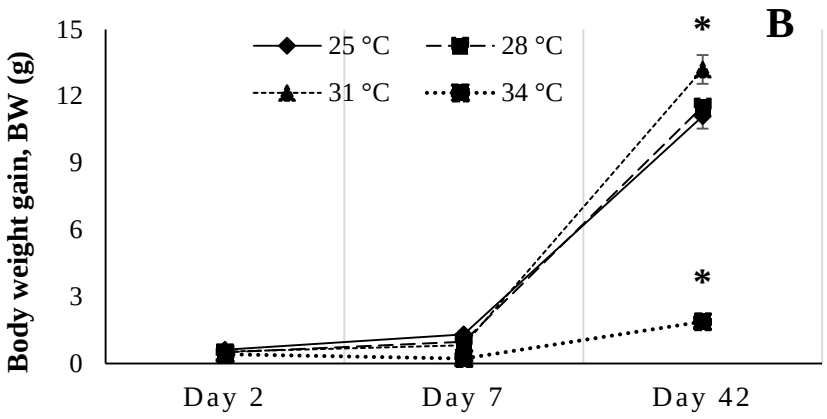
3.1. Effect of temperature on growth performance, feed utilization, and biometric indices

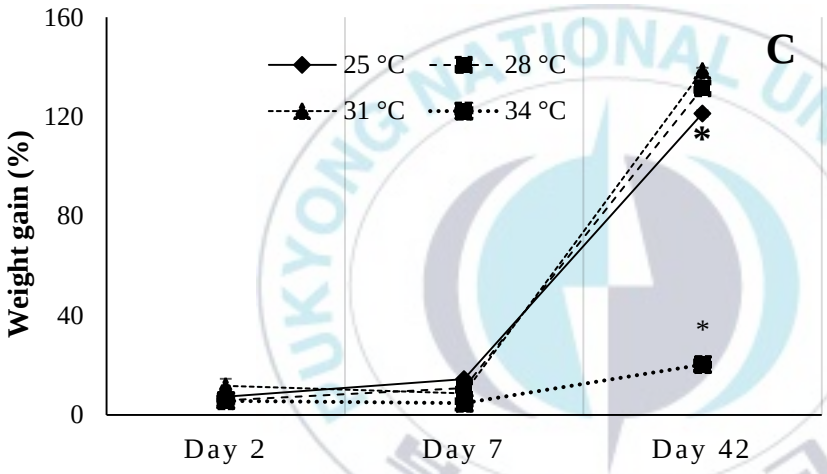
The growth performance of red-spotted grouper in response to different temperatures (25 °C, 28 °C, 31 °C, and 34 °C) is presented in Figure 2.4.1. Fish treated with 28 °C and 31 °C

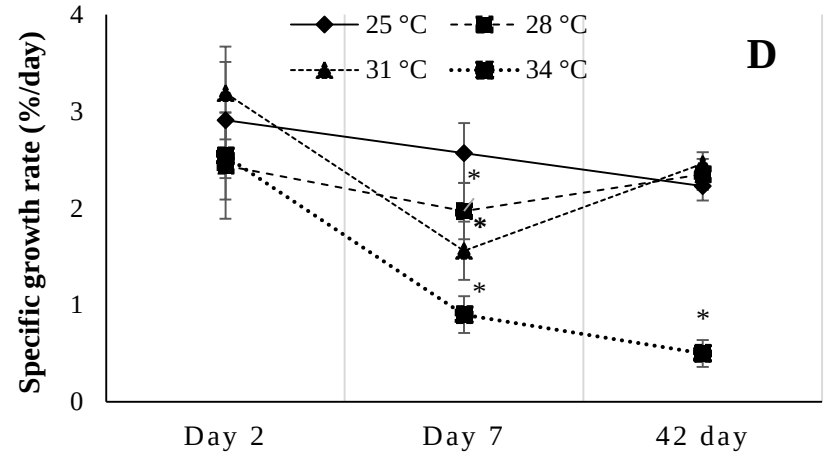
showed a significant tendency ($P < 0.05$) to have better growth performance compared with 34 °C at the end of the experiment. Besides, the response to various temperatures was significantly ($P < 0.05$) variant. The improvements in total length, body length, body weight, weight gain percentage and specific growth rate (SGR) were maximal in the fish group treated with 28 °C and 31 °C, while the lowest values were observed in fish treated with 34 °C (Fig 2.4.1 A-D).

The higher feed intake (FI) was recorded in the groups treated with 28 °C and 31 °C, which was associated with a significant enhancement in feed efficiency ratio (FER) and reduction in feed conversion ratio (FCR). The protein efficiency ratio (PER) was also improved in the fish group of 28 °C and 31 °C when compared with the 25 °C and 34 °C (Table 3). At the end of the experiment, no significant difference in condition factor (CF) was observed among the different temperature groups ($P < 0.05$); however, mortality occurred at 34 °C when fish were exposed to thermal stress for 42 days (Table 2.4.3).









Thermal exposure

Fig. 2.4.1. Growth performance of juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. Data are presented as mean ± SEM of three replicates (n=15). Asterisks (*) denote significant difference among treatment groups at equivalent time points (ANOVA, Duncan's multiple range test; $P < 0.05$).

Table 2.4.3. Feed utilization and biometric indices of juvenile red-spotted grouper, *Epinephelus akaara* exposed with different water temperatures (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days.

Parameters	Treatments	Exposure time (day)		
		2	7	42
FI (g fish^{-1} day^{-1})	25 °C	0.18 ± 0.01^{a}	0.21 ± 0.01^{a}	0.40 ± 0.03^{a}
	28 °C	0.18 ± 0.01^{a}	0.20 ± 0.01^{a}	0.42 ± 0.03^{a}
	31 °C	0.18 ± 0.00^{a}	0.21 ± 0.01^{a}	0.46 ± 0.03^{a}
	34 °C	0.18 ± 0.01^{a}	0.18 ± 0.01^{b}	0.23 ± 0.01^{b}
FER	25 °C	1.17 ± 0.07^{ab}	1.16 ± 0.06^{a}	0.76 ± 0.04^{a}
	28 °C	1.02 ± 0.12^{b}	0.91 ± 0.09^{b}	0.79 ± 0.04^{a}
	31 °C	1.46 ± 0.08^{a}	0.71 ± 0.08^{b}	0.82 ± 0.03^{a}
	34 °C	0.97 ± 0.06^{b}	0.24 ± 0.02^{c}	0.22 ± 0.01^{b}
FCR	25 °C	0.74 ± 0.13^{b}	1.51 ± 0.21^{c}	1.37 ± 0.08^{b}
	28 °C	0.63 ± 0.12^{b}	2.68 ± 0.21^{bc}	1.29 ± 0.06^{b}
	31 °C	0.87 ± 0.08^{b}	3.61 ± 0.71^{b}	1.23 ± 0.05^{b}
	34 °C	1.58 ± 0.32^{a}	5.72 ± 0.89^{a}	7.67 ± 1.29^{a}
PER	25 °C	2.25 ± 0.12^{ab}	2.24 ± 0.13^{a}	1.46 ± 0.07^{a}
	28 °C	2.53 ± 0.22^{a}	1.75 ± 0.19^{b}	1.52 ± 0.07^{a}
	31 °C	2.80 ± 0.16^{a}	1.37 ± 0.17^{b}	1.58 ± 0.06^{a}
	34 °C	1.86 ± 0.17^{b}	0.46 ± 0.04^{c}	0.43 ± 0.04^{b}
CF	25 °C	1.46 ± 0.05^{a}	1.40 ± 0.02^{ab}	1.55 ± 0.05^{a}
	28 °C	1.40 ± 0.04^{a}	1.33 ± 0.05^{b}	1.53 ± 0.03^{a}
	31 °C	1.27 ± 0.01^{b}	1.37 ± 0.01^{ab}	1.53 ± 0.01^{a}
	34 °C	1.45 ± 0.04^{a}	1.46 ± 0.04^{a}	1.61 ± 0.02^{a}
Mortality (%)	25 °C	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}
	28 °C	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}
	31 °C	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}
	34 °C	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	19.05 ± 4.76^{a}

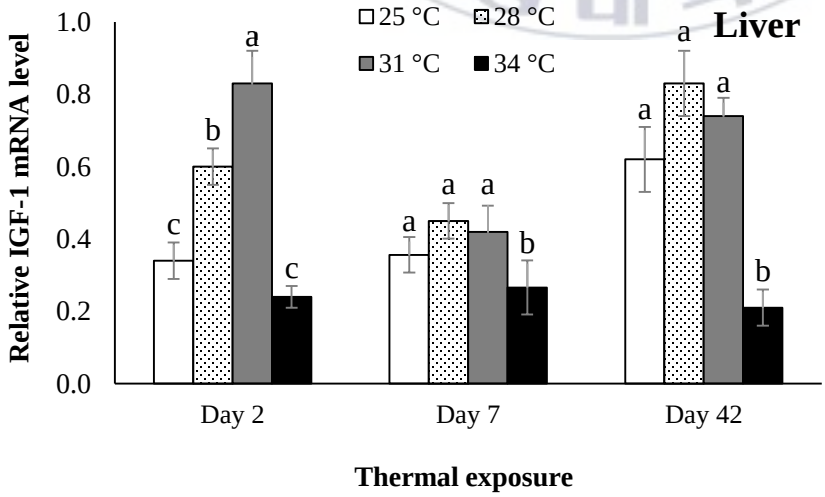
Data are presented as mean ± SEM of three replicates (n=15). Means with different lowercase letters in the same column indicate the significant difference among treatment groups at equivalent time points (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.2. Relative expression of growth-related genes

3.2.1. IGF1 mRNA expression

IGF1 mRNA expression levels in each of the three tissues is shown in Figure 2.4.2. In liver, IGF1 mRNA expression gradually increased after water temperature increased to 31 °C, then rapidly decreased at 34 °C after 2 days of exposure and showed a significantly higher level of expression at 25 °C, 28 °C and 31 °C compared to 34 °C after 7 and 42 days of exposure ($P < 0.05$) (Fig. 2.4.2 A).

In muscle and brain, the transcript level of IGF1 was found lower after 2 days of exposure; significantly increased at 28 °C and 31 °C after 7 days of exposure and higher expression was recorded to 25 °C and 28 °C at 42 days of exposure (Fig 2.4.2 B-C).



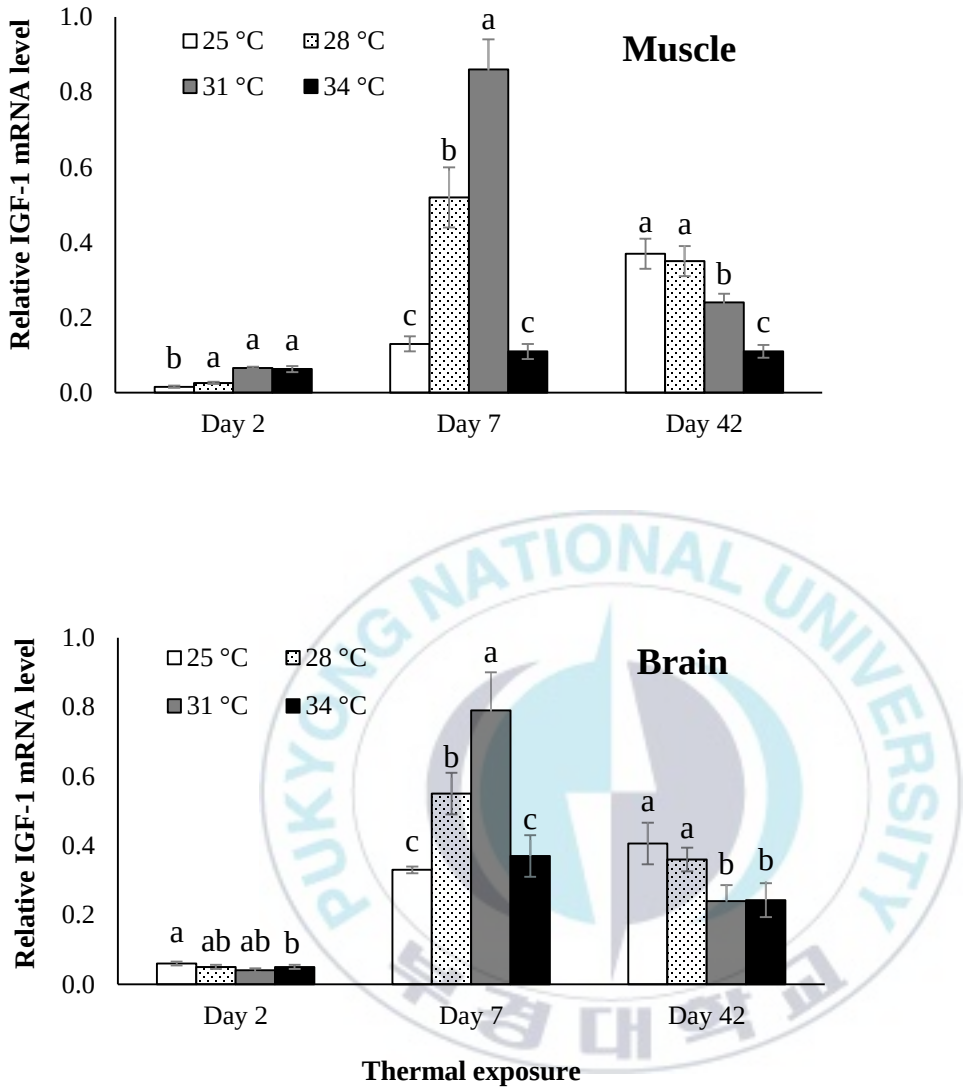
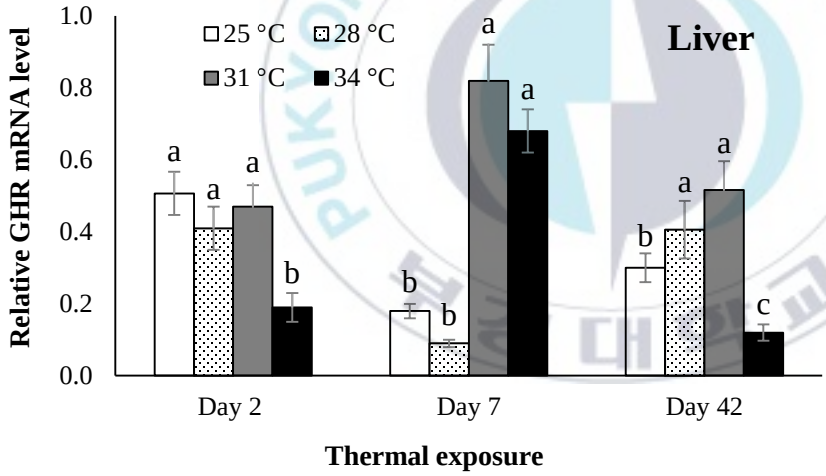


Fig. 2.4.2. Relative mRNA expression of IGF1 in the liver (A), muscle (B), and brain (C) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperature (25 °C, 28 °C, 31 °C and 34 °C) for 42 days. The expressions of the target gene were normalized by 18s rRNA expressions. Data represent the mean ± SEM of three replicates (n=7). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

3.2.2. GHR mRNA expression

GHR mRNA expression levels in each of the three tissues is shown in Figure 2.4.3. In liver, the GHR gene expression was down-regulated at 34 °C when exposed for 2 days, then significantly higher expression was observed at 31 °C and 34 °C after 7 days of exposure and found the higher level of expression at 25 °C and 28 °C after 42 days of exposure ($P < 0.05$) (Fig. 2.4.3 A).

In muscle and brain, GHR showed lower mRNA expression at 2 days of exposure; however, significantly increased at 28 °C and 31 °C after 7 and 42 days of thermal exposure (Fig 2.4.3 B-C). In addition, 34 °C group exhibited the lowest GHR expression among the temperature groups throughout the trial.



