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Effect of Various Light Spectra on the Physiological Responses of Juvenile Rock Bream *Oplegnathus fasciatus* by Changes in Water Temperature

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by

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List of Abbreviations

ANOVA	analysis of variance
AlaAT	alanine aminotransferase
AspAT	aspartate aminotransferase
cDNA	complementary deoxyribonucleic acid
CAT	catalase
ELISA	enzyme-linked immunosorbent assay
GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HPI	hypothalamic-pituitary-interrenal
HSP	heat shock protein
IgM	immunoglobulin M
LED	light-emitting diode
LPO	lipid hydroperoxide
mRNA	mitochondrial ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
SOD	superoxide dismutase
TUNEL	transferase dUTP nick end labeling

고수온 노출에 따른 돌돔 *Oplegnathus fasciatus*의 생리적 반응에 미치는 다양한 빛 파장의 영향

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요 약

본 연구는 고수온 환경에 돌돔 *Oplegnathus fasciatus*를 노출시킨 후, 다양한 파장의 빛을 조사하면서 돌돔 체내에서 스트레스와 면역 반응을 포함한 생리학적 변화를 확인하기 위하여 수행되었다. 다양한 빛 파장에 의한 돌돔의 생리학적 반응을 관찰하기 위하여 분자생물학적 실험 방법을 통하여 비교·분석하였다.

1. 고수온 노출에 따른 돌돔의 항산화 반응 및 면역시스템에 미치는 빛 파장의 영향

본 연구에서는 고수온 환경에 노출된 돌돔의 체내에서 생성된 산화스트레스의 조절 및 면역지표 그리고 세포사멸에 미치는 특정 빛 파장의 효과를 확인하기 위하여, 돌돔을 고수온 환경(25 및 30°C)에 노출시킨 후 특정 빛 파장(형광등, 녹색 및 적색)을 세기별(0.25 및 0.5 W/m^2)로 조사하면서 생리학적 변화를 관찰하였다.

하루에 수온을 1°C씩 상승시키는 수온 변화 환경(20 → 30°C)에 돌돔을 노출시킨 후, 수온 스트레스 요인에 의하여 어체 내에서 생성되는 활성산소를 제거하기 위한 항산화 물질[superoxide dismutase (SOD), catalase (CAT) 및 glutathione peroxidase (GPX)] 및 면역 지표(melatonin 및 lysozyme)의 변화를 분석하였다. 또한, 급격한 수온 변화에 따른 돌돔의 세포사멸 반응을 확인하기

위하여 caspase-3 및 hydrogen peroxide (H_2O_2)를 측정하였으며, 간 조직 내에서의 세포사멸 정도는 terminal transferase dUTP nick end labeling (TUNEL) assay을 통하여 확인하였다.

급격한 수온 변화는 돌돔 체내에서 SOD, CAT, GPX mRNA 및 단백질 발현량 그리고 효소 활성을 유의적으로 증가시켰지만, 녹색 LED 파장을 조사한 실험구에서는 대조구에 비하여 빛의 세기(0.25 및 0.5 W/m^2)와는 상관없이 항산화 유전자 및 단백질 발현 그리고 효소 활성은 유의적으로 감소되었음이 확인되었다. 또한, 본 연구에서는 고수온 노출에 따른 돌돔의 면역력 변화를 광원별로 비교한 결과, 녹색 LED 파장 실험구에서는 대조구에 비하여 빛의 세기(0.25 및 0.5 W/m^2) 와는 상관없이 항산화 유전자 및 단백질 발현 그리고 효소 활성이 유의적으로 감소되는 경향이 확인되었다. 혈장 내 caspase-3 및 H_2O_2 농도 수준을 분석한 결과에서도 고수온(25 및 30°C) 환경에 노출된 돌돔의 혈중 caspase-3 및 H_2O_2 농도는 고수온에 노출된 시간이 경과함에 따라 증가하는 경향을 보였으나, 녹색 LED 파장 실험구에서는 대조구에 비하여 빛의 세기(0.25 및 0.5 W/m^2)와는 상관없이 유의적으로 감소하는 경향을 확인할 수 있었다. 고수온 노출이 돌돔 간세포의 세포사멸에 미치는 영향을 확인하기 위하여 TUNEL assay를 실시한 결과, 30°C 고수온 실험구의 돌돔 간세포에서 세포사멸이 가장 많이 유발되었으나, 녹색 LED 파장을 조사한 30°C 실험구에서는 오히려 세포사멸이 감소된 것을 확인할 수 있었다.

이상의 연구 결과를 종합하여 보면, 25°C 이상의 고수온 환경에 돌돔을 노출시킨 경우, 어체 내에서는 산화스트레스가 유발되었으며 이러한 산화스트레스에 대응하기 위하여 항산화 물질인 SOD, CAT, GPX mRNA 및 단백질 발현 그리고 효소 활성이 유의적으로 증가된 것으로 사료된다. 그러나 녹색 LED 파장의 조사는 오히려 산화스트레스를 감소시키는 것으로 나타났다. 또한, 25°C 이상으로의 수온 변화는 돌돔 체내에서 H_2O_2 농도를 증가시켜 caspase-3 활성을 유도함은 물론 결국에는 세포사멸까지도 유발시키는

것으로 사료된다. 즉, 고수온 환경은 어류에게 생리적 스트레스를 유발시키지만, 녹색 파장의 빛은 오히려 산화스트레스를 감소시키는 역할을 하며, 0.25 W/m^{2}의 낮은 광량(세기)만으로도 항산화 능력 및 면역력을 증진시키는데 효과적인 것으로 판단되었다.

2. 고수온 노출에 따른 돌돔의 생리적 스트레스 및 DNA 손상에 미치는 빛 파장의 영향

본 연구에서는 고수온 환경에 노출된 돌돔의 체내에서 생성된 생리적 스트레스 및 핵 내 DNA의 손상에 미치는 특정 빛 파장의 효과를 확인하기 위하여, 돌돔을 고수온 환경(25 및 30°C)에 노출시킨 후 특정 빛 파장(형광등, 녹색 및 적색)을 세기별(0.25 및 0.5 W/m^2)로 조사하면서 생리학적 변화를 관찰하였다.

하루에 수온을 1°C씩 상승시키는 수온 변화 환경(20 → 30°C)에 돌돔을 노출시킨 후, 수온 스트레스 요인에 의하여 손상된 단백질을 복구하기 위하여 생성되는 heat shock protein (HSP) 70의 변화를 분석하였다. 그리고 hypothalamus-pituitary-interrenal axis (HPI 축)을 중심으로 조절되는 생리학적 스트레스 반응(cortisol 및 glucose)과 스트레스로 인하여 발생되는 간 손상 정도를 확인하기 위하여 aspartate aminotransferase (AspAT)와 alanine aminotransferase (AlaAT) 값을 측정하였다. 또한, 고수온 노출에 따른 간세포 내 핵 DNA의 손상 정도를 확인하기 위하여 comet assay를 실시하여 분석하였다.

수온이 변화함에 따라 돌돔 체내에서는 HSP70 mRNA의 발현과 혈장 내 cortisol, glucose, AspAT 및 AlaAT 농도가 유의적으로 증가하였으며, 특히 30°C 고수온 실험구에서 가장 높게 증가하였다. 그러나 녹색 LED 파장을 조사한 실험구에서는 대조구에 비하여 빛의 세기(0.25 및 0.5 W/m^2)와는 관계없이 HSP70 mRNA의 발현과 cortisol, glucose, AspAT 및 AlaAT 농도가 유의적으로 감소하는 경향이 확인되었다. 또한, 고수온에 노출된 돌돔의 간세포 내 핵 DNA 손상 정도를 대조구와 비교해 본 결과, 30°C 실험구의 돌돔 간세포에서 더

많은 손상이 발생하였지만, 녹색 LED 파장을 조사한 30°C 실험구에서는 핵 DNA의 손상 정도가 감소된 것이 관찰되었다.