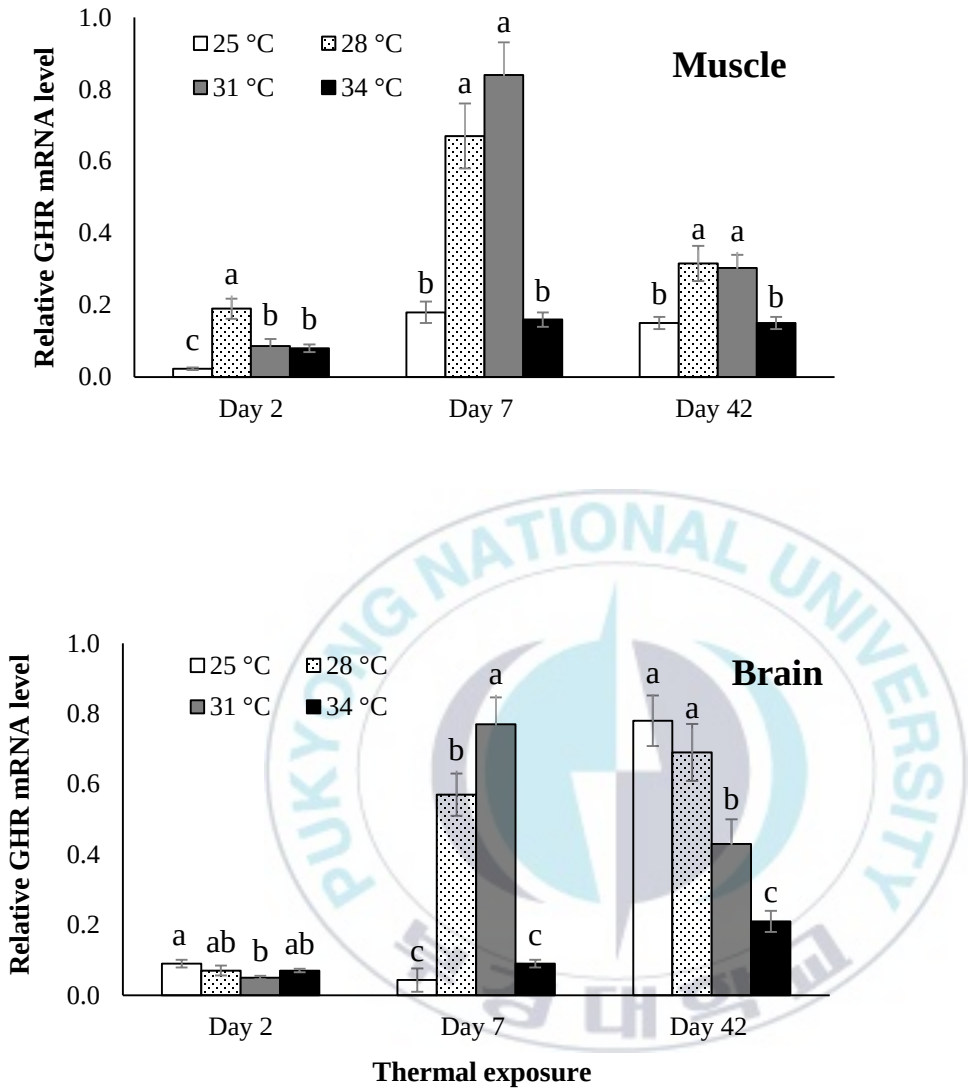


Fig. 2.4.3. Relative mRNA expression of GHR in the liver (A), muscle (B), and brain (C) of red-spotted grouper, *Epinephelus akaara*, after exposure to different water temperature (25 °C, 28 °C, 31 °C, and 34 °C) for 42 days. The expressions of the target gene were normalized by 18s rRNA expressions. Data represent the mean ± SEM of three replicates (n=7). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

4. Discussion

Water temperature plays an important role in controlling fish growth via its effect on feeding and metabolism. Higher temperature contributes to increase food intake and feed utilization in fish. The fish raised at higher temperature have increased metabolic rates that produce faster growth rates (Musuka et al., 2009; Schulte et al., 2011). In contrast, cool water temperature generally slows down the growth rates of fish (Brett, 1979). In this study, the growth rate of *E. akaara* was greatly influenced by the fluctuations of water temperature. Our results suggest that *E. akaara* show better growth responses to temperature regimes at 28 °C and 31 °C. Thermal responses in terms of growth performances have been studied in many teleosts including *Lophiosilurus alexandri* (Costa et al., 2016); Nile tilapia, *Oreochromis niloticus* (Qiang et al., 2012); European sea bass, *Dicentrarchus labrax* (Person-Le Ruyet, 2004); *Tilapia rendalli* (Musuka et al., 2009); and orange spotted grouper, *Epinephelus coioides* (Lin et al., 2008).

Overall, the highest growth response was observed at 31 °C; in contrast, fish reared at 34 °C had the lowest growth rate. It indicated that the upper threshold temperature for growth and development of *E. akaara* juveniles is around 31 °C. The suitable water temperature ranges, at which maximum growth rates were observed in *O. niloticus* was 29-31 °C (Qiang et al., 2012). It can be seen that fish at optimal water temperature used less energy to maintain its internal requirement, and thus growth was effectively promoted. High water temperature, above the suitable range, may pose physiological stress and the energy produced from the metabolism is mainly used to sustain the homeostasis of the fish body. Thus, the high temperature may lead to retard growth and feed utilization of fish (Elliot, 1972).

In the present study, fish reared at 34 °C had low appetite as because the food intake was significantly decreased (0.23 ± 0.01 g fish^{-1} day^{-1}) compared with 28 °C (0.42 ± 0.03 g fish^{-1} day^{-}

^{1}) and 31 °C (0.46 ± 0.03 g fish^{-1} day^{-1}). The inhibited growth at 34 °C could be due to the hypoxia as because the DO level was significantly declined (4.16 ± 0.03 mg/L) at 34 °C. It has been reported that hypoxic conditions can inhibit growth by reducing appetite, reducing assimilation efficiency (increasing FCR) shift in energy balance for air-breathing (Lefevre et al., 2014).

In general, the specific growth rate (SGR) is considered as a better growth performance indicator as it is used to do comparisons of growth among different species. Considerable variations in weight-specific growth rate have been reported in the teleost, ranging from < 1 to 79 % day^{-1} (Otterlei et al. 1999). In this study, the effects of water temperature on SGR in *E. akaara* juveniles were proved very significantly. The SGR of fish reared at water temperature 28 °C and 31 °C was 2.35 ± 0.18 % day^{-1} and 2.46 ± 0.15 % day^{-1} respectively, notably higher than the fish raised at water temperature 34 °C (0.50 ± 0.02% day^{-1}). Our results are consistent with those of Islam et al. (2019), who observed that Thai pangas (*Pangasianodon hypophthalmus*) reared at 28 °C and 32 °C have higher SGR values compare to those reared at 36 °C.

The feed conversion value (FCR) and feed efficiency value (FER) fluctuated significantly in accordance with temperature treatments. The term FCR is defined as the amount of feed administered to obtain a kilogram of fish. The FER value is inversely related to FCR value. The fish reared at a suitable temperature range have a low FCR value and a high FER value. In this study, the fish reared at 28 °C and 31 °C had improved FCR (28 °C: 1.29 ± 0.06; 31 °C: 1.23 ± 0.05) and FER (28 °C: 0.79 ± 0.04; 31 °C: 0.82 ± 0.03) compared to 34 °C (FCR: 7.67 ± 1.29; FER: 0.22 ± 0.01). The high FCR and low FER value indicated the growth depression at 34 °C compared with the 28 °C and 31 °C. The prolonged exposure to 34 °C has declined the growth rate that is reflected in the FCR and FER values. This finding indicated that 34 °C is toxic

temperature for the growth of *E. akaara* juveniles. Person-Le Ruyet et al. (2004) observed similar results in European sea bass. *Dicentrarchus labrax,* after exposure to 29 °C water temperature.

GH is a peptide hormone that is secreted from the pituitary gland. GH level can directly affect the secretion of IGF-1 in the liver. The actions of GH are initiated by its binding to GHR on the cell membrane of target tissues. Thus, the GH-IGF-1 axis plays an important and critical role in the regulation of both growth and development. IGF-1 is a key regulatory hormone that stimulates somatic growth by affecting different tissues (Reinecke et al., 1997). IGF-1 expression is well related to the growth rate as has already been observed in several fish species (Qiang et al., 2012; Gabillard et al., 2003b; Nakano et al., 2013; Nakano et al., 2015), suggesting that IGF-1 might be directly involved in overall growth regulation.

The involvement of IGF-1 in the endocrine regulation of growth by temperature was examined by measuring the IGF-1 mRNA levels in the liver. The highest level of hepatic mRNA was observed at the water temperature of 28 °C and 31 °C, while the level was significantly lower ($P > 0.05$) at 34 °C. This result indicated that the expression of the hepatic mRNA level was significantly correlated with water temperature conditions. The suppressed hepatic IGF-1 mRNA level at high temperature (34 °C) indicates that *E. akaara* possibly exceeding its suitable thermal range (28-31 °C) for growth. The higher temperature may interfere with the expression of IGF-1 level in the liver (Gabillard et al., 2005). We also measured the IGF-1 mRNA levels in muscle and brain tissues to determine whether it could play a role in growth as in mammals (Florini et al., 1996). The muscle and brain of *E. akaara* showed the transitory increase in the IGF-1 mRNA level at 28 °C and 31 °C. The results suggest that hepatic IGF-1 play a better role to regulate the temperature-induced growth in *E. akaara*. The similar result was reported in Nile tilapia, *O.*

niloticus (Qiang et al., 2012; Vera Cruz et al., 2006); rainbow trout, *Oncorhynchus mykiss* (Gabillard et al., 2003b); coho salmon, *Oncorhynchus kisutch* (Nakano et al., 20015).

In this study, GHR mRNA levels were highly expressed ($P < 0.05$) at 28 °C and 31 °C in liver; however, similar to IGF-1, GHR mRNA levels were transiently increased in brain and muscle. High water temperature (34 °C) inhibited ($P < 0.05$) the expression of GHR mRNA levels in all the studied tissues. These results suggest that the GHR and IGF-1 levels may increase simultaneously at a water temperature of 28 °C and 31 °C to regulate the growth of *E. akaara*. However, high water temperature (34 °C) may produce the physiological stress that suppresses the expression of GHR mRNA levels in muscle and brain of *E. akaara*. Gabillard et al. (2006) reported that high temperature led to a lower GH binding capacities in the liver of rainbow trout, *O. mykiss* reared at different water temperature (8 °C, 12 °C or 16 °C). Physiological stress resulted from acute handling and thermic shock also decreased the mRNA expression of GHR in coho salmon, *O. kisutch* (Nakano et al., 2013, 2015) and silver promfret, *Pampus argenteus* (Sun et al., 2017).

5. Conclusions

In conclusion, the results of the present study suggest that the juveniles of *E. akaara* show better growth responses at 28-31 °C water temperature. The upper threshold temperature for growth and development of red-spotted grouper is around 31 °C as because the growth rate was ceased at 34 °C. Our results also indicate that rearing water temperature of 28 °C and 31 °C can maximize the IGF-1 and GHR mRNA levels in liver. The combined action of IGF-1 and GHR in liver may regulate the growth of *E. akaara* rather than the muscle and brain. This fine information might be helpful for the grouper farmers to maximize the production as well as to improve the fitness in cultured fish.

Chapter 2.5

Critical thermal maxima (CTmax) of red-spotted grouper acclimated to different temperatures

Abstract

Quantifying a species thermal tolerance is important to assessing biological impacts of anticipated temperature increase due to climate change effect. Hence, the present study aimed to determine the critical thermal maximum (CTmax) of red-spotted grouper, *Epinephelus akaara* under different acclimation temperatures (Ta). Fish were acclimated at 24 °C, 28 °C, and 32 °C water temperature for 2 weeks. Water temperature was increased at a rate of 1 °C h^{-1} and thermal tolerance (CTmax) level was measured following the critical thermal methodology (Paladino et al., 1980). The results showed that CTmax values of *E. akaara* were 35.61 °C, 36.83 °C, and 37.65 °C for fish acclimated at 24 °C, 28 °C, and 32 °C, respectively. The acclimation response rate (ARR) of *E. akaara* was 0.26. The body size of *E. akaara* was significantly correlated with CTmax values. Collectively, it can be said that red-spotted grouper acclimated to a higher temperature has a higher thermal tolerance. Besides, the CT_{max} value 35.61-37.65 °C suggests that rearing temperature of *E. akaara* must never rise above 35 °C for its successful aquaculture especially during the dry warm summer months. Understanding the critical thermal tolerance limit of *E. akaara* is of ecological significance in the conservation and aquaculture of this species.

Keywords: *Epinephelus akaara*, Critical thermal maxima, Acclimation response ratio, Acclimation temperature, Red-spotted grouper

1. Introduction

Temperature is to be one of the most important criteria considered in the species selection for aquaculture. Temperature tolerance in fish varies with species, and acclimation time and temperature (Das et al., 2004). Assessment of thermal tolerance limit can help to understand the adaptive biology of aquatic animals to any particular habitat. Understanding of thermal acclimation and critical temperatures are two important aspects in evaluating the thermal tolerance of fish (Herrera et al., 1998). The critical thermal maxima (CTmax) is considered as an effective indicator of the thermal tolerance of an organism that allows the identification of the temperature at which the first signs of stress occur (Beitinger et al., 2000). CTmax is the temperature for a given species, above which most individuals respond with unorganized locomotion, subjecting to likely death. The CTmax procedure does not rely on sacrificing the fish as an endpoint (Lutterschmidt and Hutchison, 1997), and thus this may be more preferable for working with endangered or threatened species.

One of the common methods used to determine the upper-temperature tolerance limit (CTmax) of an aquatic organism is the critical thermal methodology. It was first described by Cowles and Bogert (1944) and then used by various researchers (Lutterschmidt and Hutchison, 1997; Beitinger et al., 2000) on fish or other aquatic animals. Critical thermal methodology data provide a relative comparison of the thermal tolerance between fish species in extreme temperature. Acclimation response ratio (ARR) is the mathematical expression of the reaction of aquatic animals to temperature change, which is also used in temperature tolerance studies (Claussen, 1977; Diaz-Herrera et al., 1998).

The red-spotted grouper (*Epinephelus akaara*) is a subtropical species, whose optimal temperature for growth is reported to be 24-28 °C (Lee and Baek, 2018). However, in recent years,

the natural landing of *E. akaara* has reduced to an alarming level due to environmental degradation, indiscriminate fishing, and anthropogenic activities. This species has been enlisted as an endangered species by IUCN (International Union for the Conservation of Nature) (Baillie et al. 2004; Tupper and Sheriff 2008). The rising water temperature due to climate change effect is an important threat to this species, which is anticipated to affect its productivity in wild as well as in aquaculture conditions.

Determining the thermal tolerance of a species is important for understanding its physiology and ecology, as well as predicting the long-term effects of climate change (Portner and Peck, 2010). This knowledge is required to improve management and conservation efforts for endangered species (Deslauriers et al., 2016). The effects of acclimation temperature (Ta) on thermal tolerance have been explored in many fish species (Currie et al., 1998; Beitinger et al., 2000; Zhang and Kieffer, 2014; Yanar et al., 2019); however, the thermal acclimation effects on *E. akaara* has so far not been studied. Therefore, the purpose of the present study was to assess the CTmax of red-spotted grouper under different Ta.

2. Materials and methods

2.1. Experimental fish

The study was carried out in the Marine Biology Department, Pukyong National University (PKNU), Busan, Korea. Fish were obtained from the Marine Science Institute, Jeju National University, Korea. The experiment was conducted following the fish maintenance, handling, and sampling guidelines of the Animal Ethics Committee of PKNU (Regulation No. 554).

2.2. Acclimation of fish

The experimental fish were gradually acclimated to 120 L aquariums for 2 weeks at 24 °C, 28 °C, and 32 °C water temperature. These temperatures were maintained by using thermostat

(OKE-6422H, Korea). Each aquarium had equal size and height (75cm × 45cm × 45cm) and was equipped with a recirculating filtration system. Air blower through airstone continuously aerated the aquariums. Water temperature, pH, salinity, and dissolved oxygen (DO) were monitored daily using a multi-parameter water quality meter (HI9829; Hanna Instrumentals, USA). During the acclimation period, salinity, pH, DO, and photoperiod were 33.67–33.81 g/L, 7.89–7.97, and 5.62-6.96 mg/L, 12L : 12D, respectively. Fish were fed 2 times daily (09:00 and 18:00 h) at a rate of 2% of their body weight. Uneaten food was removed after 30 minutes of feeding and 10% of the water in each aquarium was replaced daily with filtered clean seawater. At the end of the acclimation period, 10 fish from each temperature group were randomly captured and weighted individually to determine the average weight.

2.3. Determination of CTmax

The fish were starved for 1 day before CTmax test. 7 fish from each acclimation temperature were randomly selected and determined the CTmax value individually. Thus, 21 individuals from three acclimation temperature were used in this process. The thermal tolerance (CTmax) levels were assessed using the critical thermal methodology as described previously by Paladino et al. (1980). The individual fish were transferred into 40L aquarium and subjected to thermal stress by using a thermostatically controlled aquarium heater. Water temperature was constantly increased at a rate of 1 °C h^{-1} (Wedemeyer and McLeay, 1981) until loss of equilibrium (LOE) was reached. LOE was designated as the endpoint used for the CTmax, at which fish were first time unable to keep the position in dorsoventrally for 10 sec (Ziegeweid et al., 2008). Once the fish reached the endpoint (LOE), the final temperature was recorded (Fig. 2.5.1). The temperature was carefully monitored before and during the thermal test. Continuous aeration was provided during each test to maintain adequate DO levels in the test tank. Immediately after the

CTmax test, the fish were transferred to their respective acclimation temperature and monitored for survival for the next 24 h. During the CTmax trial, the fish were noted for behavioral changes such as extreme excitement, mobility, rapid opercular movement, mucus production, and finally loss of balance. The acclimation response rates (ARR) were calculated according to Claussen (1977).

2.4. Statistical analysis

All the data were presented as mean ± SEM and analyzed by using SPSS statistics software (ver. 21.0; IBM Corp., USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to assess the significant differences between the means. Regression analysis was used to estimate the relationship between acclimation water temperatures and other studied parameters. Statistical significance value was set to $P < 0.05$.

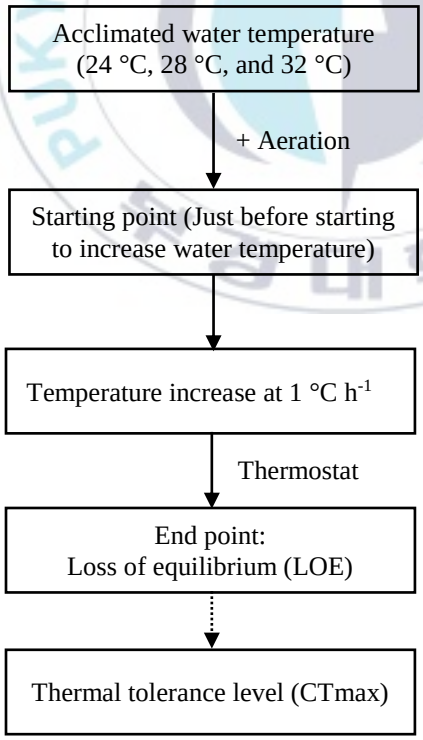


Fig. 2.5.1. Flow diagram of the experimental design for the determination of CTmax of red-spotted grouper, *Epinephelus akaara.*

3. Results

3.1. Effects of T_a on thermal tolerance (CT_{max}) level

CT_{max} was significantly influenced by T_a (ANOVA, $P < 0.05$; Fig. 2.5.2). A significant positive relationship existed between CT_{max} and T_a ($R^2 = 0.965$, $P < 0.01$, $CT_{max} = 29.55 + 0.26T_a$). Fish acclimated to 24 °C had the lowest CT_{max} (35.61 °C) and fish acclimated to 32 °C had the highest CT_{max} (37.65 °C) (Fig. 2.5.2). The acclimation response ratio (ARR = $\Delta CT_{max}/ \Delta T_a$; Claussen 1977) was 0.26 between 24 °C and 32 °C.

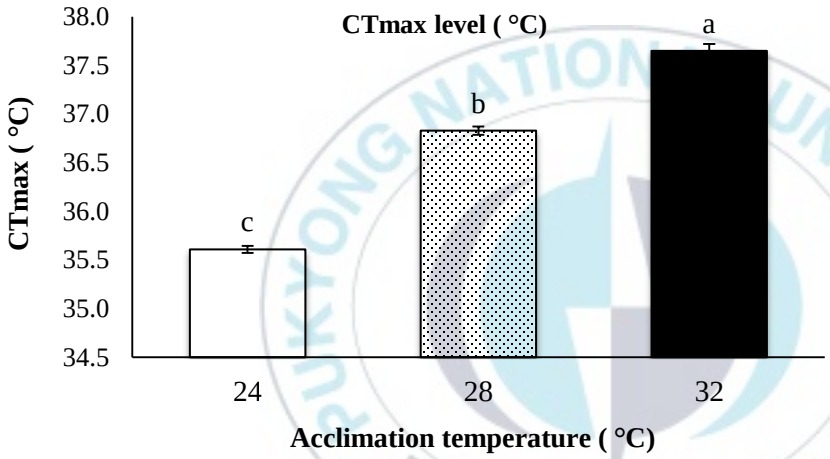


Fig. 2.5.2. Critical thermal maximum (CT_{max}) level of red-spotted grouper, *Epinephelus akaara*, under different acclimation water temperatures (24 °C, 28 °C, and 32 °C). Values are presented as mean ± SEM (n=7). Different lowercase letters indicate the significant difference across temperatures (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.2. Effects of T_a on thermal tolerance period (TT_p)

The thermal tolerance period was significantly affected by T_a (ANOVA, $P < 0.05$; Fig. 2.5.3). Fish acclimated at 32 °C had higher TT_p (388.57 min) compared with those acclimated at 24 °C (318.15 min) and 28 °C (325.14 min). In addition, a positive relationship was also observed between TT_p and T_a ($R^2 = 0.352$, $P<0.01$, $TT_p = 97.45 + 8.8T_a$).

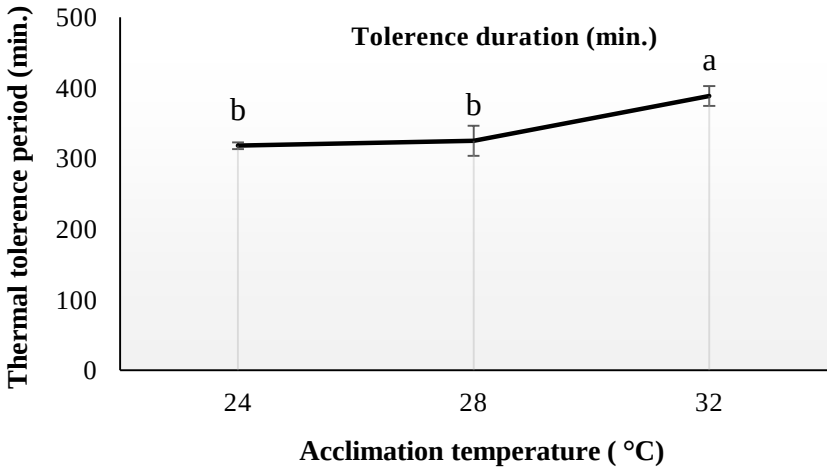


Fig. 2.5.3. Thermal tolerance period (TT_P) of red-spotted grouper, *Epinephelus akaara*, under different acclimation water temperatures (24 °C, 28 °C, and 32 °C). Values are presented as mean ± SEM (n=7). Different lowercase letters indicate the significant difference across temperatures (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.3. Effects of T_a on operculum movement number (OPMN)

The operculum movement was significantly influenced by T_a (ANOVA, $P < 0.05$). Fish acclimated to 24 °C and 28 °C had higher OPMN in comparison to the fish acclimated at 32 °C (Fig. 2.5.4).

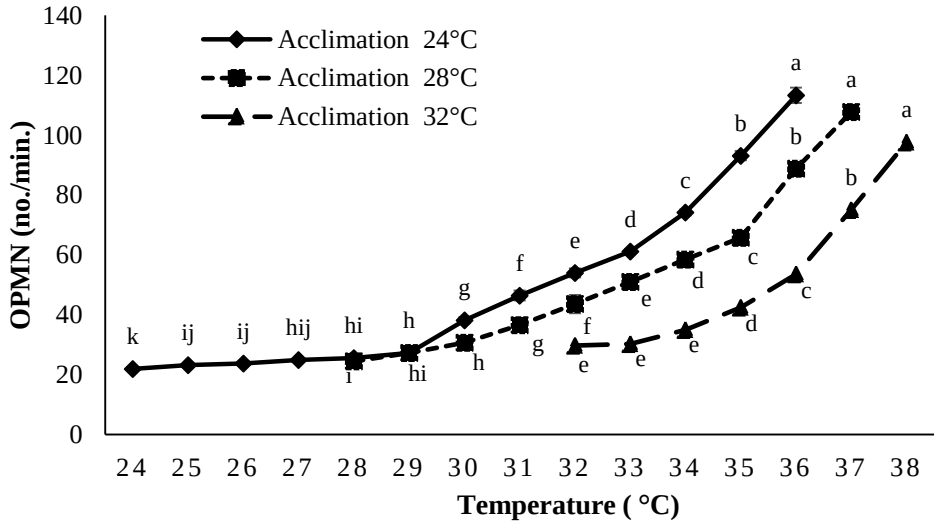
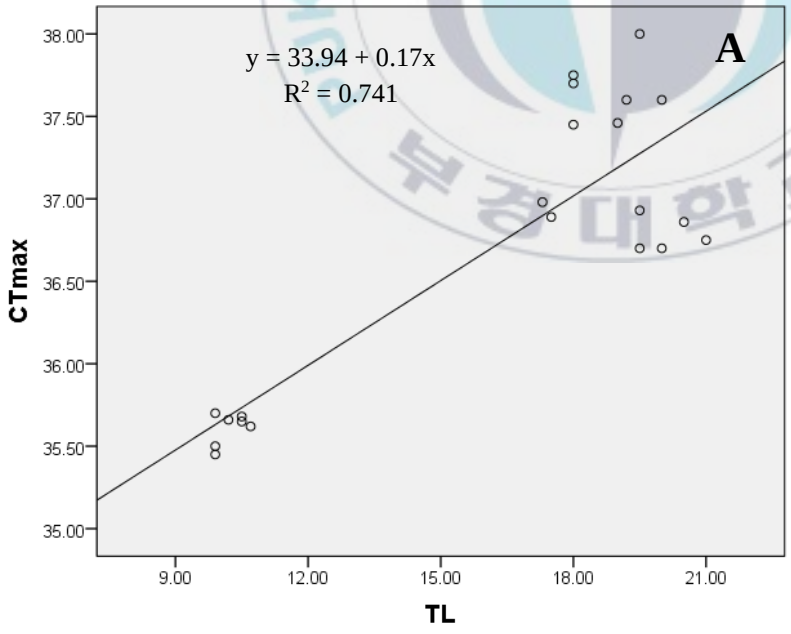


Fig. 2.5.4. Operculum movement number (OPMN) of red-spotted grouper, *Epinephelus akaara*, under different acclimation water temperatures (24 °C, 28 °C, and 32 °C). Values are presented as mean ± SEM (n=7). Different lowercase letters indicate the significant difference across temperatures (ANOVA, Duncan's multiple range test; $P < 0.05$).

3.4. Correlation between body size and CT_{max} level

The total length (TL) and body weight (BW) of *E. akaara* at different acclimated temperature are shown in Table 2.5.1. A significant positive relationship exists between CT_{max} and body size of red-spotted grouper. CT_{max} was found positively correlated with the total length (T_L, $R^2 = 0.741$; $P<0.01$) (Fig 2.5.5A) and body weight (B_W, $R^2 = 0.695$; $P<0.01$) (Fig 5B) of *E. akaara*. The relationships are described by the equations, $CT_{max} = 33.94 + 0.17T_L$ and $CT_{max} = 35.56 + 0.01B_W$.



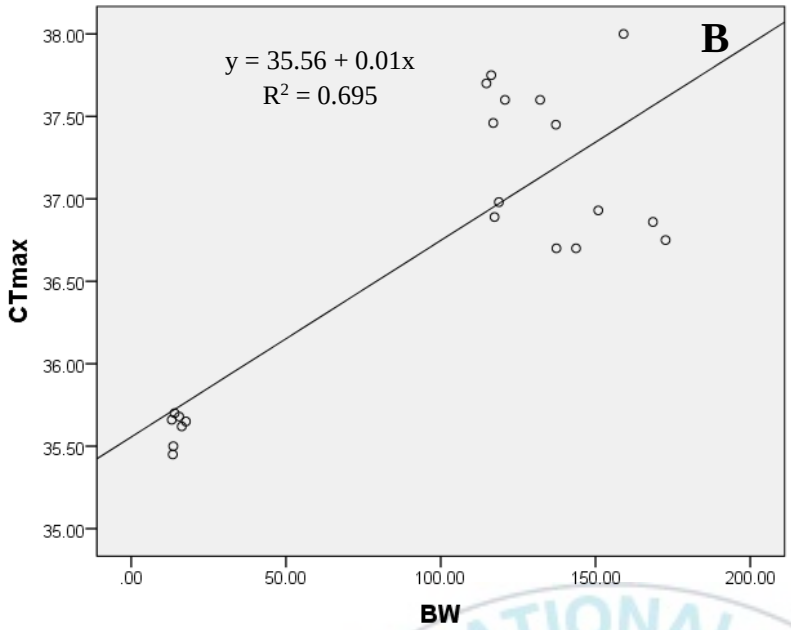


Fig. 2.5.5. Correlation between (A) total length (T_L) and CT_{max}, (B) body weight (B_W) and CT_{max} of red-spotted grouper, *Epinephelus akaara*, under different acclimation water temperatures (24 °C, 28 °C, and 32 °C). Values are presented as mean ± SEM (n=7). Different lowercase letters indicate the significant difference across temperatures (ANOVA, Duncan's multiple range test; $P < 0.05$).

Table 2.5.1. Total length and body weight of *E. akaara* at different acclimation temperature. Values are expressed as mean ± SEM (n=10).

Parameters	Temperature groups		
	24 °C	**28 °C**	**32 °C**
Total length, TL (cm)	10.23 ± 0.13	19.33 ± 0.54	18.81 ± 0.31
Body weight, BW (g)	14.80 ± 0.65	144.19 ± 8.25	128.16 ± 0.68

4. Discussions

Thermal tolerance in animals can be studied in using various approaches; of these, the critical thermal maximum test (CTmax) is the most relevant (Beitinger et al., 2000). CTmax is often used to provide an ecologically and physiologically valuable reference point that can signal an early sign of thermal stress (Stewart and Allen, 2014). Surprisingly, little research has been performed on the critical thermal maximum in *E. akaara*. This is the first study that has examined the thermal tolerance, measured as CTmax in red-spotted grouper. The results of the present study showed that acclimation temperature (Ta) significantly affects the CTmax level of *E. akaara*.

The Ta has been suggested to be the most important factor that affects the thermal tolerance of fish (Beitinger and Lutterschmidt, 2011). In addition to the Ta, a variety of factors like size and condition factor (Baker and Heidinger, 1996; Zhang and Kieffer, 2014); the presence of toxic chemicals (Beitinger et al., 2000) and species (Das et al., 2004) also influence the thermal tolerance limits. The relationship between thermal tolerance (CTmax) and Ta have been reported in many aquatic species (Akhtar et al., 2012; He et al., 2014; Zhang and Kieffer, 2014; Yanar et al., 2019) as obtained in the present study for *E. akaara*. In this study, the highest CTmax value observed at 32 °C Ta compared with the 24 °C and 28 °C indicating that CTmax level increased significantly with increasing Ta. It confirms that thermal tolerance is largely dependent on fish's prior thermal exposure history or acclimation. Our result is in accordance with the findings of Cheng et al. (2013), who reported that the CTmax value of brown-marbled grouper (*Epinephelus fuscoguttatus*) was ranged from 35.90 to 38.30 °C under different Ta. Murchichie et al., (2011) reported that CTmax values for the tropical bonefish (*Albula vulpes*) were 36.4 °C and 37.9 °C for fish acclimated at 27.3 °C and 30.2 °C, respectively. The CTmax values in different Ta were also observed in many fish species including rohu (*Labeo rohita*) (Das et al., 2004), climbing perch (*Anabas testudineus*)

(Sarma et al., 2010), carp (*Cyprinus carpio*) (Chatterjee et al., 2004), and shortnose sturgeons (*Acipenser brevirostrum*) (Zhang and Kieffer, 2014).

The acclimation water temperature exerts a major effect on thermal tolerance and it has a strong linear correlation with CTmax (Beitinger et al., 2000). In this study, the linear regression slope indicates that, for each 1 °C Ta, the CTmax of *E. akaara* increased by 0.26 °C. This indicates a gain in thermal tolerance with the increase in Ta. However, this is fairly smaller than the previously studied shortnose sturgeons (*A. brevirostrum*), where CTmax temperature increment rate was 0.52 °C for each 1 °C Ta (Zhang and Kieffer, 2014). This may be due to the regional difference as *E. akaara* is a subtropical species and *A. brevirostrum* is a cold-water species.

The acclimation response rate (ARR) is defined by Claussen (1977) as the change in CTmax with the changes of Ta. ARR indicates the physiological response of aquatic organisms to a change in the temperature (Diaz et al., 2002). In the present study, the ARR values of *E. akaara* was 0.26. Similar ARR values have been reported in many warm water fishes such as 0.40 °C for channel catfish (*Ictalurus punctatus*), 0.32 °C for large mouthbass (*Micropterus salmoides*) and 0.36 °C for *Pelteobagrus vachelli* (Currie etal., 1998; Wang, 2009). However, the present study had smaller ARR value in comparison with the shortnose sturgeons (*A. brevirostrum*) (Zhang and Kieffer, 2014). The ARR values are dependent on the geographic zone where the organisms dwell (Diaz eta al., 2002). The smaller ARR values of *E. akaara* indicate that they have narrower tolerance range and weaker acclimation ability than species with larger ARR.

In this experiment, Ta significantly affected the thermal tolerance period. The fish acclimated at high temperature have the higher thermal tolerance period. Similarly, acclimated at low temperature have a higher tolerance period at low temperature (Aziz and Greenwood, 1981). Zhang and Kieffer (2014) reported similar variation in thermal tolerance duration in shortnose

sturgeons (*A. brevirostrum*). In this study, the body size of *E. akaara* was significantly correlated with CTmax value. CTmax increases with fish size because larger fish have a lower surface area to volume ratios, which causes longer period to penetrate the heat into fish's body (Ziegeweid et al., 2008). The similar result was observed in shortnose sturgeons (*A. brevirostrum*) (Zhang and Kieffer, 2014).

5. Conclusion

Collectively, it can be said that red-spotted grouper acclimated to a higher temperature has a higher thermal tolerance (CTmax). The CTmax value 35.61-37.65 °C suggest that rearing temperature of *E. akaara* must never rise above 35 °C for its successful aquaculture especially during the dry warm summer months. Understanding the critical thermal tolerance limit of *E. akaara* is of ecological significance in the conservation and aquaculture of this species.

Chapter 3

Growth performance and sex reversal of red-spotted grouper by 17α-methyltestosterone (MT) treatment

Chapter 3.1

Evaluation of growth parameters and growth-related gene expression in red-spotted grouper

Abstract

The influence of 17α-methyltestosterone (MT) on growth performance and growth-related genes expression were investigated in red-spotted grouper to evaluate the growth promoting potentiality of MT. 90-day-old juveniles of *E. akaara* (total length: 5.98 ± 0.14 cm, body weight: 3.67 ± 0.20 g) were fed MT incorporated diets at different concentration (0 as control, 10mg, and 40mg MT/Kg diet) for a period of 9 week. Growth depression was observed in MT-feeding groups during hormonal treatment. The growth performance in terms of average length and body weight gain of the control groups were significantly higher than those of MT-fed groups. The mRNA levels of IGF-1 and GHR were also under-expressed in MT treated groups. However, compensatory growth was observed in MT-fed groups after completion of MT treatment. The average body weight, weight gain percentage, and specific growth rate of 40mg MT/Kg diet group were significantly ($P < 0.05$) improved compared with the control and 10mg MT/Kg diet groups. IGF-1 and GHR mRNA expression were also up-regulated in 40mg MT/Kg diet group. Collectively, our results suggest that MT incorporation in the diets can yield a highly significant increase in growth rate and food conversion rate (FCR) in red-spotted grouper. Besides, MT treatment (40mg MT/Kg diet) can improve the growth performance of *E. akaara* by modulating the GH/IGF-1 system.

Keywords: 17α-methyltestosterone, Growth performance, Insulin-like growth factor, Growth hormone receptor, Red-spotted grouper, *Epinephelus akaara*

1. Introduction

Groupers are one of the most important commercially groups of marine fishes and are widely distributed in tropical and subtropical regions including South Korea, China, Japan, and other Asian countries (Liao and Leano, 2008; Rimmer and Glamuzina, 2019). Most groupers including the red-spotted grouper (*Epinephelus akaara*) are protogynous hermaphrodites: function as female at a young age and then later undergo sex changes to become the male when they have reached a larger size (Brusle-Sicard et al., 1992; Bhandari et al., 2003; Liu and Sadovy, 2009; Sao et al., 2012). Thus, a significant amount of the absorbed energy has been used for maintaining gonadal development, which has ultimately slow down growth performance. The complicated reproductive characteristics of groupers have made their aquaculture less profitable.