이상의 연구 결과를 종합하여 보면, 25°C 이상의 고수온 환경에 돌돔을 노출시킨 경우, 어체 내에서는 HSP70 mRNA의 발현 그리고 cortisol, glucose, AspAT 및 AlaAT 농도가 유의적으로 증가하였을 뿐만 아니라 간세포 내 핵 DNA가 손상되는 등 생리적 스트레스가 다량으로 발생되었다. 그러나 녹색 LED 파장의 조사는 스트레스와 간세포 내 핵 DNA의 손상을 감소시키는 것으로 나타났으며, 특히 0.25 W/m^2의 낮은 광량(세기)의 조사만으로도 고수온에 의한 스트레스를 감소시키는데 효과적인 것으로 판단되었다.

Chapter 1.

General Introduction

Water temperature is an important factor involved in the growth, immunity, maturation, and in the physiological adjustment of fish (Maule et al., 1989; Bly and Clem 1992; Bowden 2008). Rapid change in temperature is an environmental stress for fish that causes an increase in reactive oxygen species (ROS), such as superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and single oxygen ($^1O_2$) (Roch 1999). The excessive production of ROS as a result of environmental stress induces physiological disorders, such as a decrease in disease resistance and reproductive ability because of denaturation of cellular nucleic acids and proteins and loss of their functions, as well as because of the promotion of lipid peroxidation that adversely damages cell membrane and affects cell viability (Kim and Phyllis 1998; Pandey et al., 2003). In addition, ROS is known to have a negative effect on immune function because it decreases the activity of lysozyme (Wang et al., 2008).

Living organisms possess antioxidant defense mechanisms to protect themselves from oxidative stress caused by ROS and to maintain homeostasis. These antioxidant defense mechanisms mainly involve the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Mcfarland et al., 1999).

The immunity of fish is particularly influenced by external factors, such as change in water temperature due to the environmental characteristics of the habitat (Magnadottir 2010). Lysozyme, which is one of the important indicators of the level of immunity, is known to destroy the invading bacterial pathogens by damaging their cell wall during the process of phagocytosis (Saurabh et al., 2008; Shin et al., 2014). In addition, melatonin, a powerful antioxidant, has been reported to

function in the direct removal of ROS as well as in enhancing immunity (Reiter et al., 1997; Gülçin et al., 2009; Carrillo-Vico et al., 2013).

Apoptosis, which is characterized by DNA fragmentation and cellular shrinkage, akin to morphological incidents, is regulated by caspases belonging to the cysteine protease family (Alnemri 1997). Caspase-3 is known to play an important role in the process of apoptosis, which is influenced by DNA damage and inflammation caused by biochemical and morphological processes (Häcker 2000).

When fish are exposed to rapid changes in water temperature, heat shock proteins (HSPs) are produced in large quantities. In particular, HSP70 is highly expressed in various tissues and is known to play an important role in cellular metabolism (Nakano et al., 2002).

In addition, cortisol which is a well-known indicator of the stress response is produced in the fish body to maintain homeostasis when the fish is exposed to environmental stress, such as rapid changes in water temperature (Beato et al., 1996; Bonga, 1997). Cortisol is secreted through activation of the hypothalamus - pituitary - interrenal gland axis (HPI axis); the first reaction in this process is the release of corticotropin-releasing hormone from the hypothalamus (Bonga, 1997), followed by secretion of adrenocorticotropic hormone from the anterior pituitary gland, inducing cortisol synthesis in the inter-renal cells of the head kidney (Bonga, 1997; Flik et al., 2006). Cortisol has been reported to directly increase the plasma concentrations of glucose, which is used as an energy source in cell metabolism, by promoting cortisol-mediated gluconeogenesis in the body in response to cell damage caused by stress (Begg and Pankhurst, 2004; Small, 2004).

In addition, stress caused by the external environment may cause liver damage (Cheng et al., 2005; Choi et al., 2015). Aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) are well known indicators of liver damage (Nemcsók et al., 1990).

The physiological stress responses of fish are mainly controlled by the endocrine system. In particular, light is an environmental factor that greatly influences the endocrine system (Pierce et al., 2008; Jin et al., 2009). Recently, various physiological effects of different wavelengths of light from light-emitting diodes (LEDs) on fish have been studied (Villamizar et al., 2009; Choi et al., 2012; Song et al., 2016). LEDs have been shown to be effective for influencing fish culture (Villamizar et al., 2009; Choi et al., 2015).

In the present study, I investigated the effects of specific wavelengths of light in the regulation of oxidative stress induced by an environment of high temperature, by analyzing the changes in mRNA and protein expression and the enzymatic activities of SOD, CAT, and GPX in juvenile rock bream exposed to a high temperature environment (25 and 30°C) and different sources (fluorescent, green, and red LEDs) as well as different intensities (0.25 and 0.5 W/m^2) of light. In addition, I measured H_2O_2 levels to determine the level of fluctuation in stress under the different conditions, and also measured the concentrations of lysozyme and melatonin as immunological indicators. And I also investigated environmental stress (high temperature) by analyzing changes in HSP70 mRNA and cortisol and glucose levels to determine fluctuations in stress under different conditions and measured the concentrations of AspAT and AlaAT to determine the effects of stress on the liver. Finally I also measured the DNA damage and in liver cells of fish as a consequence of apoptotic activity by analyzing the changes in the expression and activity of caspase-3 mRNA and nuclear DNA damage by conducting a terminal transferase dUTP nick end labeling (TUNEL) assay and comet assay.

Chapter 2.

Effect of Different Wavelengths of Light on the Antioxidant and Immunity Status of Juvenile Rock Bream *Oplegnathus fasciatus* Exposed to Thermal Stress

Abstract

I investigated the effect of light wavelengths on antioxidant and immunity parameters in juvenile rock bream *Oplegnathus fasciatus* exposed to thermal stress (25 and 30°C). I exposed the fish to light emitting diodes (LEDs) emitting green (520 nm) and red light (630 nm) of 0.25 and 0.5 W/m^2 intensity, and measured the activity, and mRNA and protein expression levels of the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase. I also determined the levels of plasma hydrogen peroxide (H_2O_2), melatonin, and lysozyme. Furthermore, the mRNA and protein levels of caspase-3 were measured and terminal transferase dUTP nick end labeling (TUNEL) assays were performed. I observed that mRNA expression and activities of antioxidant enzymes and plasma H_2O_2 levels were significantly higher after exposure to high temperatures. However, increases in these parameters were significantly lower after exposure to green LED light. The plasma melatonin and lysozyme levels were significantly lower in the different groups after exposure to high temperatures; however, in groups exposed to green LED light, their levels were significantly higher than those in the control group. The expression pattern of caspase-3 mRNA was similar to that of H_2O_2. The TUNEL assay showed that apoptosis was markedly higher at higher water temperatures than

that at 20°C. These results indicate that high water temperatures induce oxidative stress and decrease the immunity in juvenile rock bream but green LED light inhibits the rise in oxidative stress and combats the decrease in immunity and should, thus, be useful in the culture of rock bream.

1. Introduction

Water temperature is an important factor involved in the growth, immunity, maturation, and in the physiological adjustment of fish (Maule et al., 1989; Bly and Clem 1992; Bowden 2008). Rapid change in temperature is an environmental stress for fish that causes an increase in reactive oxygen species (ROS), such as superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and single oxygen ($^1O_2$) (Roch 1999). The excessive production of ROS as a result of environmental stress induces physiological disorders, such as a decrease in disease resistance and reproductive ability because of denaturation of cellular nucleic acids and proteins and loss of their functions, as well as because of the promotion of lipid peroxidation that adversely damages cell membrane and affects cell viability (Kim and Phyllis 1998; Pandey et al., 2003). In addition, ROS is known to have a negative effect on immune function because it decreases the activity of lysozyme (Wang et al., 2008).