The naturally-occurring or synthetic androgens and estrogens have shown growth-promoting effect in many cultured species (James and Sampath, 2006). Steroids both androgenic and estrogenic increase growth and food conversion efficiency when administered in food (Jensi et al., 2016). 17α-methyltestosterone (MT) is a synthetic male hormone, which has an androgenic effect. Synthetic androgens are used in fish culture either by immersion or through the diet as sex controlling agents or growth promoters if energy is shut away from developing ovaries towards the growth of somatic tissues (Rizkallah et al., 2004). MT treatment has been reported to accelerate the growth performance of many species including coho salmon (Shelbourn et al., 1992), *Channa punctatus* and *Cirrhinus mrigala* (Muniasamy et al., 2019). However, it has been reported in few studies that MT treatment has negative effects on survival, growth or gonadal development if a particular threshold dose is surpassed (Shen et al., 2015; Fatima et al., 2016; Luckenbach et al., 2017). The dose and duration of MT used for growth enhancement are species-specific. We assumed that MT administration through the diet might accelerate the growth performance of *E.*

akaara. Therefore, the effects of androgens as growth promoters need to be clarified in red-spotted grouper.

Endocrinological knowledge is important to understand the growth patterns induced by MT-treatment. Growth hormone (GH)/Insulin-like growth factor 1 (IGF-1) system is associated with body growth and nutrient metabolism in mammals and teleost (Moriyama et al., 2000). GH is a peptide hormone produced by the pituitary gland that can bind to the growth hormone receptor (GHR) in the target tissues. The IGFs are mainly produced in fish liver under the control of growth hormone (GH). IGF-1 is a key regulatory hormone that stimulates somatic growth by affecting different tissues (Reinecke et al., 1997). GHR mRNA is expressed in hepatic and other tissues (reviewed by Canosa et al., 2007). Likewise, IGF-1 mRNA expression has also been observed in both hepatic and non-hepatic tissues of fish, including tilapias, *Oreochromis mossambicus* (Reinecke et al., 1997), *Oreochromis niloticus* (Yue et al., 2018), and rainbow trout (*Oncorhynchus myskiss*) (Norbeck and Sheridan, 2011)

In recent years, the steroids, which are known to enhance growth parameters and reduce the feed-cost in animal husbandry, have attracted the attention of fish farmers. MT is very much recommended in the livestock industry. Previous studies have been conducted on the red-spotted grouper, examining feeding frequency (Kayano Nakagawa, 1993), spawning behavior and early life history (Park et al., 2016), sex differentiation (Lee et al., 2014) and growth performance related to stress (Lee and Baek, 2018). However, the impact of MT-feeding on the growth performance of *E. akaara* remains unexplored. Therefore, the present study was conducted to evaluate the growth-inducing potential of MT incorporated diet on the economically valuable red-spotted grouper.

2. Materials and Methods

2.1. Experimental animals and Ethics statement

90-day-old juveniles of *E. akaara* (total length: 5.98 ± 0.14 cm, body weight: 3.67 ± 0.24 g) were collected from the Marine Science Institute, Jeju National University and reared in the Marine Biology Department, Pukyong National University (PKNU), Busan, Korea. Fish were acclimated for one week before starting the experiment. The experiment was conducted following the fish maintenance, handling, and sampling guidelines of the Animal Ethics Committee of PKNU (Regulation No. 554).

2.2. Preparation of MT-containing diets

The MT powder (Sigma-Aldrich, USA) was dissolved in absolute ethanol to prepare the stock solution (mg/mL). MT stock solution was incorporated to commercial fish feed (Otohime Hirame; Marubeni Nisshin Feed Co., Ltd., Japan; 50~52% protein and 7~10% lipids) at different concentrations (10 and 40mg MT/Kg diet). The experimental diets were homogeneously mixed with the desired concentration of the hormone. The sham control diet was saturated with ethanol only and the control diet was kept untreated. The feed was dried overnight under a fume hood before being stored at 4 ºC until further use.

2.3. Feeding protocols and sampling

Fish were randomly divided into the control and MT-feeding group (10 and 40mg MT/Kg diet). The red-spotted groupers were fed MT-supplemented diets for 9 week and then fish were fed with the untreated commercial diet until termination of the experiment. Fish were sampled three times during MT treatment (3, 6, and 9 week) and three times after MT treatment termination (12, 24, and 36 week) for growth measurements. Fish were fed two times daily at the rate of 2% of their body weight during the experimental period. Fish were reared at a water temperature of

28 °C throughout the study period. All animals were fasted for 24h prior to any handling and measurements. All fish were anaesthetized with 300 mg/L 2-phenoxyethanol (Sigma Aldrich, USA) to measure the length and weight individually. The muscle and liver were collected (n = 7) after the measurement and immediately kept in RNA later (Qiagen, Germany) at -80 °C for further analysis. The sampling schedule is shown in Figure 3.1.1a.

2.4. Growth performance

At each sampling points, the biometric parameters such as body weight (BW) and body length (BL) were measured individually (n = 10). Growth performance and feed efficiency were evaluated by determining a number of growth and nutrient utilization indices. The following parameters were estimated according to below formulas:

Weight gain (WG; %) = 100 × [final body weight (g) – initial body weight (g)]

Feed efficiency ratio (FER) = weight gain (g) / feed intake (g)

Feed conversion ratio (FCR) = feed intake (g) / body weight gain (g)

Protein efficiency ratio (PER) = weight gain (g) / protein intake (g)

Specific growth rate (SGR; %WG/day) = 100 × [In final weight (g) – In initial weight (g)] / days

Condition factor (K; g/cm^3) = 100 × [fish weight (g) /fish length^3 (cm) ^{3}]

Hepatosomatic index (HIS; %) = 100 × [liver weight (g) / body weight (g)]

Viscerosomatic index (VSI; %) = 100 × [visceral weight (g) / body weight (g)]

Gonadosomatic index (GSI; %) = 100 × [gonad weight (g) / body weight (g)]

2.5. Estimation of growth retarding rate (GRR) and growth increment rate (GIR)

During the MT-feeding period, growth retarding rate (GRR) and growth increment rate (GIR) were calculated in MT treated groups compared to the control group to explore the GRR and GIR values, respectively. The body weight data were used to estimate the GRR and GIR values.

Growth retarding rate (GRR) = (BWc - BWt)/ BWc

Growth increment rate (GIR) = (BWt - BWc)/ BWc

Where BWc = mean body weight in the control group and BWt = mean body weight in the MT treated group

2.6. Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNAiso plus (Takara, Japan) from the preserved liver and muscle according to the manufacturer's protocol. Total RNA concentration was measured using a spectrophotometer (NanoDrop 1000, Thermo Scientific). The cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The synthesized cDNA was stored at -20 °C for further analysis.

2.7. Quantitative real-time PCR analysis

To evaluate the mRNA levels of insulin-like growth factor 1 (IGF-1) and growth hormone receptor (GHR), primers were designed using the sequence information available in National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.gov).

Real-time PCR was carried out with Chromo4 Real-Time PCR System (Bio-Rad, USA). The real-time PCR reaction mixture contained 2µl of the diluted cDNA sample, specific primer, and TB Green™ Premix Ex Taq™ (Takara, Japan). The PCR reaction was performed with an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The 18S rRNA gene was used as a reference gene. The primers used for the PCR and qRT-PCR are shown in Table 3.1.1.

2.8. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to identify the

significant differences among the treatment groups. P value of less than 0.05 indicates significant differences. All statistical analysis was done using SPSS program Version 21 (IBM Corp., USA).

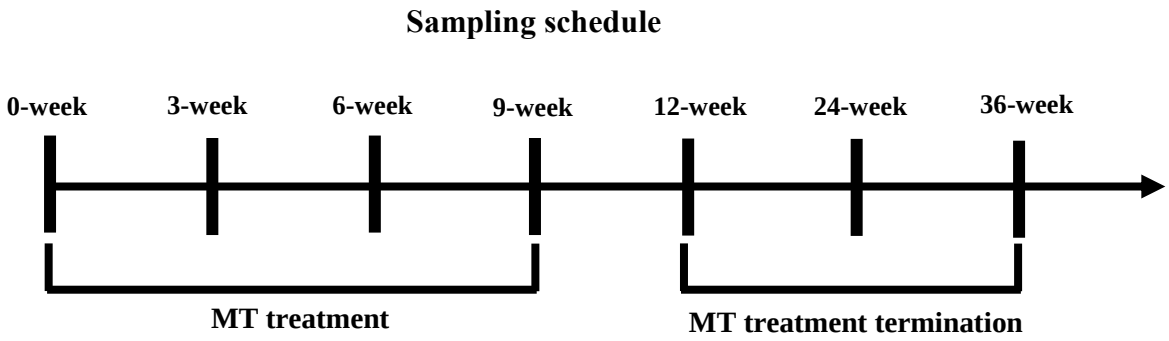


Fig. 3.1.1a. The sampling schedule during the experimental period.

Table 3.1.1. Primers used for quantitative real-time PCR of GHR and IGF-1 genes

Gene	Direction	Sequence (5' - 3')
Growth hormone receptor	Forward	TCAAACCATACACCCTCAGC
	Reverse	CTGTACCACTGTGTAGTCTGC
Insulin-like growth factor1	Forward	ATGTAGGGAAGGTGCGAATG
	Reverse	CCTTTGTCAGCATCCTCTTTG
18S rRNA	Forward	CGGTAATTCCAGCTCCAATAGCG
	Reverse	CTTAATCATGGCCCCAGTTCAGAG

3. Results

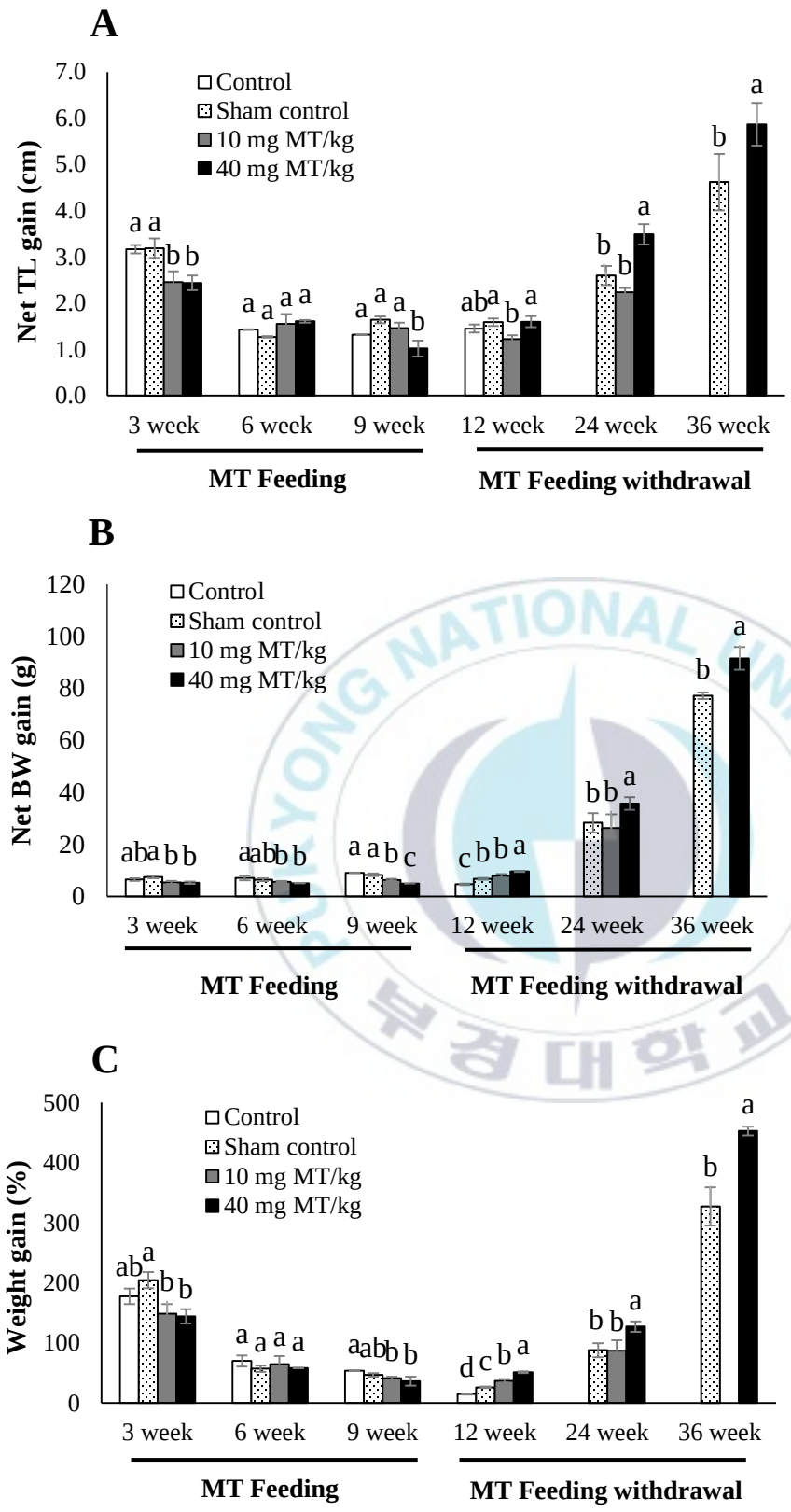
3.1. Effect of the MT feeding on growth performance, feed utilization, and biometric indices

The growth performance of red-spotted grouper in response to MT treatment was significantly ($P < 0.05$) variant. During the MT treatment period (9 week), the growth rate of MT feeding groups (10 and 40mg MT/Kg) was slow down compared with the control groups; however, fish fed MT showed better growth pattern after the withdrawal of MT treatment (after 9 weeks).

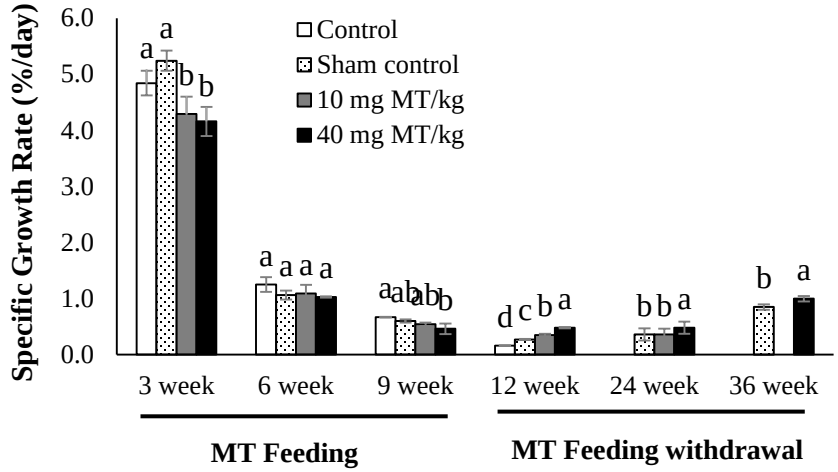
The MT treated groups (10 and 40mg MT/Kg) had significantly lower ($P < 0.05$) length, weight, weight gain percentage, and specific growth rate (SGR) in comparison to the control during the hormone treatment period (9 week); however, these values were found significantly higher ($P < 0.05$) in 40mg MT/Kg group after removal of MT treatment (Fig. 3.1.1 A-D).

The lower feed intake (FI) was recorded in the MT treated groups (10 and 40mg MT/Kg) during the hormonal treatment period (9 week), which was associated with a significant reduction ($P < 0.05$) in feed efficiency ratio (FER) and increment in feed conversion ratio (FCR). The protein efficiency ratio (PER) of the MT treated groups was also reduced when compared with the control. However, after the termination of MT treatment, no significant changes ($P > 0.05$) was observed in FI, FER, and PER values of the experimental groups except for FCR values of 40mg MT/Kg group (Fig.3.1.1 E-H).

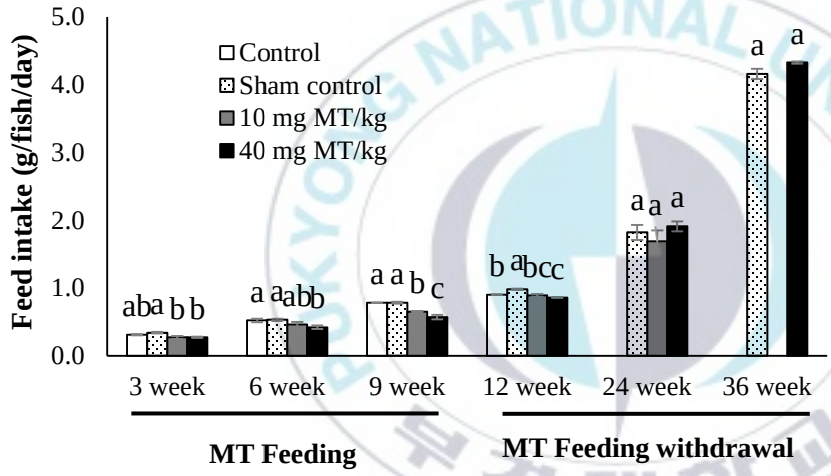
The MT feeding did not affect ($P > 0.05$) the biometric indexes (CF, HIS, VSI) of red-spotted grouper during and after the hormonal treatment period (36 week) except for gonadosomatic index (GSI). GSI values of the 40mg MT/Kg group was found significantly higher ($P < 0.05$) than the control at the end of the experiment (after 36 week) (Fig.3.1.1 I-L).


A
Net TL gain (cm)
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal
B
Net BW gain (g)
C
Weight gain (%)

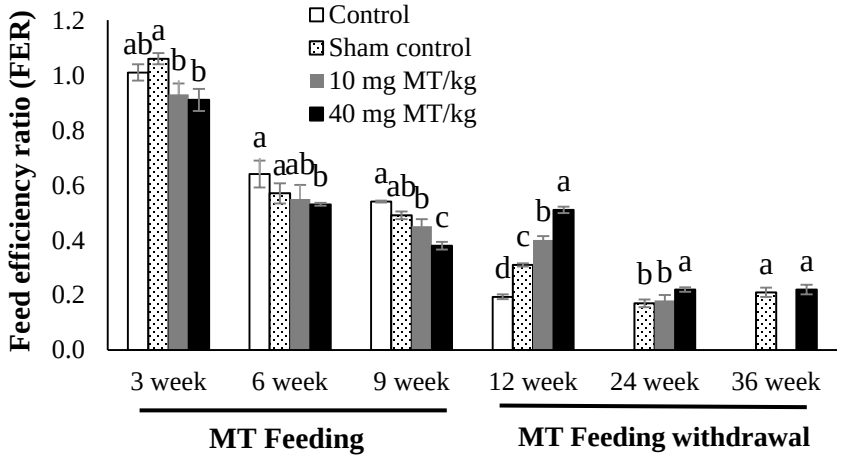
D


Specific Growth Rate (%/day)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

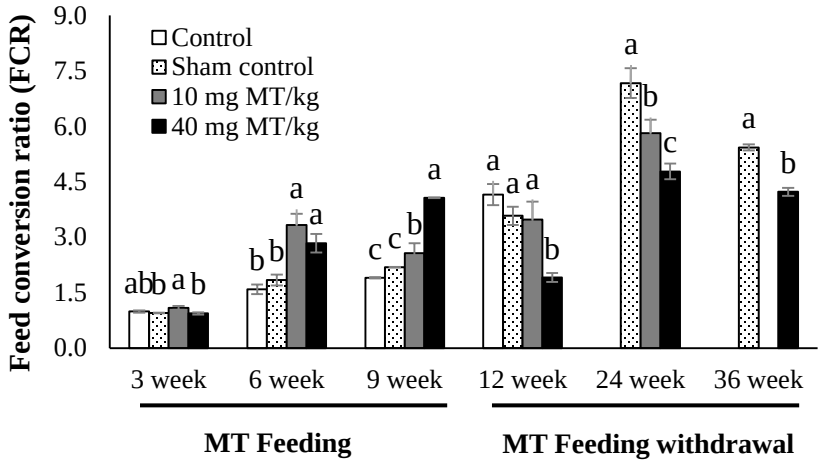
E


Feed intake (g/fish/day)
5.0
4.0
3.0
2.0
1.0
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

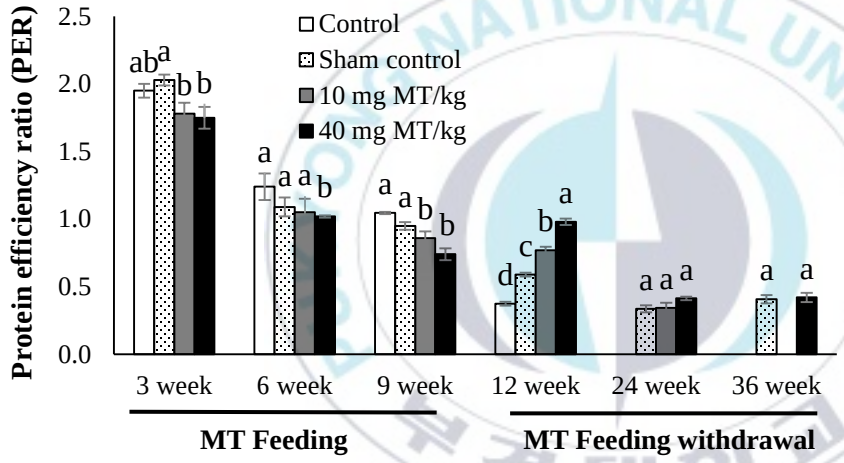
F


Feed efficiency ratio (FER)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

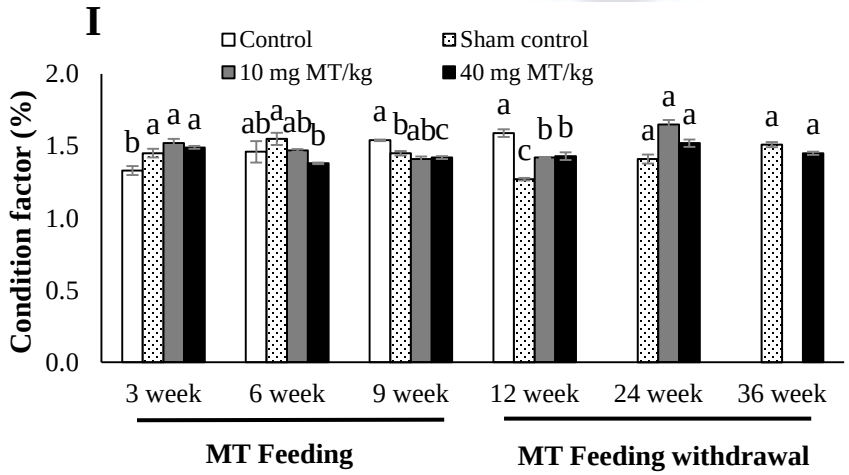
G

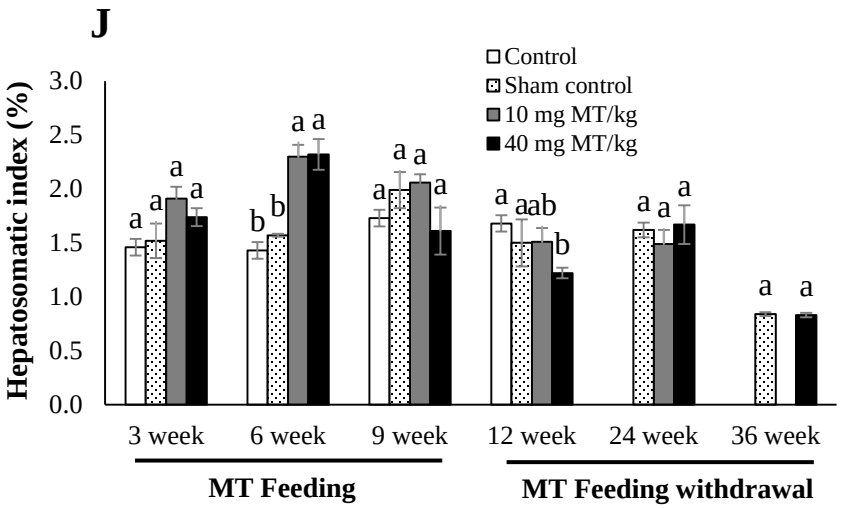

Feed conversion ratio (FCR)
9.0
7.5
6.0
4.5
3.0
1.5
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

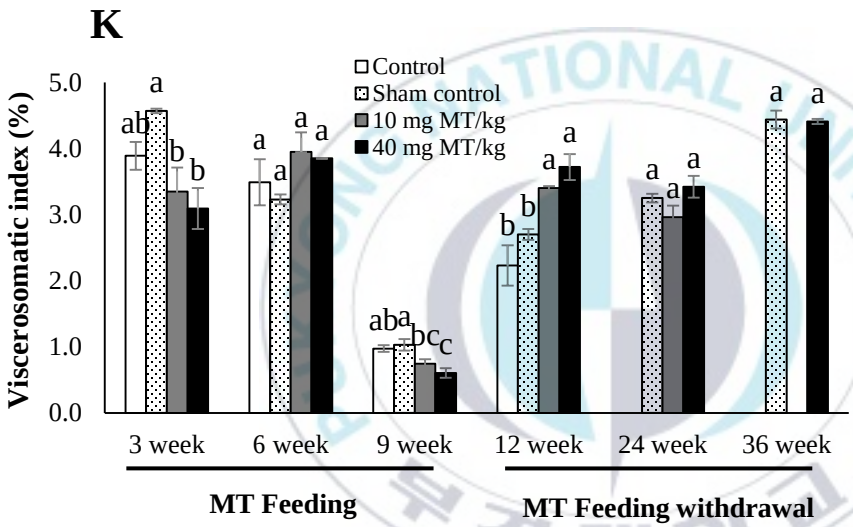
H


Protein efficiency ratio (PER)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

I


Condition factor (%)
2.0
1.5
1.0
0.5
0.0
Control
Sham control
10 mg MT/kg
40 mg MT/kg
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal


J
Control
Sham control
10 mg MT/kg
40 mg MT/kg
Hepatosomatic index (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal


K
Control
Sham control
10 mg MT/kg
40 mg MT/kg
Viscerosomatic index (%)
5.0
4.0
3.0
2.0
1.0
0.0
3 week
6 week
9 week
12 week
24 week
36 week
MT Feeding
MT Feeding withdrawal

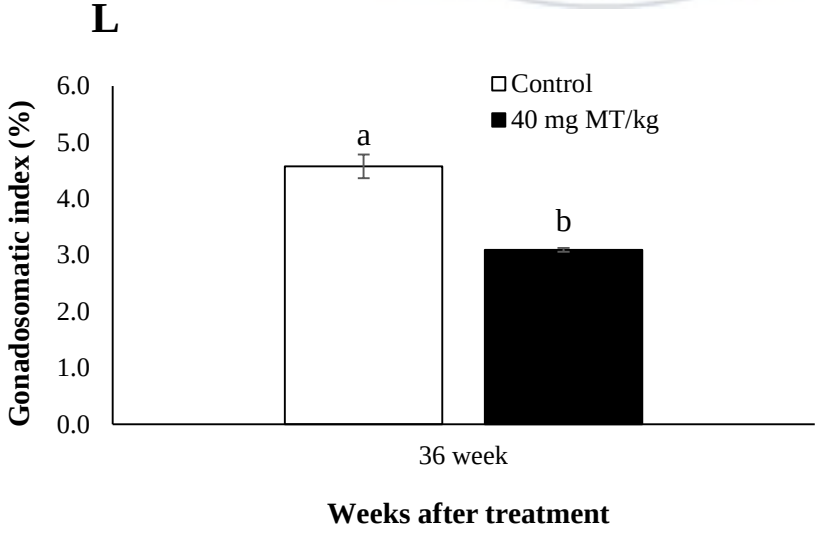
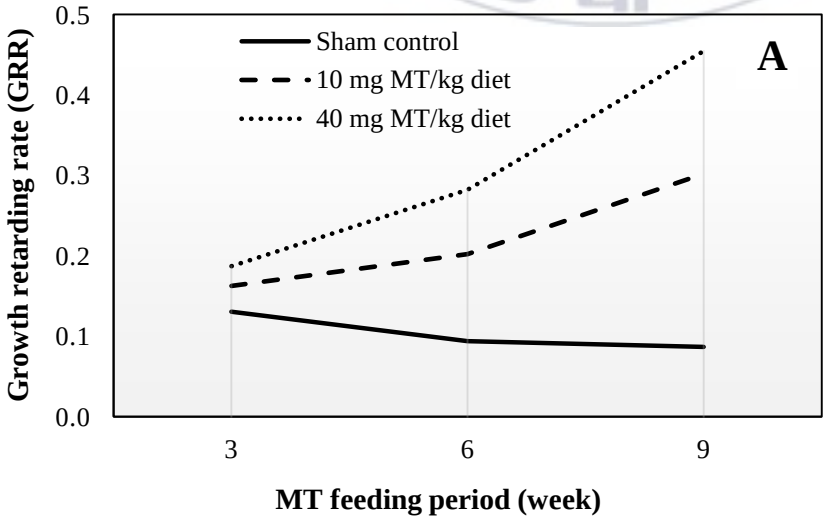

L
Control
40 mg MT/kg
Gonadosomatic index (%)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
36 week
Weeks after treatment

Fig. 3.1.1b (A-L). Growth, feed utilization, and biometric indices of red-spotted grouper, *Epinephelus akaara* during MT-feeding and MT-feeding withdrawal period. Data are presented as mean ± SEM (n=10). Different letters indicate the significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

3.2. Growth retarding rate (GRR), and growth increment rate (GIR) during and after MT treatment

In the MT treatment groups (10 and 40mg MT/Kg diet), growth suppression was observed from 3 to 9 week when compared with control; however higher growth was noticed in 40mg MT/Kg diet group as compared with the control and 10mg MT/Kg group after removal of MT feeding (Fig. 3.1.2A).

Similarly, growth-retarding rate (GRR) increased in the MT treated groups (10 and 40mg MT/Kg diet) in comparison with the sham control during the treatment period (9 weeks). The highest retardation was recorded in 40mg MT/Kg Mt group (Fig. 3.1.2B). On the other hand, growth increment rate (GIR) was increased after completion of MT treatment. The highest GIR value was observed in 40mg MT/Kg diet group (Fig. 3.1.2C).



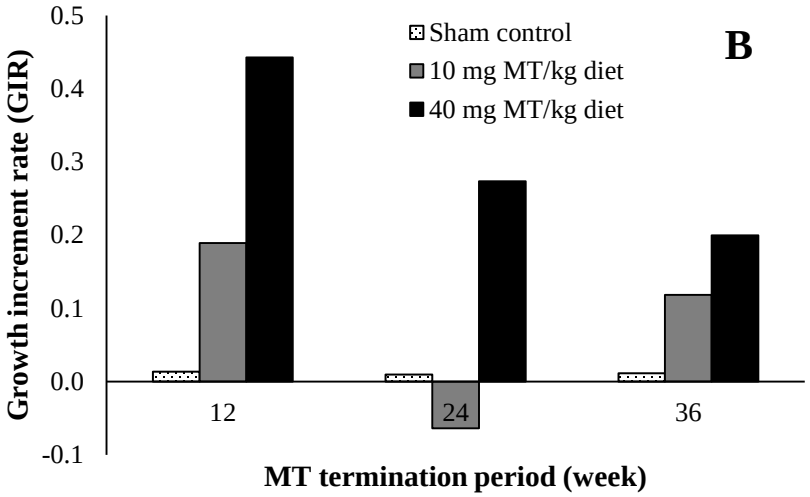


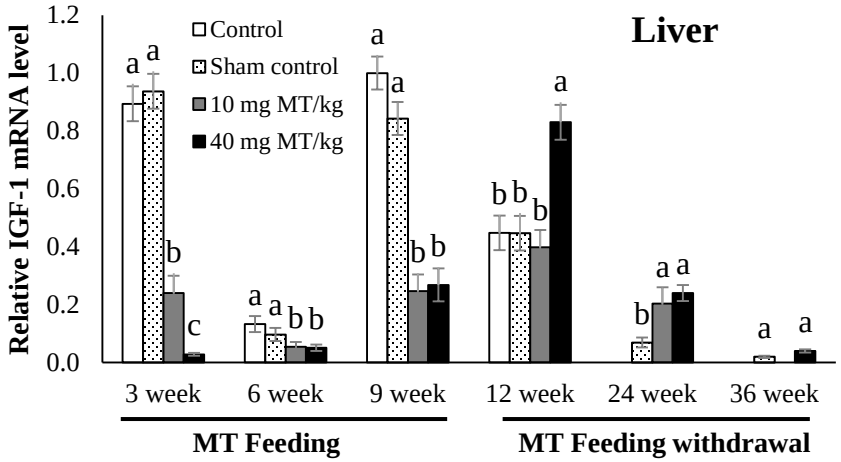
Fig. 3.1.2 A-B. Growth retarding rate (GRR) during MT treatment period and growth increment rate (GIR) after MT treatment withdrawal period in red-spotted grouper, *Epinephelus akaara*. Mean body weight data were used to estimate the GRR and GIR values.

3.3. Relative expression of growth-related genes

3.3.1. IGF1 mRNA expression

IGF1 mRNA expression levels in each of the two tissues is shown in Figure 3.1.3. In liver, the IGF1 mRNA level was under-expressed in MT treated groups (10mg MT/Kg and 40mg MT/Kg) during the MT feeding period (9 week) as compared with the control; however, highly ($P < 0.05$) expressed in 40mg MT/Kg diet group after completion of MT treatment (Fig. 3.1.3 A).

In muscle, IGF1 transcript level was found lower in MT treated group during hormonal treatment period (9 week); but, the expression was significantly increased ($P < 0.05$) in 40mg MT/Kg diet group after completion of MT treatment (Fig. 3.1.3 B).



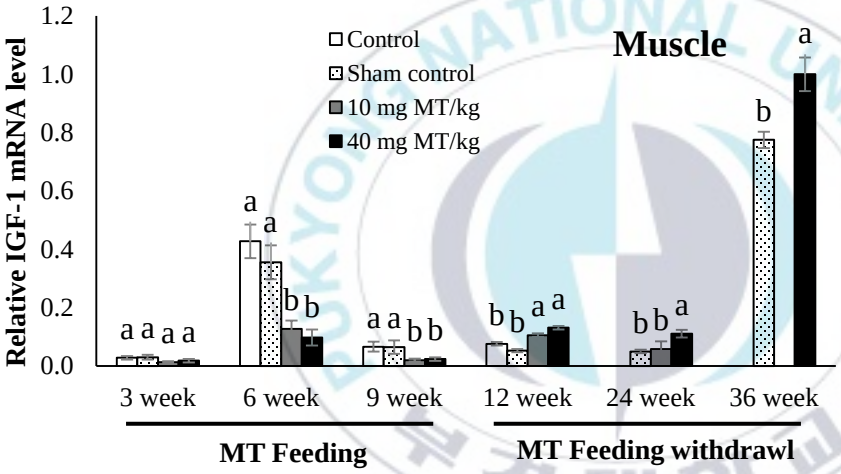


Fig. 3.1.3. Relative mRNA expression of IGF1gene in the liver (A) and muscle (B) of red-spotted grouper, *Epinephelus akaara* during MT-feeding and MT-feeding withdrawal period. The expressions of the target gene were normalized by 18s rRNA expressions. Data are presented as mean ± SEM (n=7). Different letters indicate the significant differences among the treatment groups at equivalent times (ANOVA, Duncan's multiple range test, $P < 0.05$).

3.3.2. GHR mRNA expression

GHR mRNA expression levels in each of the two tissues is shown in Figure 3.1.4. In liver, the GHR mRNA level was significantly down-regulated ($P < 0.05$) in 40mg MT/Kg group

compared with the other groups (control and 10mg MT/Kg) during the hormonal treatment period (9 week); however, after completion of MT treatment, GHR was up-regulated in 40mg MT/Kg diet group compared with other groups (Fig. 3.1.4 A).