Living organisms possess antioxidant defense mechanisms to protect themselves from oxidative stress caused by ROS and to maintain homeostasis. These antioxidant defense mechanisms mainly involve the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Mcfarland et al., 1999). Such antioxidant enzymes are known to exert an anti-oxidative action, mainly in the liver and kidney of an organism (Basha and Rani 2003; Hansen et al., 2006). Firstly, SOD temporarily eliminates the active oxygen by converting O_2^- into O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$), and, there after, H_2O_2, which is also an active oxygen species, is converted to non-toxic H_2O and O_2 by CAT ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Kashiwagi et al., 1997).

The immunity of fish is particularly influenced by external factors, such as change in water temperature due to the environmental characteristics of the habitat (Magnadottir 2010). Lysozyme, which is one of the important indicators of the

level of immunity, is known to destroy the invading bacterial pathogens by damaging their cell wall during the process of phagocytosis (Saurabh et al., 2008; Shin et al., 2014). In addition, melatonin, a powerful antioxidant, has been reported to function in the direct removal of ROS as well as in enhancing immunity (Reiter et al., 1997; Gülçin et al., 2009; Carrillo-Vico et al., 2013).

Apoptosis, which is characterized by DNA fragmentation and cellular shrinkage, akin to morphological incidents, is regulated by caspases belonging to the cysteine protease family (Alnemri 1997). Caspase-3 is known to play an important role in the process of apoptosis, which is influenced by DNA damage and inflammation caused by biochemical and morphological processes (Häcker 2000).

The stress and immune responses of fish are mainly controlled by the endocrine system. Light is one of the environmental factors that greatly influence the endocrine system (Pierce et al., 2008; Jin et al., 2009). Recently, various physiological effects of different wavelengths of light, obtained from light emitting diodes (LEDs), on fish have been studied (Villamizar et al., 2009; Choi et al., 2012; Kim et al., 2016). Studies have shown that the wavelength and intensity of a specific LED play a role in homeostasis, immunity, maturation, and growth of fish (Karakatsouli et al., 2008; Kim et al., 2016). LEDs have been demonstrated to be effective for use in fish culture (Villamizar et al., 2009; Choi et al., 2015).

Rock bream is important to the aquaculture industry as one of the major species in East Asian coasts, including those of Korea and Japan. It is a typical sub-tropical fish that lives in shallow coastal areas with water temperatures around 20-22°C (Oh et al., 2007; Park et al., 2015). This species is sensitive to temperature changes; especially in the summer, immunity is reduced due to high temperature stress, and this frequently causes widespread death (Choi et al., 2010). Thus, this study was conducted to investigate the effects of high temperature changes on juvenile rock bream, because the juveniles are more sensitive to

temperature changes than the adult fish (Zhang et al., 2013).

In the present study, I investigated the effects of specific wavelengths of light in the regulation of oxidative stress induced by an environment of high temperature, by analyzing the changes in mRNA and protein expression and the enzymatic activities of SOD, CAT, and GPX, in juvenile rock bream exposed to a high temperature environment (25 and 30°C) and different sources (fluorescent, green, and red LEDs) as well as different intensities (0.25 and 0.5 W/m^2) of light. In addition, I measured H_2O_2 levels to determine the level of fluctuation in stress under the different conditions, and also measured the concentrations of lysozyme and melatonin as immunological indicators. I also measured the DNA damage in liver cells of fish as a consequence of apoptotic activity by analyzing the changes in the expression and activity of caspase-3 mRNA and by conducting a terminal transferase dUTP nick end labeling (TUNEL) assay.

2. Materials and methods

2.1. Experimental fish and environmental conditions

For each experiment, juvenile rock bream (n = 225; length, 10.6 ± 1.1 cm; mass, 8.7 ± 0.7 g) were purchased from a commercial aquarium (Jeju, Korea) and were allowed to acclimate in eleven 100-L circulation filter tanks in the laboratory. There were 45 tanks (three tanks each for exposure to 5 different wavelengths at 20, 25, and 30°C), with five fish in each tank. The fish in the control group were exposed to a white fluorescent bulb. For the experimental groups, the fish were exposed to either green (520 nm) or red (630 nm) LEDs (Daesin LED Co. Kyunggi, Korea), maintained at an intensity of approximately 0.25 or 0.5 W/m^2 in both cases (Fig. 1). The LEDs were placed 50 cm above the water surface and the depth of the water column was 50 cm. The irradiance level in the water column, in the tanks illuminated with the external light source was maintained at approximately 0.3 or 0.6 W/m^2, as determined using a spectrometer (MR-16; Rainbow Light Technology Co. Ltd., Taoyuan, Taiwan) and PHOTO-RADIO METER (HD2102.1; Delta OMH CO., Caselle di Selvazzano, Italy). The photoperiod consisted of a 12-h light (L):12-h dark (D) cycle, with the photo-phase lasting from 07:00 to 19:00 h (the lights were turned on at 07:00 h and turned off at 19:00 h). The juvenile rock bream were reared in the presence of an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and were allowed to acclimatize to the conditions for 24 h after transfer to the tanks. The fish were acclimated in the tanks for 24 hours, and the control group was sampled immediately after adaptation. Thereafter, the tanks were illuminated with light of different wavelengths in 12-h light:12-h dark cycles. The water temperature was increased by 1°C per day 20°C to 25 and 30°C in each tank. There was no death of fish in any of the groups. Moreover, because the period of this study was short (10 days), no change in the growth of rock bream was observed. The fish

received commercial feed twice daily until the day prior to sampling. The sampling was performed at the experimental temperatures (20, 25, and 30°C). All the fish were anesthetized using tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) and were decapitated prior to tissue collection. Liver samples were collected, immediately frozen in liquid nitrogen, and stored at -80°C until total RNA was extracted for analysis. Blood samples were separated by centrifugation (4°C, 10,000 × g for 5 min) and stored at -80°C until the analysis.

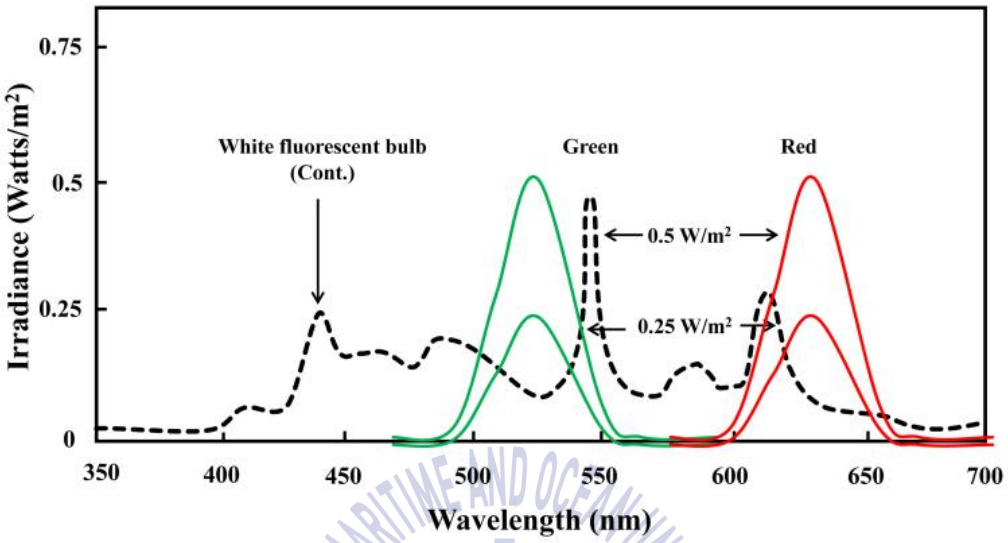


Fig. 1. Spectral profiles of light emitting diodes (LEDs; green, 520 nm; red, 630 nm) and white fluorescent bulb (Cont.) in this study. Each LEDs light source was set two intensity (low, 0.25; high, 0.5 W/m^2). Reprinted from Shin et al., (2011), with permission from Comparative Biochemistry and Physiology, Part-A.