In muscle, the GHR mRNA level was under-expressed in MT-treated groups during the hormonal treatment period. On the other hand, after completion of MT treatment, GHR transcript level was significantly elevated ($P < 0.05$) in 40mg MT/Kg diet group (Fig. 3.1.4 B).

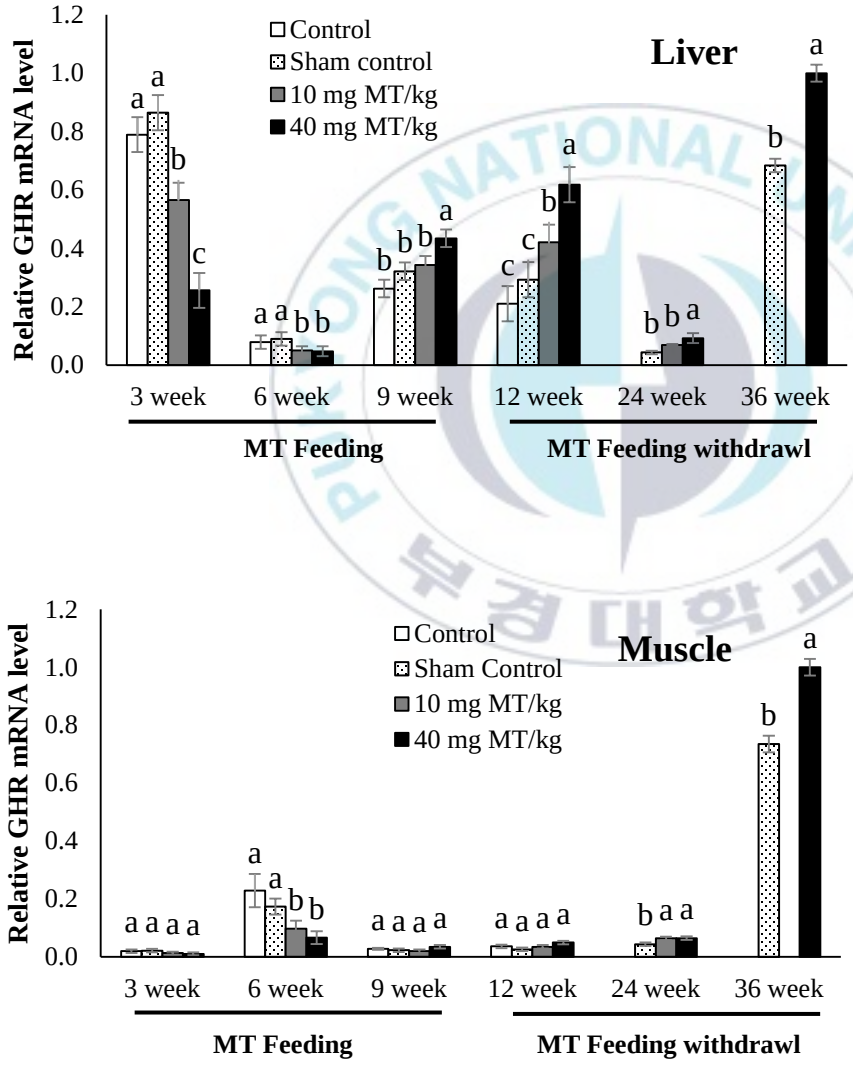


Fig. 3.1.4. Relative mRNA expression of GHR gene in the liver (A) and muscle (B) of red-spotted grouper, *Epinephelus akaara* during MT-feeding and MT-feeding withdrawal period. The

expressions of the target gene were normalized by 18s rRNA expressions. Data are presented as mean ± SEM (n=7). Different letters indicate the significant differences among the treatment groups at equivalent times (ANOVA, Duncan's multiple range test, $P < 0.05$).

4. Discussion

Sex steroids affect reproduction and multiple biological functions. 17α-methyltestosterone (MT) is a synthetic male sex steroid that closely mimics the naturally-produced testosterone hormone. It is considered an effective androgen, which can accelerate both muscle growth and the development of male sexual characteristics (Phumyu et al., 2012). The present study investigated the growth performance and growth-related gene expression in *E. akaara* in response to feeding of different concentrations of MT incorporated diets. Our results showed that dietary administration of MT at a dose of 40 mg/Kg diet could enhance better growth effect in red-spotted grouper compared with the control and 10 mg/Kg diet. The doses (10 and 40mg MT/Kg diet) of MT hormone used in this experiment were based on the supplementation level applied to the other species including Nile tilapia, *O. niloticus* (El-Greisy and El-Gamal, 2012); Silver perch, *Bidyanus bidyanus* (Fotedar, 2017); and orange spotted grouper, *Epinephelus coioides* (Wang et al., 2018).

In this study, the fish treated with 40mg MT/Kg diet had higher weight gain than the control and 10mg MT/Kg diet groups after 24 and 36 week, indicate that MT feeding have higher growth effect compared with the control group and 10mg MT/Kg diet. Our results are in agreement with the findings of El-Greisy and El-Gamal, (2012), Jensi et al., (2016) and Yue et al. (2018), who observed faster growth and better food conversion efficiency in hormone-treated *O. niloticus*. Dietary MT has also proven to be an effective growth promoter in coho salmon (Shelbourn et al., 1992); European sea bass, *D. labrax* (Navarro-Martin et al., 2009); Silver perch, *B. bidyanus*

(Fotedar, 2017); and spotted snakehead, *Channa punctatus* and white carp, *Cirrhinus mrigala* (Muniasamy et al., 2019).

In the present study, the specific growth rate (SGR) was higher in the MT treated groups when compared to the control. The reasons for the enhanced growth performance might have been a result of the direct feeding of MT which led to increasing appetite, resulting in a higher specific growth rate (SGR). The higher mean weights could also be attributed to the improvement of food conversion rate (FCR) (El-Greisy and El-Gamal, 2012). This observation of high weight and SGR values showed a close resemblance with the results obtained by Muniasamy et al. (2019). However, the effect of MT on growth performances have shown certain discrepancies in previous investigations. MT treatment has shown a negative effect on growth in some species including European sea bass, *Dicentrarchus labrax* (Blazquez et al., 1995) and sablefish, *Anoplopoma fimbria* (Luckenbach et al., 2017). Shen et al. (2015) reported that treatment with MT did not affect the growth of yellow catfish (*Pelteobagrus fulvidraco*). The effect of MT on growth seems to vary greatly between species. The dosage, duration and protocol used for MT treatment are likely to influence the results of growth studies. Hence, it can be stated that the response to a given steroid hormone is species-specific which could promote higher growth rate through increased food conversion and activate the formation of other androgenic hormones (Kuwaye et al., 1993).

We introduced two new terms, growth-retarding rate (GRR) and growth increment rate (GIR), deduced from the growth difference between MT-treated and control groups, to explore the growth profiles during and after MT treatment. GRR and GIR values were increased in MT-treated groups during and after MT-feeding. The higher GRR values observed in the MT-fed groups indicate the growth suppression that may arise from physiological disturbances in response to MT treatment. The higher GIR values in the MT-treated groups indicates the growth recovery happen

after completion of hormonal treatment as compensatory growth. An accelerated growth phase may arise in fish after a period of adverse conditions (Ali et al., 2003). Therefore, the growth inhibition effects of MT were temporary rather than permanent. The similar growth depression was observed in yellow catfish (*Pelteobagrus fulvidraco*), when treated with MT and aromatase inhibitor letrozole (Shen et al., 2015).

The GH/IGF axis is important for regulating fish growth. The activity of GH is initiated when it binds to GHR. GHs from the pituitary stimulates the liver to synthesize and release IGF-1, which is thought to directly affect somatic tissue growth (Phumyu et al., 2012). The ideal growth performance is thought to be achieved with low plasma GH (low GH mRNA level in the pituitary) in combination with a high concentration of hepatic GHRs and high circulating levels of IGFs (high IGF mRNA in the liver) (Perez-Sanchez and Le Bail, 1999). Our results conform to this ideal model.

In this study, liver and muscle of MT treated group showed higher IGF-1 and GHR mRNA expression compared to control after completion of MT treatment. Our results are in consistent with those of Yue et al. (2018), who observed the higher hepatic expression of IGF-1 and GHR genes in Nile tilapia (*O. niloticus*) when injected with testosterone (T). Ma et al. (2015) reported that Nile tilapia treated with MT showed higher GHR-1 mRNA expression. However, both IGF-1 and GHR-1 mRNA levels were increased in rainbow trout (*O. myskiss*) after treated with T (Norbeck and Sheridan, 2011). The upregulation of GHR in MT-treated group may represent a mechanism for increasing the GH signaling in the liver. The IGFs IGF1 and IGF2 are mainly produced in fish liver, the main source of circulating (endocrine) IGF1 and IGF2, under the effect of GH. The IGF-1 gene plays a central role in the complex system that regulates growth, differentiation, and reproduction.

5. Conclusion

In conclusion, the results of the present study indicate that MT incorporation in the diets can yield a highly significant increase in growth rate and food conversion rate in red-spotted grouper. MT feeding at 40 mg/Kg diet can promote higher growth than 10 mg/Kg diet. Moreover, MT treatment promote growth by modulating the GH/IGF-1 system. This fine information might be helpful for the grouper farmers to make the culture more extensive and profitable.

Chapter 3.2

Impermanent female-to-male sex change in red-spotted grouper

Abstract

17α-methyltestosterone (MT) has been used to artificially induce male fate in many grouper species. In this study, the potentiality of MT-feeding to induce the gonadal sex change in red-spotted grouper (*Epinephelus akaara*) was investigated. 90-day-old red-spotted groupers (total length: 5.98 ± 0.14 cm, body weight: 3.67 ± 0.2 g) were fed MT-supplemented diets (10mg and 40mg MT/Kg diet) at the sex differentiation stage for 9 week and then, fed with a normal commercial diet until termination of the experiment. Gonadal histology and sex steroid (estradiol, E2; testosterone, T) levels were evaluated during the experimental period. MT-feeding induced to form efferent duct-like structure, bypassing the formation of an ovarian cavity, and decrease the plasma E2 levels in the MT treated group. Over the long-term, we observed the sex-reversed testis in the MT-fed group with numerous spermatozoa. A significant increase in plasma T levels and subsequent decrease in E2 levels were also observed. Plasma cortisol level was found higher during the gonad transformation phase and return to the basal level after completion of sex change. Collectively, our results suggest that MT-feeding at 40 mg/Kg diet can induce impermanent sex change from female-to-male in *E. akaara*, by increasing the plasma T levels.

Keywords: Red-spotted grouper, *Epinephelus akaara*, 17α-methyltestosterone, Gonadal sex change

1. Introduction

Groupers (Pisces: Perciformes, Serranidae) are one of the most commercially important groups of marine fishes that are widely distributed throughout the tropical and subtropical waters of the world (Craig et al., 2011; Sadovy de Mitcheson et al., 2013). Sexual patterns in grouper species are diverse including gonochorism, protogynous hermaphroditism, and bi-directional sex change (Sadovy de Mitcheson & Liu, 2008). The red-spotted grouper (*Epinephelus akaara*) is a protogynous hermaphroditic fish that is economically valuable and widely cultured in southern Asia (Rimmer and Glamuzina, 2019). Most of the red-spotted grouper first developed the ovaries at a young age and then undergo sex changes to become the male later in life (Brusle-Sicard et al., 1992; Bhandari et al., 2003; Liu & Sadovy, 2009; Sao et al., 2012). The minimum age and size of sexual maturation for male *E. akaara* is 5 to 6 years old and 34 cm, respectively (Li et al., 2006). Mature males are an important prerequisite in artificial seed production of grouper; however, the shortage of mature male broodstock has limited the success of artificial propagation. The unreliable and limited supply of seed has slow down the culture of red-spotted grouper. Mass production of seed requires quality male brood fish, which will help to reduce the generation gap as well as improve the aquaculture of *E. akaara*.

In red-spotted grouper, the primordial germ cells were appeared around 6 weeks after hatching (WAH) and the gonad was first observed at 27 WAH. The gonad differentiation was started at around 21 WAH (Liu et al., 2016). Xu et al. (2020) reported that ovarian differentiation of *E. akaara* began at 80 day after hatching (DAH) and finished at 120 DAH. Generally, endogenous sex steroid hormones have a pivotal role in the natural gonadal sex differentiation of gonochoristic teleost. Therefore, controlling the endogenous sex steroid levels during the sex differentiation period could be effective for an artificial sex change.

Artificial sex change has been successfully performed in many grouper species to overcome the deficiency of mature males for breeding. Treatments with exogenous sex steroids can induce permanent sex change in some fish, regardless of their genetic sex. Several studies suggest that treatment with exogenous 17α-methyltestosterone (MT) or aromatase inhibitor (AI) can induce sexually females to become functional males of some grouper species by downregulating the endogenous estrogen synthesis (Yeh et al., 2003; Bhandari et al., 2004; Bhandari et al., 2006; Shi et al., 2012); but, these permanent sex reversal in groupers were performed after completion of ovarian cavity formation. Sex inversion by MT-treatment in immature Malabar grouper (*Epinephelus malabaricus*) (Murata et al., 2014), and orange spotted grouper (*E. coioides*) (Wang et al., 2018) may not be maintained and create transient or impermanent sex change; however, whether MT-treatment can induce a permanent sex change in red-spotted grouper at sex differentiation stage remains unexplored.

Lee et al. (2014) observed the MT immersion effect in *E. akaara* juveniles during gonadal sex differentiation stage; but the study has examined only the gonadal histology at the end of the experiment. The details about the gonadal sex change pattern and correlative sex steroid profiles have not been studied. Therefore, in this study, we reinvestigated the gonad histology and sex sterod levels levels during and after MT-feeding in red-spotted grouper.

2. Materials and methods

2.1 Collection of experimental fish

Juveniles of *E. akaara* were collected 90-days after hatching (DAH) from Marine Science Institute, Jeju National University and reared in Marine Biology Department, Pukyong National University (PKNU), Korea. Fish were acclimated for 1 week before starting the experiment. Fish

maintenance, handling, and sampling were conducted according to the guidelines of the Animal Ethics Committee of PKNU (Regulation No. 554).

2.2 Preparation of MT-containing diets

The MT powder (Sigma-Aldrich, USA) was dissolved in absolute ethanol to prepare the stock solution (mg/mL). MT stock solution was incorporated to commercial fish feed (Otohime Hirame; Marubeni Nisshin Feed Co., Ltd., Japan) at different concentrations (10 and 40mg MT/Kg diet). The hormone was homogenously mixed with the experimental diets. The sham control feed was saturated with ethanol only and the control feed was kept untreated. The feed was dried overnight under a fume hood before being stored at 4 ºC until further use.

2.3 MT-feeding protocols and sampling

For initial control, seven fish were anaesthetized, and their gonads were collected to determine the gonadal status before the start of the experiment. Fish were randomly divided into the control and MT-feeding group (10 and 40mg MT/Kg diet). Fish were fed MT-supplemented diets for 9 weeks. After MT treatment, fish were fed with a normal diet until termination of the experiment. Fish were sampled at 3, 9, 24, and 36 weeks of experiment. Gonadal tissues were collected (n = 7) at each sampling points.

All fish were anaesthetized with 300 mg/L 2-phenoxyethanol (Sigma Aldrich, USA) before sampling. Fish were fed two times daily at the rate of 2% of their body weight during the experimental period. Fish were reared at a water temperature of 28 °C throughout the study period. Size and gonadal status of experimental fish is given in Table 3.2.1.

2.4 Measurement of steroid profiles

Blood samples were obtained from the control and MT-treated groups (10 and 40mg MT/Kg diet; n = 7) at each sampling times, except for 36 week. The blood was collected from

control and 40mg MT/Kg diet group due to the culture system error occurred at 10mg MT/Kg diet group. Blood samples were collected from the caudal vein of fish using heparinized capillary tubes and stored in 1.5 mL centrifuge tubes. Plasma samples were immediately separated after centrifugation at 4 °C (15 min at 13,000 *g*) and the supernatant was stored at –70 °C for further analysis. Plasma estradiol (E2), testosterone (T) and cortisol levels were determined by radioimmunoassay (RIA) (Kobayashi and Mikuni, 1987). Steroids were extracted twice using 2 mL diethyl ether, dried under liquid nitrogen gas and re-suspended in phosphate buffer (pH = 7.5). The antiserum was purchased from Cosmo-Bio Co. Ltd. (Japan). Non-radioactive steroid standards were procured from Steraloids Inc. (Wilton, USA). Radiolabelled steroids, $[^3H]^-$ were purchased from Amersham Life sciences (Piscataway, NJ, USA).

2.5 Gonadal histology

Fish were anaesthetized and their gonads were dissected, fixed in 10 % neutral formalin, dehydrated through a graded series of ethanol concentrations, and embedded in paraffin. All tissue blocks were sectioned at 4-5 μm and stained with Mayer's hematoxylin-eosin. Gonadal histology was observed under light microscopy (BX-50; Olympus, Tokyo, Japan).

2.6 Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to identify the significant differences among the treatment groups. P-value of less than 0.05 indicates significant differences.