2.2. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

Total RNA was extracted from each sample (15 fish per experimental group) using the Trizol kit (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Two micrograms of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-d(T) anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4°C for use in quantitative PCR. The qPCR analysis was conducted to determine the relative expression levels of the mRNAs of the antioxidant enzymes, SOD, CAT, GPX, and caspase-3, using the total RNA extracted from the liver of juvenile rock bream. The qPCR primer pairs were designed to span the spliced exon–exon junctions using the known juvenile rock bream sequences (Table 1). The qPCR amplification was performed using a Bio-Rad iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and the iQ SYBR green Supermix (Bio-Rad), following the manufacturer's instructions. As a control, β-actin gene was also amplified for each sample, and all the data were expressed in terms of their difference with the corresponding values calculated for the β-actin threshold cycle (Ct). The Ct values of the PCR products formed the basis for all the analyses. The Ct values were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) per sample and that for their internal control (β-actin) were calculated as follows: [$\Delta\Delta Ct = 2^{-(\Delta Ct_{sample}-\Delta Ct_{internal\ control})}$]. The qPCR data from three replicate samples were analyzed using CFX96^{TM} Real Time System (Bio-Rad) to estimate the transcript copy numbers in each sample.

Table 1. Primers used for qPCR amplification

Genes (accession no.)	Primer	DNA sequences
SOD (JN593103)	Forward	5′-TGA CCT GAC CTA CGA CTA TG-3′
	Reverse	5′-GCC TCC TGA TAT TTC TCC TCT-3′
CAT (AY734528)	Forward	5′-GTG CTG AAC GAA GAG GAG-3′
	Reverse	5′-TTG TTG AGA AGA GTC TGA ACC-3′
GPX (AY734530)	Forward	5′-GAT GTG AAC GGA CAG GAT G-3′
	Reverse	5′-ACT GAC GGG ACT CCA AAT-3′
Caspase-3 (JQ315116)	Forward	5′-CTT CTT CTA CGC CTT CTC-3′
	Reverse	5′-TGA GTA GTA GCC TGT GGA-3′
β-actin (FJ975145)	Forward	5′-CAG AGC AAG AGA GGT ATC C-3′
	Reverse	5′-TCG TTG TAG AAG GTG TGA TG-3′

2.3. Western blot analysis

The total protein isolated from liver samples of juvenile rock bream (15 fish per experimental group) was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. A total of 30μg protein was loaded in each lane of Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used as a reference. The samples were electrophoresed at 180V, and were immediately transferred from the gels onto a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85V for 3 min using a Trans-Blot® Turbo™ Transfer System. The membranes were subsequently blocked with 5% milkin Tris-buffered saline (TBS) (pH7.4) for 45min, after which they were washed in TBS. The membranes were then incubated with antibodies against SOD (1:2000 dilution, NBP1-47443, Novus Biologicals, USA), CAT (1:2000 dilution, SC-58332, Santa Cruz Biotechnology, USA), and GPX (1:2000 dilution, CPBT-35941RH, Creative Diagnostics, USA), and were incubated, thereafter, with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:2000 dilution, Bio-Rad) for 60min. β-tubulin (probed using an anti-β-tubulin antibody, ab6046, Abcam, UK, at 1:4000 dilution) was used as an internal control. The bands were detected using Western Bright TMECL (Advansta, Menlo Park, CA, USA) and were visualized by 30-s exposure in a Molecular Imager® (ChemiDoc™XRS + Systems, Bio-Rad). The images of the blot were scanned using a high-resolution scanner and the band density was estimated using a computer program (ImageLab™ Software, version3.0, Bio-Rad).

2.4. Analysis of plasma parameters

Plasma (from five fish per experiment group in triplicate) was separated from blood samples by centrifugation (4°C, 10,000 × g, for 5 min). The H_2O_2 levels were measured using a modified version of the methods described by Nouroozzadeh

et al (1994), and in the instruction manual of PeroxiDetect kit (Sigma). The absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. The concentrations were expressed as nM/mL.

The SOD, CAT, and GPX activities were determined by immunoassays using specific ELISA kits (SOD, CSB-E15929fh; CAT, CSB-E15928fh; Cusabio Biotech Co., Ltd., China; GPX, MBS0924388; Mybiosource Inc., San Diego, California, USA).

The plasma melatonin, lysozyme, and caspase-3 levels were analyzed using immunoassay ELISA kits (melaotnin, MBS013211; Mybiosource; lysozyme, CSB-E17296Fh, Cusabio Biotech; caspase-3, MBS012786, Mybiosource). The absorbance was read at 450 nm.

2.5. Terminal transferase dUTP nick end labeling (TUNEL) assay

To evaluate the apoptotic response of the fish liver cells to green LED light, I performed the TUNEL assay using a commercially available in situ cell death detection kit (catalogue number, 11 684 795 910, Roche, Switzerland). Polylysine-coated slides were used to prevent the loss of adherence of the apoptotic cells to the slides. The fish liver tissue was washed and fixed with 4% buffered paraformaldehyde, and was permeabilized with freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate solution. This liver tissue was then incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. The slides were washed three times with phosphate-buffered saline (PBS), and the incorporated biotin-dUTP was detected under a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). For the paraffin-embedded tissue sections, the slides were dewaxed and fixed according to standard protocols, and then treated as described above. The green fluorescent cells indicated apoptosis.

2.6. Statistical analysis

All the data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A two-way ANOVA followed by Tukey's post-hoc test was used to compare the differences in the data ($P < 0.05$). The values are expressed as means ± standard error (SE).

3. Results

3.1. Expression and activities of antioxidants (SOD, CAT, and GPX) in the liver

I investigated the changes in the enzymatic activities and in the expression of mRNAs and proteins of SOD, CAT, and GPX in liver tissues in response to the changes in water temperature (Fig. 2 and 3).

The mRNA and protein expression as well as the activities of SOD, CAT, and GPX were increased significantly with the rise in temperature in all the experimental groups. However, the mRNA and protein expression levels, and the activities of SOD, CAT, and GPX in the green LED irradiation groups were significantly lower than in the control groups exposed to illumination with white fluorescent bulb and there were no significant differences between the groups exposed to green light intensities of 0.25 and 0.5 W/m^2. However, the groups exposed to red LED showed significant increase in the measured parameters with the increase in light intensity.

3.2. Plasma H_2O_2 level

The plasma H_2O_2 levels in all the experiment groups were increased at high water temperatures. However, the plasma H_2O_2 levels in the groups irradiated with green LEDs were significantly lower than in the control groups. However, the levels in the groups exposed to red LEDs were significantly higher than in the control groups (Fig. 4).

3.3. Plasma melatonin and lysozyme levels

The plasma melatonin and lysozyme levels were decreased significantly with the rise in temperature in all the experiment groups (Fig. 5). However, the levels of plasma melatonin and lysozyme in the green LED light groups were significantly higher than in the control groups, and there were no significant differences between

the groups exposed to green light of different intensities. In contrast, the groups exposed to red LED light showed significant decrease in the plasma melatonin and lysozyme levels with the increasing intensity of light.

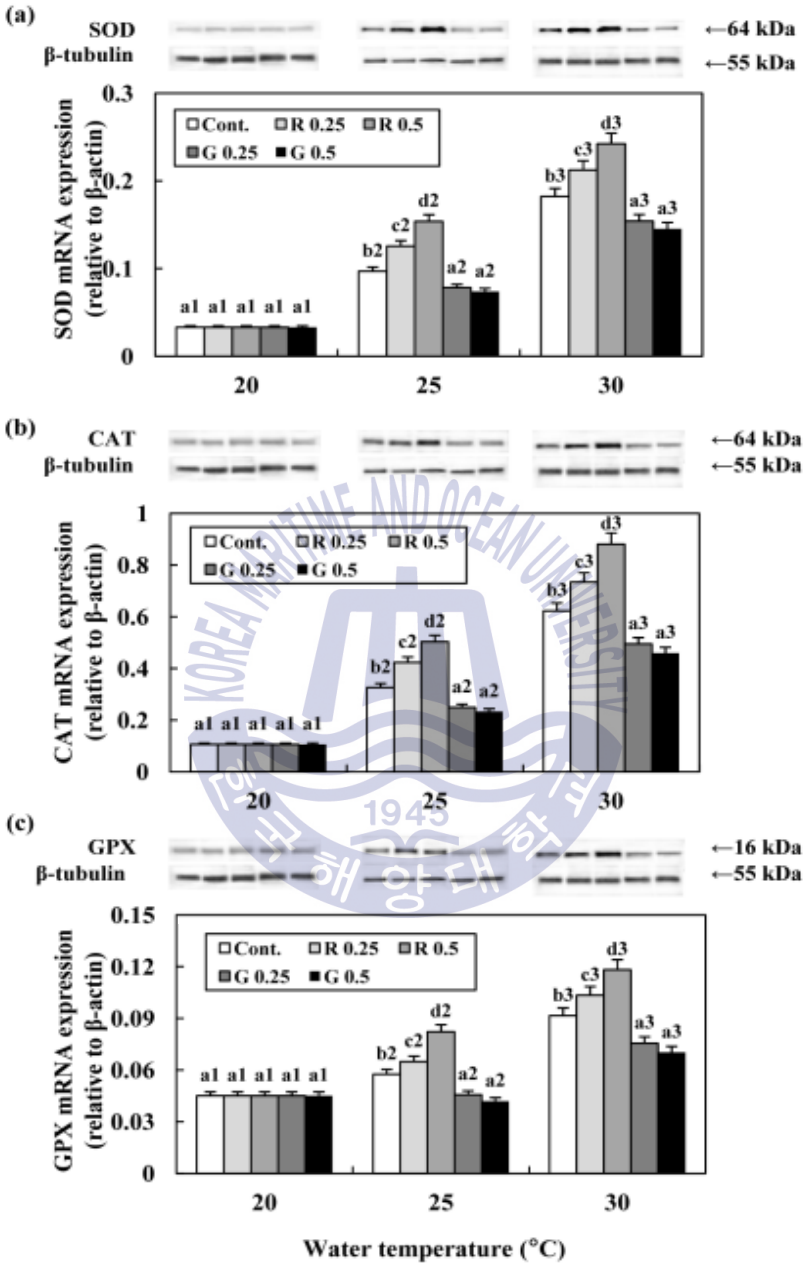


Fig. 2. Expression levels of SOD (a), CAT (b), and GPX (c) mRNAs and proteins in juvenile rock bream liver tissue during thermal changes under different lighting conditions using red (R) and green (G) LEDs at two light intensities (0.25 and 0.5 W/m^2), and white fluorescent bulb (Cont.), as measured by qPCR and western blotting. Total liver RNA (2 μg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Western blots showing the expression of antioxidant enzymes [SOD (18 kDa), CAT (64 kDa), and GPX (16 kDa)] in the liver of juvenile rock bream; β -tubulin (55 kDa) was used as an internal control. Values with numbers are significantly different at the temperature within the same LED spectra ($P < 0.05$). The lowercase letters indicate significant differences between the different LED spectra within the same temperature ($P < 0.05$). All the values are means ± SE ($n = 15$).

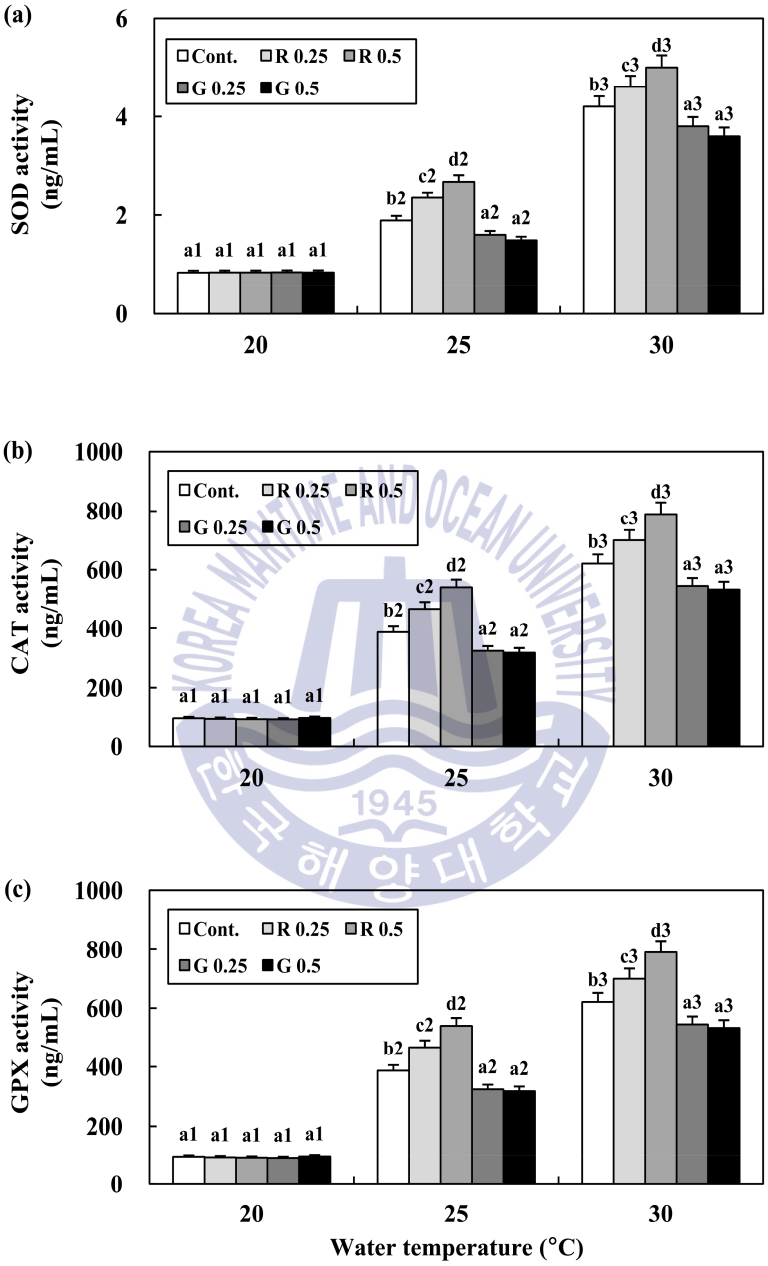


Fig. 3. Activities of plasma SOD (a), CAT (b), and GPX (c) in juvenile rock bream during thermal changes under different lighting conditions using red (R) and green (G) LEDs at two light intensities (0.25 and 0.5 W/m^2), and white fluorescent bulb (Cont.), as measured using micro plate reader. Values with numbers are significantly different at the temperature with in the same LED spectra ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All the values are means ± SE ($n = 15$).

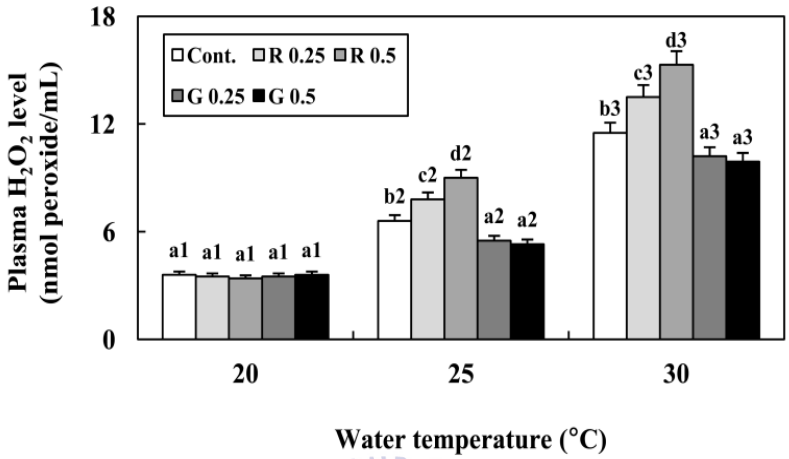


Fig. 4. Activities of plasma H_2O_2 in juvenile rock bream during thermal changes under different lighting conditions using red (R) and green (G) LEDs at two light intensities (0.25 and 0.5 W/m^2), and white fluorescent bulb (Cont.). Values with numbers are significantly different at the temperature with in the same LED spectra ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All the values are means ± SE ($n = 15$).