Table 3.2.1. The size and gonadal status of red-spotted grouper, *Epinephelus akaara* during experimental period

Experimental groups	Week after treatment	Treatment	Sample No	Total length[1] (cm)	Body weight[1] (g)	Gonadal status				
						Ovary[2]	Ovary[3]	Ovary[4]	Intersex[5]	Testis[6]
Initial	0-week	Initial control	07	5.98 ±0.14	3.67 ± 0.24	7	0	0	0	0
MT-feeding	3 week	Control	05	9.17 ± 0.36	11.17 ± 0.91	0	5	0	0	0
		10mg MT/kg	05	8.44 ± 0.26	9.13 ± 0.62	0	5	0	0	0
		40mg MT/kg	05	8.42 ± 0.27	8.97 ± 0.77	0	5	0	0	0
	9 week	Control	05	12.15 ± 0.11	26.12 ± 0.84	0	5	0	0	0
		10mg MT/kg	05	11.53 ± 0.16	21.72 ± 0.90	0	5	0	0	0
		40mg MT/kg	05	10.99 ± 0.20	18.91 ± 1.05	0	5	0	0	0
MT-feeding withdrawal	24 week	Control	09	16.14 ± 0.34	60.54 ± 4.95	0	7	2	0	0
		10mg MT/kg	03	15.05 ± 0.15	56.46 ± 9.14	0	0	2	1	0
		40mg MT/kg	06	16.08 ± 0.33	63.81 ± 3.82	0	0	2	4	0
	36 week	Control	11	20.73 ± 0.46	137.68 ± 8.80	0	8	3	0	0
		40mg MT/kg	07	22.23 ± 0.88	155.32 ± 14.77	0	0	0	6	1

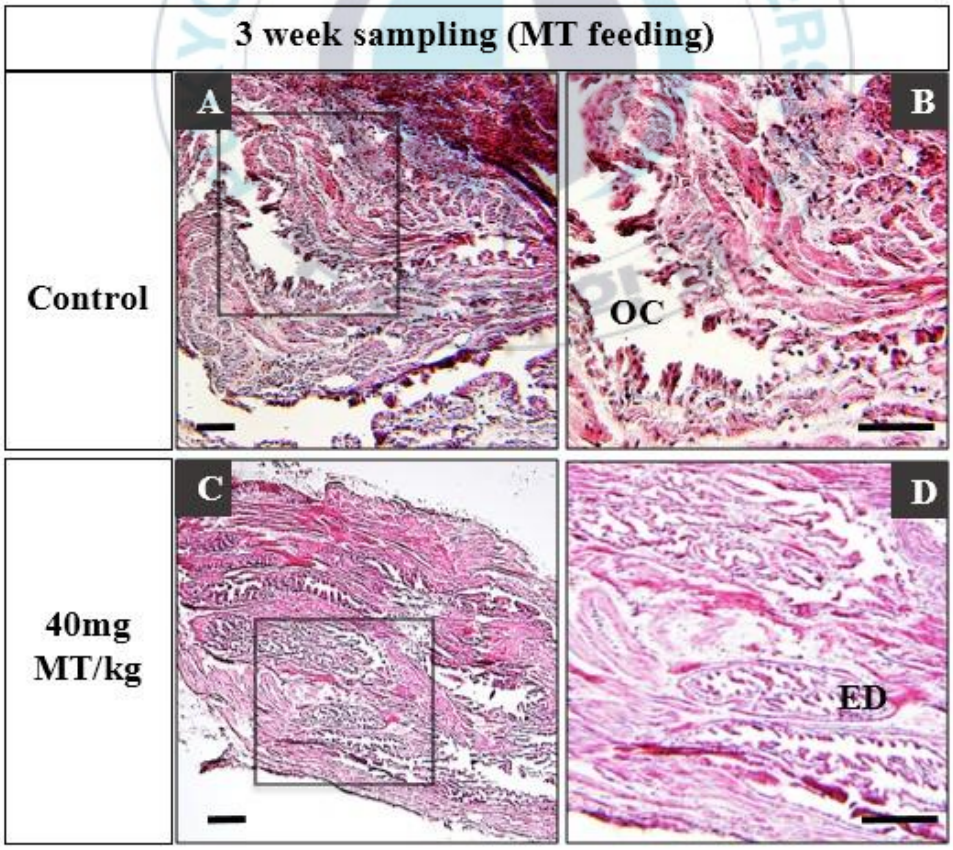
[1]Mean±SEM; [2]Initial ovarian cavity; [3]female phase; [4]Degenerative oocytes; [5]intersex-transitional phase; [6]male phase

3. Results

3.1. Gonadal histology during MT-feeding

We investigated the effect of androgen treatment on the sexual differentiation in 90 DAH fish fed containing MT at a concentration of 40 mg/kg diet. The gonad development induced by 10mg MT/Kg (data not shown) was more or less similar to the control. We compared the gonadal development phases between control and 40mg MT/Kg diet group. At 3 week after treatment (WAT), an ovarian cavity was observed in the untreated control group (Fig. 3.2.1A-B); however, an efferent duct (ED) was appeared in the MT-fed group (Fig. 3.2.1C-D).

9 week after treatment, large number of oogonia (Og) and primary oocytes (POc) were appeared in the control group (Fig. 3.2.1 E-F), whereas spermatogenic cyst (Sgc) was formed in the MT-fed group (Fig. 3.2.1 G-H).



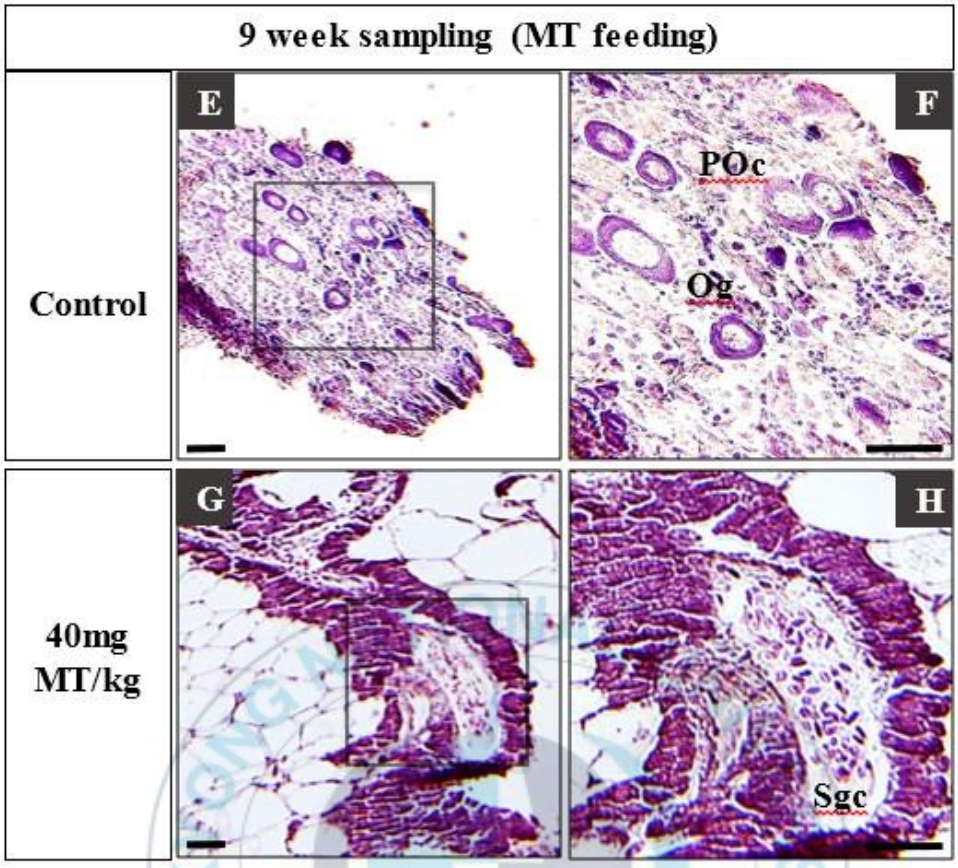


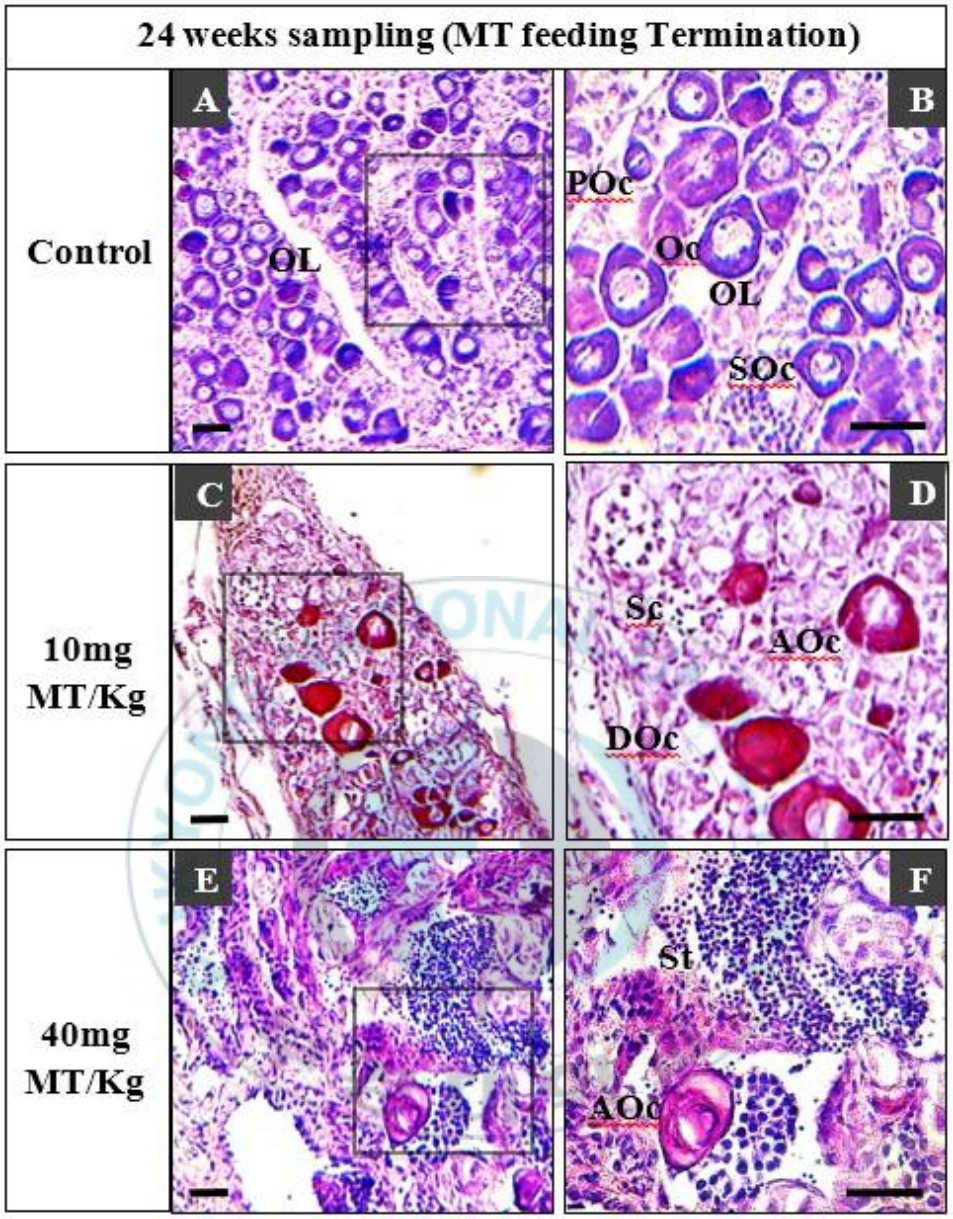
Fig. 3.2.1. Gonad histology of red-spotted grouper, *Epinephelus akaara* during 9 weeks of MT treatment. The gonadal status is shown as follows: (A-B) Control gonad at 3W; (C-D) MT-feeding (40mg MT/kg diet) gonad at 3W; (E-F) Control gonad at 9W; (G-H) MT-feeding (40mg MT/kg diet) gonad at 9W. OC: ovarian cavity; ED: efferent duct-like structure; Og, oogonia; POc: primary oocytes; Sgc, spermatogenic cyst. Scale bars, 25μm.

3.2. Gonadal changes after completion of MT treatment

We investigated the gonadal status after completion of MT treatment. Gonad samples were collected at 24 and 36 week of experiment.

At both the 24-week and 36-week sampling points, control group contained numerous primary oocytes (POc), secondary oocytes (SOc), and complete oocytes (Oc). Moreover, few spermatogenic cyst (Sgc) were also observed (Fig. 2 A-B, G-H).

After 24 week, 40mg MT/Kg diet group gonad contained atretic oocyte (AOc), degenerated oocytes (DOc), numerous spermatocytes (Sc), and spermatids (St). 10mg MT/Kg diet group contained atretic oocyte (AOc), degenerated oocytes (DOc) and few spermatogenic cyst (Sgc) (Fig. 2 C-F). After 36-week, the gonad of the 40mg MT/Kg diet group showed numerous spermatozoa (Sz), and St. Moreover, AOc and DOc were also observed (Fig. 2 K-L).


24 weeks sampling (MT feeding Termination)
Control
10mg MT/Kg
40mg MT/Kg
A
B
C
D
E
F
OL
POc
Oc
OL
SOc
Sc
AOc
DOc
St
AOc

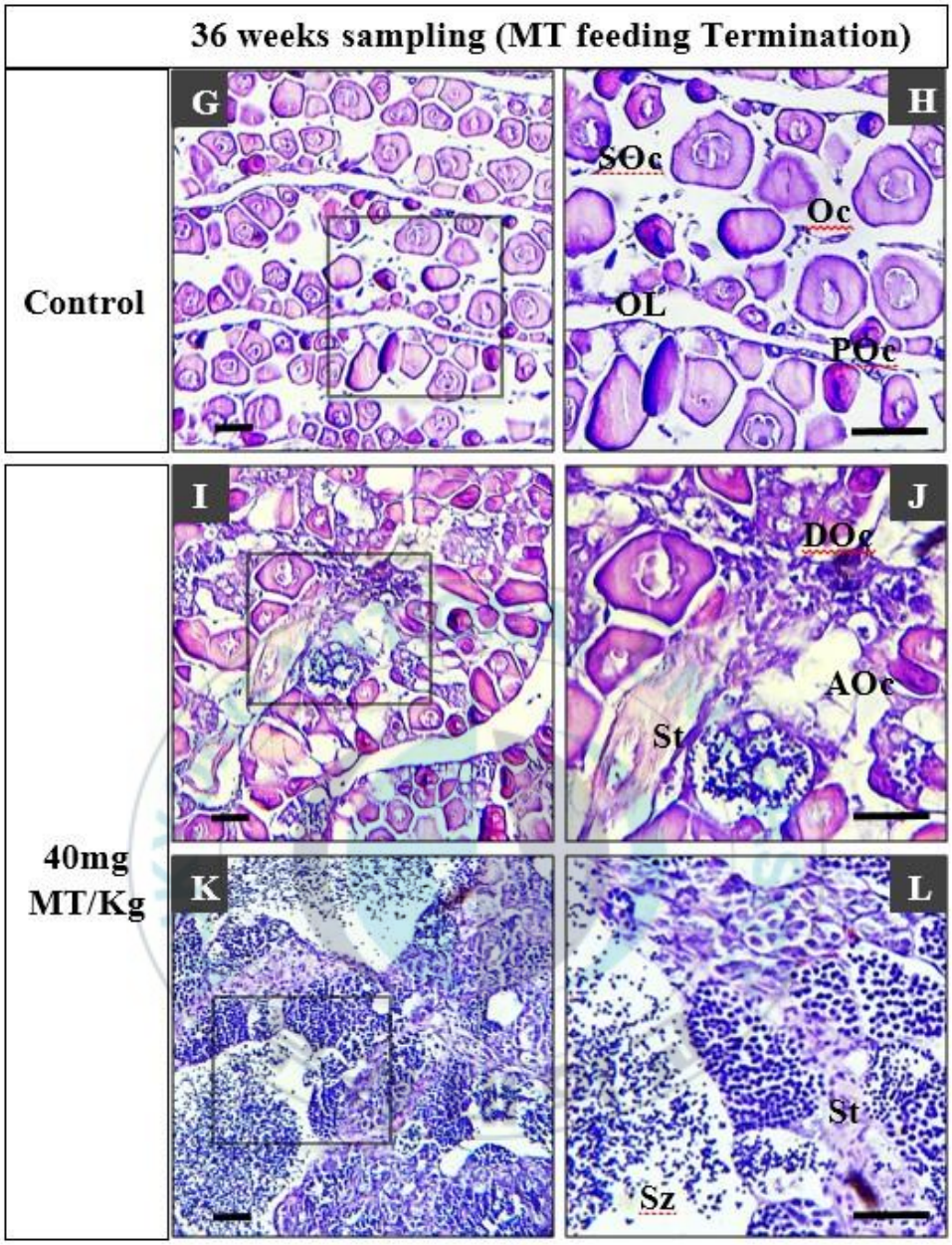


Fig. 3.2.2. Gonad histology of red-spotted grouper, *Epinephelus akaara* after MT feeding withdrawal period. The gonadal status is shown as follows: (A-B) Control gonad at 24W; (C-D) 10mg MT/kg diet gonad at 24W; (E-F) 40mg MT/kg diet gonad at 24W; (G-H) Control gonad at 36W; (I-J) 10mg MT/kg diet gonad at 36W; (K-L) 40mg MT/kg diet gonad at 36W. POc, primary oocytes; SOc, secondary oocytes; Oc, oocytes; OL, ovarian lumen; Sgc, spermatogenic cyst; Sc,

spermatocytes; St, spermatids; Sz, spermatozoa; AOc, atretic oocyte; DOc, degenerated oocytes. Scale bars, 25µm.

3.3. Gonad morphology after 36 weeks of sampling

There were no visual deviations in fish from any treatment groups. However, the difference in gonad morphology of control and MT-treated group was observed after 36 weeks of experiment.

In the control group, the ovary was found in bi-lobed shape, where one lobe was bigger than the other (dimorphic Shape) (Fig. 3.2.3 A-C). On the other hand, gonad in the MT-treated group was found in elongated thread-like shape, which was the indication of conversion of ovaries to testes (Fig. 3.2.3 D-F).