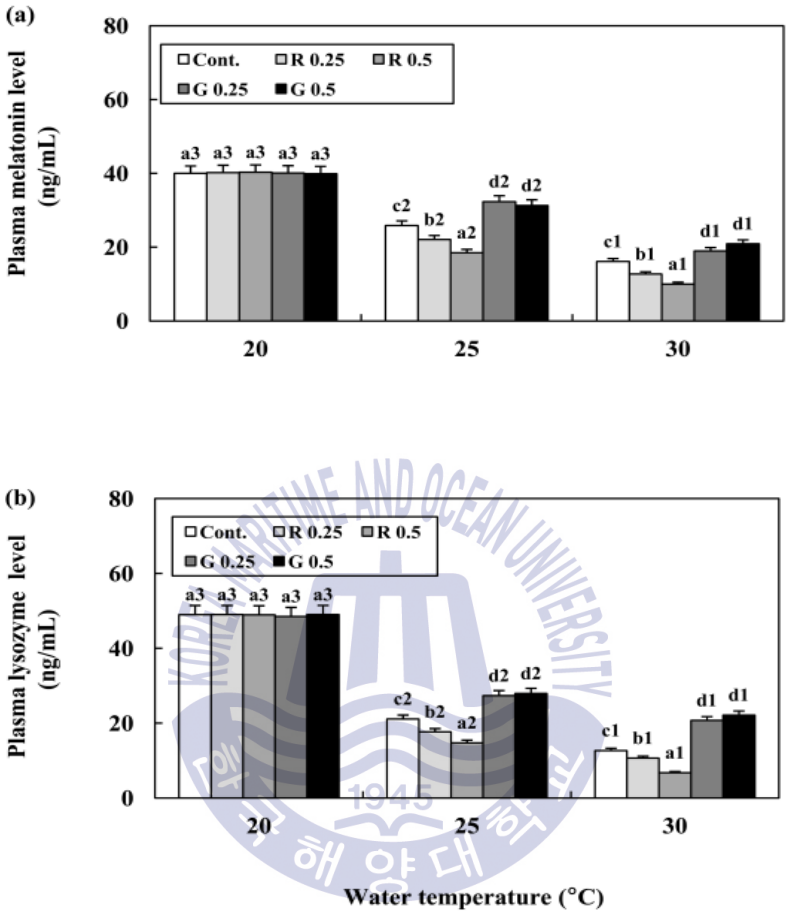


Fig. 5. Levels of plasma melatonin (a) and lysozyme (b) in juvenile rock bream during thermal changes under different lighting conditions using red (R) and green (G) LEDs at two light intensities (0.25 and 0.5 W/m^2), and white fluorescent bulb (Cont.). Values with numbers are significantly different at the temperature with in the same LED spectra ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All the values are means ± SE ($n = 15$).

3.4. Expression and activity of caspase-3 in liver

I investigated the changes in the mRNA expression and activity of caspase-3 in liver tissue in response to the changes in water temperature (Fig. 6). I observed that the levels of mRNA and activity of caspase-3 increased significantly with the rise in temperature in all the experiment groups. In addition, the mRNA levels and the activity of caspase-3 in the groups irradiated with green LEDs were significantly lower than in the control groups and there were no significant differences between the groups irradiated with 0.25 and 0.5 W/m^2 of green light. However, the groups exposed to red light showed significant increase in caspase-3 with the increasing light intensity.

3.5. TUNEL assay

The TUNEL assay was used to investigate the presence of apoptotic cells (Fig. 7). There were significant visible differences among the labeled cells in the TUNEL assay between the control (non-treated) and the experimental groups (fluorescent, red LED, and green LED) exposed to a temperature of 30°C. The frequency of apoptotic cells was lower after the exposure to green LEDs than that in the other experimental groups. In contrast, more apoptotic cells were detected after exposure to red LEDs.

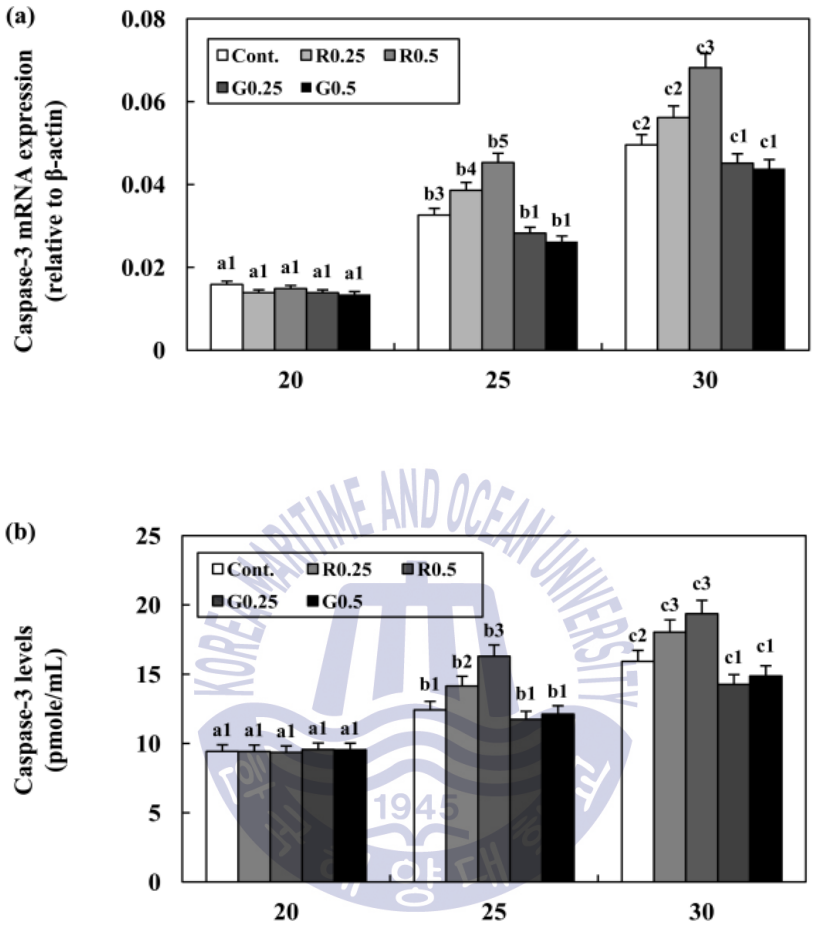
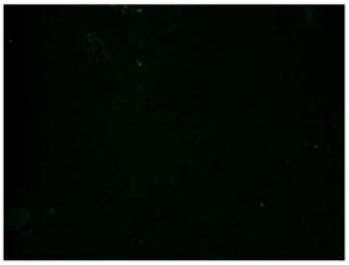
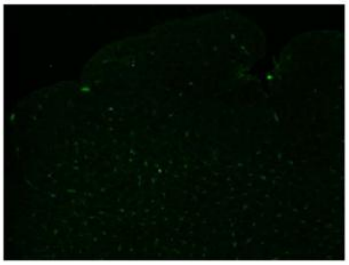


Fig. 6. Change in the levels of expression of caspase-3 mRNA (a) and plasma caspase-3 (b) during thermal changes under different lighting conditions using red (R) and green (G) LEDs at two light intensities (0.25 and 0.5 W/m^2), and white fluorescent bulb (Cont.). Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with numbers are significantly different at the temperature within the same LED spectra ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All the values are means ± SE ($n = 15$).

(a) White fluorescent bulb
20 °C

(b) White fluorescent bulb
30 °C





(c) Green LED (0.5 W/m^2)
30 °C

(d) Red LED (0.5 W/m^2)
30 °C

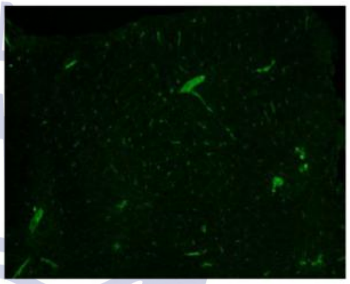


Fig. 7. TUNEL detection of juvenile rock bream liver cell apoptosis under different lighting conditions. The different panels are for the different groups, as follows: control group (Cont.) exposed to white fluorescent bulb at 20°C (a), group exposed to white fluorescent bulb at 30°C (b), group exposed to green LED (0.5 W/m^2) at 30°C (c), and group exposed to red LED (0.5 W/m^2) at 30°C (d). The cells were stained with acridine orange and visualized under a fluorescent microscope. Cells producing green fluorescence indicate apoptotic cells. Scale bars = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

4. Discussion

In this study, I investigated the effect of specific wavelengths (green, 520 nm; red, 630 nm) and intensities (0.25 and 0.5 W/m^2) of light on the antioxidant capacity and immunity of juvenile rock bream exposed to high water temperature by measuring the changes in expression and activity of antioxidant enzymes as well as in the plasma concentrations of H_2O_2, melatonin, and lysozyme. I also measured the changes in the expression and activity of caspase-3 and performed TUNEL analysis to investigate the effects of high temperature and specific wavelengths of light on cell death.

The results of the changes in the expression of protein, mRNA, and activities of SOD, CAT, GPX, which are typical antioxidative genes in organisms, revealed that these parameters were significantly increased with the increase in water temperature in each experimental group. When comparing the differences between the light sources, regardless of the light intensity (0.25 or 0.5 W/m^2), these values for parameters were observed to be significantly lower upon exposure to the green LED than their values in the control groups. However, the values for these parameters were observed to increase with the intensity of red light.

In a similar study, Kim et al., (2014) investigated the effects of various wavelengths (red, green, blue, and purple) on the oxidative stress in goldfish exposed to a high temperature environment. They observed that as the temperature increased, the expression of mRNAs and proteins, and the enzymatic activity of the antioxidant enzymes, SOD, CAT, and GPX showed an increasing trend. These parameters were significantly lower upon exposure to the green wavelength of light than their values in the control group, whereas they were significantly increased in the group exposed to the light of red wavelength.

Based on the results obtained in the present study, which are in agreement with those obtained in previous research, I suggest that the antioxidant gene expression

was significantly increased due to the oxidative stress induced in the juvenile rock bream exposed to a high temperature environment, and that the green LED wavelength effectively reduced the oxidative stress regardless of the intensity of light. However, the red LED wavelength was observed to increase the oxidative stress.

I also determined the plasma H_2O_2 concentrations in juvenile rock bream exposed to a high temperature environment and observed that the H_2O_2 concentrations in plasma were significantly increased with the increase in water temperature. In addition, the plasma concentrations of H_2O_2 were significantly lower in the group exposed to green LED wavelengths than in the control. There was no significant difference between the different intensities of the green light used. However, the plasma concentrations of H_2O_2 increased significantly with the increase in the intensity of red light in the same range.

It was also demonstrated by Kim et al., (2014) that in goldfish exposed to a high temperature environment (25 and 30°C), as the water temperature increased, plasma H_2O_2 concentrations were significantly increased. However, the concentrations of H_2O_2 were significantly lower in the green and blue LED groups than in the control whereas they were significantly increased in the red LED groups. Shin et al., (2011) reported that the concentration of H_2O_2 in the green LED wavelength group was significantly lower than in the control group, when *Amphiprion clarkii* was exposed to different LED wavelengths (red, green, and blue). Therefore, in this study, green LED wavelength was found to be effective in reducing the plasma H_2O_2 concentrations by reducing the oxidative stress, as reported in previous studies.

Furthermore, I investigated the effect of high water temperature on the immunity of fish in the different groups. I measured the changes in the levels of lysozyme and melatonin in plasma and used them as indicators of immunity in the body. I

observed that the concentrations of lysozyme and melatonin in the plasma were significantly decreased with the increase in water temperature. However, the lysozyme and melatonin concentrations were significantly higher in the green LED wavelength groups than that in the group exposed to the fluorescent bulbs. In a similar study, Choi et al., (2012) investigated the concentration of lysozyme in plasma after starvation of clownfish *Amphiprion melanopus* by inducing oxidative stress. They reported that the lysozyme concentration was significantly higher in the groups exposed to green LEDs.

I also analyzed the changes in the expression of mRNA and in the activity of caspase-3, which is a typical apoptosis marker in organisms. These parameters were significantly increased with the increase in water temperature in each experimental group. When comparing the differences between the different light sources, regardless of the light intensity, these parameters were significantly lower in the green LED group than their values in the control groups. However, these parameters were significantly increased with the increase in the intensity of red light.

In a similar study, Kim et al., (2016) showed that when individuals of the Olive flounder *Paralichthys olivaceus* were exposed to a high temperature environment, the expression of caspase-3 mRNA increased significantly with the increase in water temperature. However, compared to that in the control group, the expression of caspase-3 mRNA in the green LED group was significantly lower when compared to that in the light of other wavelengths (fluorescent bulbs, red and blue LEDs). In contrast, in the red LED wavelength group, the caspase-3 mRNA levels were significantly higher with respect to the expression levels in groups exposed to other wavelengths of light (fluorescent bulbs, green and blue LEDs). Yabu et al., (2001) showed that when zebrafish *Danio rerio* were exposed to a high temperature for an hour, apoptosis was significantly higher than that in the control groups.

In this study, when juvenile rock bream were exposed to a high water temperature (30°C), the caspase-3 mRNA expression and apoptosis were increased. Because the caspase-3 mRNA expression and apoptosis were decreased in the group exposed to green LEDs, I suggest that green LED wavelength has a role in reducing apoptosis.

In conclusion, the results of this study suggest that light of green wavelength has a role in reducing the oxidative stress in juvenile rock bream and it is also effective in increasing antioxidative capability as well as enhancing immunity even at alow intensity of 0.25 W/m^2. Moreover, the red wavelength causes oxidative stress that increases with the increasing intensity of light. In addition, the results show that light of green wavelength decreases apoptosis in contrast to that of red wavelength, which probably plays a role in increasing the apoptosis. Our results should aid in the judicious use of LED light of green and red wavelength in juvenile rock bream culture, especially under conditions of high temperature.

Chapter 3.

Effects of Various Light Spectra on Physiological Stress and DNA Damage by Thermal Stress in Rock Bream *Oplegnathus fasciatus*

Abstract

In this study, I investigated the effects of light spectra on antioxidant stress and immunity in rock bream *Oplegnathus fasciatus* using light-emitting diodes (LEDs; green, 520 nm; red, 630 nm) at two intensities (0.25 and 0.5 W/m^2) with application of thermal stress (25 and 30°C). I measured the mRNA expression of heat shock protein 70 (HSP70) and the levels of plasma cortisol, glucose, aspartate aminotransferase (AspAT), and alanine aminotransferase (AlaAT). Additionally, DNA damage was measured using comet assays. Our findings showed that HSP70 mRNA expression and plasma cortisol, glucose, AspAT, and AlaAT levels were significantly higher after exposure to high temperatures and were significantly lower after exposure to green LED light. Thus, although high water temperatures induced stress in rock bream, green LED light inhibited stress. In particular, green LED light reduced stress and DNA damage to a greater degree than other light sources.