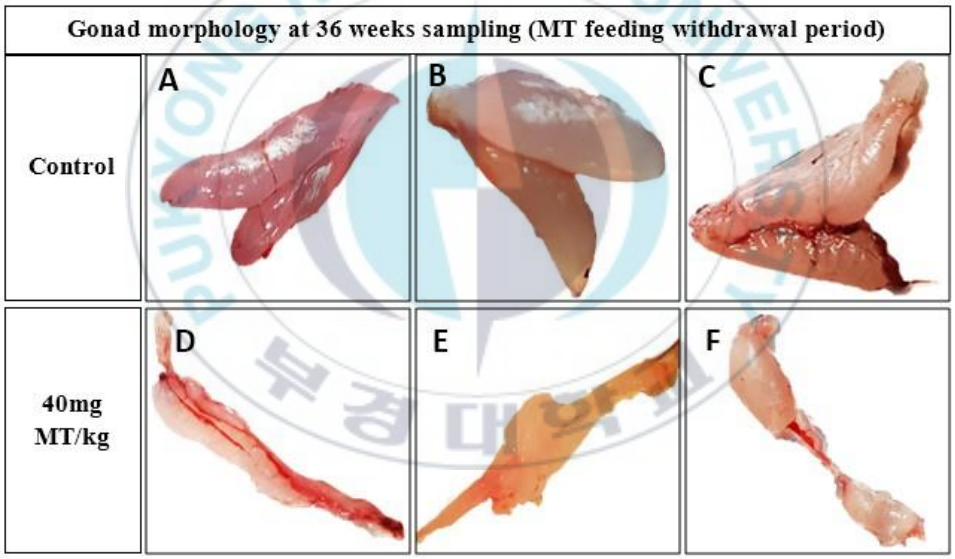


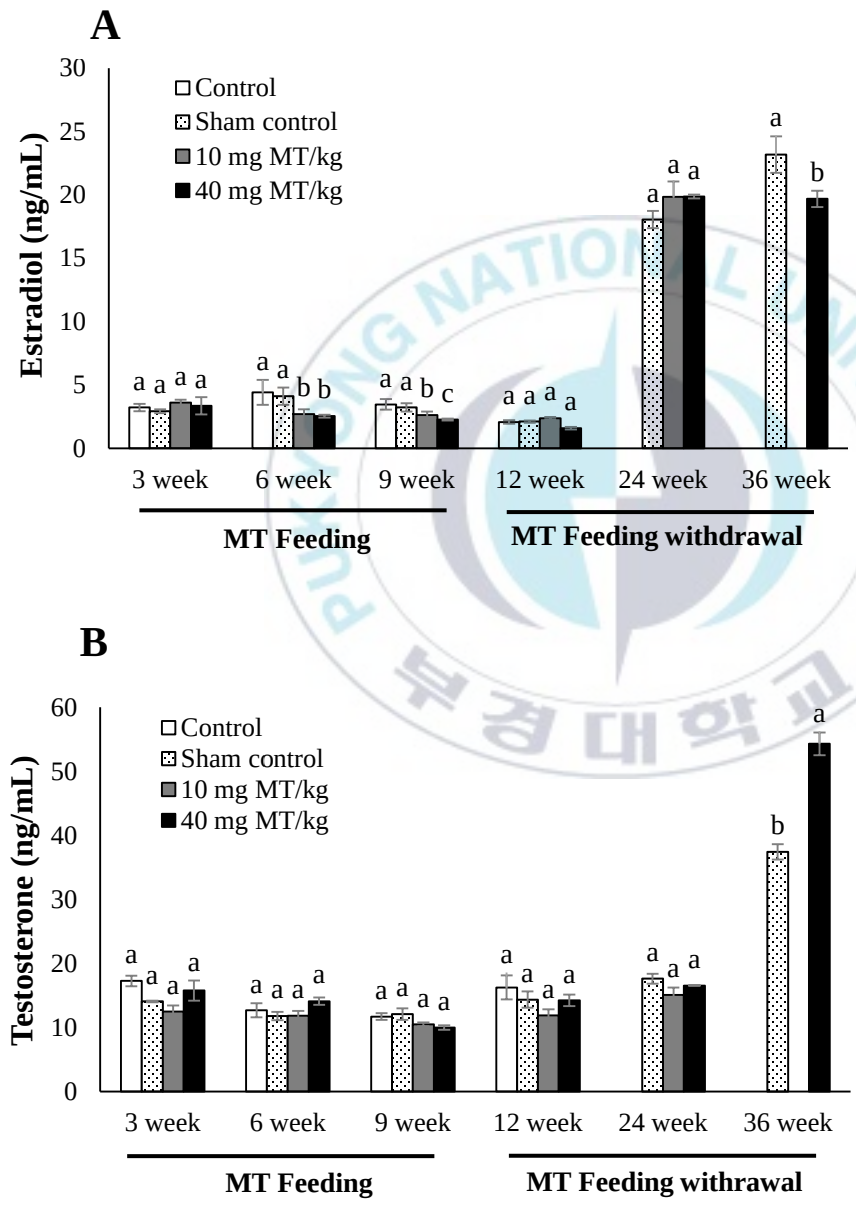
Fig. 3.2.3. Gonad morphology of red-spotted grouper, *Epinephelus akaara* at the end of experiment (36 week sampling).

3.4. Hormonal changes during MT-feeding and MT-feeding withdrawal

The plasma E2 levels were significantly ($P < 0.05$) decreased in the MT-fed group compared to the control during MT treatment period, but no significant changes were marked after completion of MT-treatment (Fig. 3.2.4 A).

The plasma T level was unchanged during MT-feeding period; however, high level of T was observed in 40mg MT/Kg group after 36 weeks of experiment (Fig.3.2.4 B).

The increased cortisol level was observed in MT-fed groups (10 and 40mg MT/Kg) during MT treatment period, but no significant changes ($P < 0.05$) were detected after 36 week of experiment (Fig. 3.2.4 C).



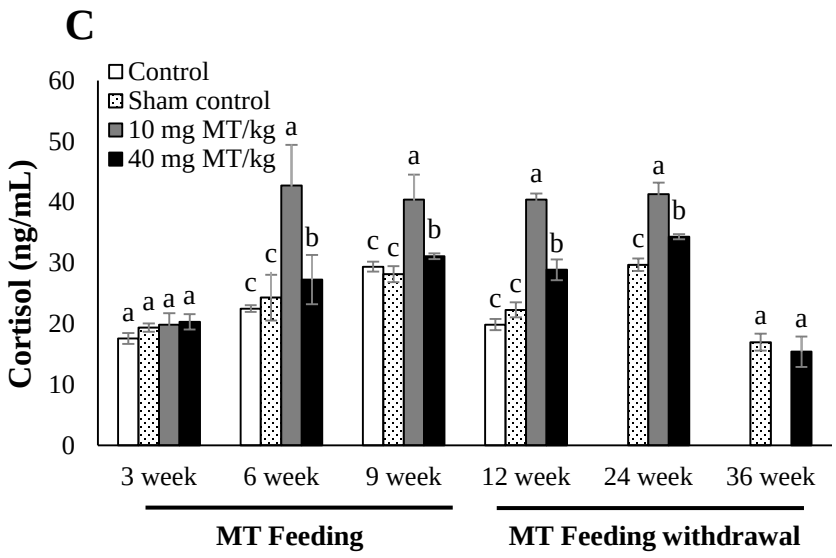


Fig. 3.2.4. Changes in plasma sex steroid (A) estradiol (E2), (B) testosterone (T) and stress hormone (C) cortisol level of red-spotted grouper, *Epinephelus akaara* during during MT feeding and MT feeding withdrawal period.. Data are expressed as the mean ± SEM for seven fish (n=07). Different letters represent statistically significant differences among the treatment groups at equivalent time points (ANOVA, Duncan's multiple range test, $P < 0.05$).

4. Discussion

MT treatment has been reported to induce sex reversal from female-to-male in many grouper species (Bhandari et al., 2006; Hu et al., 2011; Shi et al., 2012; Sun et al., 2014). The present study investigated the potentiality of MT treatment to induce gonadal sex change from female to male in juvenile *E. akaara*. In this study, our results demonstrated that MT treatment with a dose of 40 mg/Kg diet during the sex differentiation stage could induce impermanent sex change from female-to-male in the red-spotted grouper.

The MT-induced sex change is a continual process of restructuring the ovarian tissue to testicular tissue (Alam et al., 2007; Sun et al., 2014; Wang et al., 2017; Huang et al., 2019). In the present study, the observed efferent duct-like structure in MT-treated group 3 week after treatment

(WAT), indicates that MT-feeding can induce to bypass the formation of the ovarian cavity (OC). The formation of OC is an important step to fish developing towards a female fate; however, MT-feeding abolished the OC formation in the MT-treated group. The formation of ED is a key step for testis development. Similar results were observed in 90 DAH orange spotted grouper and 120 DAH Malabar grouper after fed with MT-treated diet for 12 days and 60 days, respectively (Murata et al., 2014; Wang et al., 2018). Therefore, MT-feeding can initiate the gonadal sex change by disrupting the OC formation. In this study, the formation of spermatogenic cyst (Sgc) in MT-treated group at 9 WAT, indicating the onset of gonadal sex change in the red-spotted grouper. Similar results were observed in 90 DAH orange spotted grouper, when treated with 5mg MT/Kg diet for 60 days and 10mg MT/Kg diet for 36 days (Huang et al., 2019; Wang et al., 2018). Therefore, MT-feeding can induce male fate by transforming the OC to testicular tissue during the sex differentiation stage.

In the present study, we investigated the gonadal status during the MT-termination period and gonad was sampled after 24-week and 36-week of the experiment. The fish from the control group showed the bisexual phase gonads, indicates the co-existing of ovarian and testicular tissues in the gonad. Liu et al. (2016) reported that post-larvae of *E. akaara* reared in tanks show the bisexual phase gonad after 34 week of hatching.

The gonads collected from the MT-fed group showed the primary male characteristics at 24-week sampling points. The presence of AOc and DOc indicates that the gonad was in the transition phase. The gonads at 36-week sampling points contained numerous spermatids (St) and spermatozoa (Sz), indicating the formation of testes. The presence of intersex with AOc indicates the late-transition period where the complete transformation of ovary-to-testes was in the process. The gonadal shape at the end of the experiment also confirmed the conversion process of ovaries

to the testis. Our results are consistent with those of Huang et al. (2019), who observed that 90-day-old orange spotted grouper treated with MT at 5 mg/Kg diet for 96 days can permanently reverse sex from female-to-male. Therefore, it can be said that MT-feeding can induce male fate from the female in red-spotted grouper, even after withdrawal of MT treatment, However, it has been reported that MT-treatment induces impermanent or transient sex change in immature Malabar grouper and orange spotted grouper (Murata et al., 2014; Wang et al., 2018). These studies used the lower MT dose, short duration of treatment and shorter sampling period compare to the present study that might be the cause of transient sex change result. The above results suggest that the effect of MT-treatment may vary with the volume of dose, duration of treatment, sampling time, age, size, and the degree of sexual maturation.

In protogynous species, a sharp drop in serum E2 levels resulted from the degeneration of oocytes and simultaneously an increment in T levels resulted from the proliferation of spermatogonia are considered as key events during the natural or induced sex reversal from female-to-male (Bhandari et al., 2003; Alam et al., 2005; Alam et al., 2006; Kokokiris et al., 2006). In the present study, we found that serum E2 levels decreased after MT treatment; however, serum T levels did not immediately show a significant increase. Estrogen plays an important role in maintaining ovarian development (Bhandari et al., 2003). Decreased E2 levels may lead to the degradation of female germ cells and be the key factor for inducing the female-to-male sex change state. This could disrupt ovarian development and initiate the sex reversal process. Similar results were reported for the MT treatment of Malabar grouper and orange spotted grouper (Murata et al., 2014; Wang et al., 2019).

During the MT-feeding termination period, we found significantly higher levels of serum T and low levels of serum E2 in the MT-feeding withdrawal group. The increase in serum T level

may play a critical role in maintaining the testes. Wang et al. (2018) observed similar findings in orange spotted grouper after treated with MT diets for a long period (96 days). The higher cortisol level was observed in the MT-fed group during and after the hormonal treatment; however, the level was normal after sex change from female-to-male. The changes in cortisol levels could result from the physiological stress arise during the transformation of ovaries-to-testis. Cortisol is the primary stress hormone that typically surges following the stress and return to basal levels after recovery (Iwama et al. 2006).

5. Conclusion

In conclusion, our study demonstrated that MT administration at 40 mg/Kg diet could induce gonadal sex change in the protogynous red-spotted grouper. Our results suggest that MT treatment triggers the sex reversal process by increasing the production of serum T. Our findings also suggest that induction of sex change by MT treatment could apply in the aquaculture field to increase the seed production of red-spotted grouper. Further studies are required to understand the molecular level changes during the MT-induced sex reversal process in *E. akaara*.

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Summary (in Korean)

붉바리 (*Epinephelus akaara*) 치어의 성장 개선을 위한 생리학적 연구

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요약문

붉바리(*Epinephelus akaara*)는 아열대 어종으로 중국, 일본, 대만 등 동남아지역에서 고급 어종으로 유통되고 있어 현재 우리나라에서 수출품종으로 개발 중인 종이다. 최근 지구 온난화로 인한 수온 상승은 붉바리의 생리 기능에 영향을 미칠 뿐만 아니라 자성선숙형인 붉바리의 성전환 과정에도 영향을 미치는 것으로 알려져 있다. 이러한 번식 특성으로 인하여 성장 속도가 느려지고 성숙한 수컷의 가용성이 제한되어 이에 대한 해결책이 요구된다.

본 연구에서는 첫째 수온상승에 대한 붉바리 치어의 생리적 반응을 조직형태학적, 혈액 생화학적 그리고 온도와 성장관련 유전자발현 정도를 분석하였다; 실험 1 에서는 부화 후 200 일 치어(TL: 9.4 ± 0.12 cm; BL: 12.89 ± 0.61 g)를 대상으로 24 ℃ (대조구), 28℃, 32℃ 그리고 36 ℃ 의 실험구를 설정하여 28 일 동안 사육하였으며, 실험 2 에서는 부화 후 130 일 치어(TL: 8.28 ± 0.10 cm, BW: 8.53 ± 0.27 g)를 대상으로 25 ℃(대조구), 28 ℃, 31℃ 그리고 34 ℃ 로 설정하여 42 일간 사육하였다. 실험 1 의 경우, 36 ℃ 에서 아가미의 상피세포 괴사, 새판의 위축, 간 세포의 공포화 및 간 세포의 수축과 괴사 등의 손상이 관찰되었다. 생화학적 분석 결과, 포도당, 글루탐산 피루브산 트랜스아미나제(GPT), 글루타민옥살초산 전이효소(GOT), 코르티솔 농도도 다른 실험구(24 ℃, 28 ℃ 그리고 32 ℃)에 비해 36 ℃ 에서 유의하게 높게 나타났다($P < 0.05$). 또한 간, 근육, 그리고 아가미 조직 내 고수온 스트레스 관련 유전자 (Hsp60, 70, 90) 발현량도 36 ℃ 실험구에서 높게 나타났다. 실험 2 의 경우, 수온 31 ℃ 와 34 ℃ 에서 적혈구(RBC) 및 백혈구(WBC) 수 증가, 적혈구 세포 형태 및 크기 이상(ECA)이 관찰되었으며, 34 ℃ 에서는 아가미와 간의 조직학적 관찰결과, 아가미 새판의 비대현상과 꼬임, 간세포의 수축과 괴사, 공포화 그리고 모세혈관 확장 등이 관찰되었다. 생화학적 분석 결과, 포도당, 코르티솔, GPT, GOT 그리고 젖산탈수소효소(LDH)의 수치가 높게 나타났으며, 트리글리세리드(TG)와 총콜레스테롤(TCHO)의 수치는 다른 실험구(25 ℃, 28 ℃ 그리고 31 ℃)에 비해 34 ℃ 에서 낮게 나타났다($P < 0.05$). Hsp60, 70, 90 mRNA 발현량도 34 ℃ 에서 높게 나타났다. 한편, 성장률(SGR)과 사료효율(FER)은 28 ℃ 와 31 ℃ 에서 유의하게 높은 수치를 보였다. 성장관련 유전자 즉, IGF-2 과 GHR mRNA 발현량도 간 조직에서 높게 나타났다($P < 0.05$).

둘째, 부화 후 90 일된 붉바리 치어(TL: 5.98 ± 0.14 cm, BW: 3.67 ± 0.24 g)를 대상으로 17α-methyltestosterone (MT)처리에 따른 잠재적 성장과 성전환 과정을 분석하였다. MT 처리 농도는 10mg 과 40mg MT/Kg diet 로 설정하여 9 주동안 급이하였다. 성장률은 MT 급이 실험구 (10mg 과 40mg MT/Kg diet) 모두에서 낮은 체중 증가량(WG)을 보였으며, IGF-1 및 GHR mRNA 발현량도 대조구에 비해 낮은 수치를 보였다. 9 주동안 MT 처리 먹이 급이 완료 이후에 40mg MT/Kg diet 실험구에서 높은 체중 증가량(WG)과 성장률(SGR)을 보였으며, IGF-1 과 GHR mRNA 발현량도 증가하였다($P < 0.05$). 실험 종료 후인 36 주째에 40mg MT/Kg diet 처리구에서 정소의 세정관과 유사한 구조 (efferent duct-like) 와 난소에서 정소로의 전환과정이 관찰되었다. MT 먹이 급이 동안에는 혈장 에스트라디올(E2) 농도가 대조구에 비해 낮게 나타났으며, MT 먹이 급이 완료 이후에 높은 혈장 테스토스테론(T) 농도를 나타냈다($P < 0.05$).

본 실험 결과를 종합해 보면 붉바리 치어의 경우, 치사 온도는 36 ℃, 아치사 온도는 34 ℃ 임을 알 수 있었으며, 수온 30 ℃ 이상, 장기간 노출은 붉바리의 스트레스 반응을 유발시키는 것으로 나타났다. 생화학적 분석 결과, 붉바리의 수온 상승으로 인한 스트레스 지표 반응은 포도당, 코르티솔, GOT 그리고 GPT 분석, RBC 그리고 WBC 개수 측정으로 확인할 가능 하였다. MT 처리 결과, 대조구와 10mg MT/Kg diet 실험구에 비해 40mg MT/Kg diet 실험구에서 성장율과 암컷에서 수컷으로의 성전환 유도 가능성이 높은 것으로 생각된다.

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