1. Introduction

Factors inducing stress in fish include water temperature, salinity, and breeding density (Beckmann et al., 1990). In particular, changes in water temperature not only cause oxidative stress in the fish body but also have negative effects on physiological phenomena, such as antioxidant control ability, immunity, and sexual maturity (Machado et al., 2014; Jung et al., 2016).

When fish are exposed to rapid changes in water temperature, heat shock proteins (HSPs) are produced in large quantities. These HSPs act as chaperone proteins that repair proteins damaged by external environmental stressors and play a role in maintaining the normal functions of the cells (Welch, 1991; Donaldson et al., 2008). In particular, HSP70 is highly expressed in various tissues and is known to play an important role in cellular metabolism (Nakano et al., 2002).

In addition, cortisol is produced in the fish body to maintain homeostasis when the fish are exposed to environmental stress, such as rapid changes in water temperature (Bonga, 1997). Cortisol is a well-known indicator of the stress response and directly affects cells through binding to the glucocorticoid receptor in the cell membrane following secretion into the blood, thereby controlling various physiological responses (Beato et al., 1996). Cortisol is secreted through activation of the hypothalamus - pituitary - interrenal gland axis (HPI axis); the first reaction in this process is the release of corticotropin-releasing hormone from the hypothalamus (Bonga, 1997), followed by secretion of adrenocorticotropic hormone from the anterior pituitary gland, inducing cortisol synthesis in the inter-renal cells of the head kidney (Bonga, 1997; Flik et al., 2006). Cortisol has been reported to directly increase the plasma concentrations of glucose, which is used as an energy source in cell metabolism, by promoting cortisol-mediated gluconeogenesis in the body in response to cell damage caused by stress (Begg and Pankhurst, 2004; Small, 2004).

In addition, stress caused by the external environment may cause liver damage

(Cheng et al., 2005; Choi et al., 2015). Aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) are well known indicators of liver damage (Nemcsók et al., 1990).

The physiological stress responses of fish are mainly controlled by the endocrine system. In particular, light is an environmental factor that greatly influences the endocrine system (Pierce et al., 2008; Jin et al., 2009). Recently, various physiological effects of different wavelengths of light from light-emitting diodes (LEDs) on fish have been studied (Villamizar et al., 2009; Choi et al., 2012; Song et al., 2016). LEDs have been shown to be effective for influencing fish culture (Villamizar et al., 2009; Choi et al., 2015).

Accordingly, in the present study, I investigated the effects of specific wavelengths of light on regulation of environmental stress (high temperature) by analyzing changes in HSP70 mRNA in rock bream. In addition, I measured cortisol and glucose levels to determine fluctuations in stress under different conditions and measured the concentrations of AspAT and AlaAT to determine the effects of stress on the liver. Finally, I analyzed nuclear DNA damage in rock bream liver cells in response to stress.

2. Materials and methods

2.1. Experimental fish and environmental conditions

For each experiment, rock bream (n = 150; length, 10.6 ± 1.1 cm; mass, 8.7 ± 0.7 g) were purchased from a commercial aquarium (Jeju, Korea) and were allowed to acclimate in ten 100-L circulation filter tanks in the laboratory. Each tank (each experimental group) contained 15 fish. The fish in the control group were exposed to a white fluorescent bulb. For the experimental groups, the fish were exposed to either green (520 nm) or red (630 nm) LEDs (Daesin LED Co., Kyunggi, Korea), maintained at an intensity of approximately 0.25 or 0.5 W/m^2 in both cases (Fig. 8). The lights were located 50cm above the surface of the water and were irradiated at the surface of water. The photoperiod consisted of a 12-h light (L) / 12-h dark (D) cycle, with the photophase lasting from 07:00 to 19:00 h (the lights were turned on at 07:00 hand turned off at 19:00 h). The rock bream were reared in the presence of an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and were allowed to acclimate to the conditions for 24h after transfer to the tanks. The water temperature was then increased from 20°C to 30°C in daily increments of 1°C. The fish received commercial feed twice daily until the day prior to sampling. The sampling was performed at the experimental temperatures (20, 25, and 30°C). All fish were anesthetized using tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) and were decapitated prior to tissue collection. Liver samples were collected, immediately frozen in liquid nitrogen, and stored at -80°C until total RNA was extracted for analysis. Plasma samples were separated by centrifugation (4°C, 10,000×g for 5 min) and stored at -80°C until analysis.

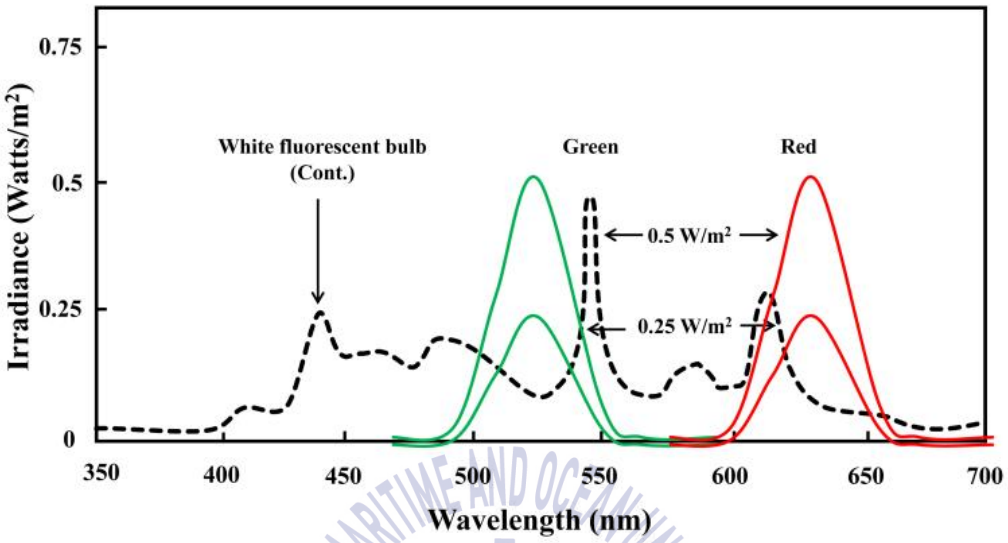


Fig. 8. Spectral profiles of light emitting diodes (LEDs; green, 520 nm; red, 630 nm) and white fluorescent bulb (Cont.) in this study. Each LEDs light source was set two intensity (low, 0.25; high, 0.5 W/m^2). Reprinted from Shin et al., (2011), with permission from Comparative Biochemistry and Physiology, Part-A.

2.2. Total RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from each sample using a TRIzol kit (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Two micrograms of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-d(T) anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4℃ for use in qPCR. The qPCR analysis was conducted to determine the relative expression level of HSP70 mRNA using the total RNA extracted from the livers of rock bream. The qPCR primer pairs were designed to span the spliced exon-exon junctions using known rock bream sequences. Primers for qPCR were designed with reference to known rock bream sequences: HSP70 forward (5′-CTA TGT GGC GTT CAC TGA C-3′) and reverse (5′-AGT CTC TTG GCA TCA AAC AC-3′) primers; and β-actin (internal control) forward (5′-CAG AGC AAG AGA GGT ATC C-3′) and reverse (5′-TCG TTG TAG AAG GTG TGA TG-3′) primers. I conducted qPCR amplification using a Bio-Rad iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix (Bio-Rad), following the manufacturer's instructions. As a control, the β-actin gene was also amplified for each sample, and all data were expressed as differences relative to the threshold cycle (Ct) of β-actin. The Ct values were defined as the PCR cycle in which the fluorescence signal crossed a set threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) per sample and that for their internal control (β-actin) were calculated as follows: ($\Delta\Delta Ct = 2\text{-}[\Delta Ct_{sample} - \Delta Ct_{internal\ control}]$). The qPCR data from three replicate samples were analyzed using a CFX96 Real-Time System (Bio-Rad) to estimate the transcript copy numbers in each

sample.

2.3. AspAT/AlaAT, glucose, and cortisol levels in plasma

Plasma AspAT/AlaAT and glucose levels were measured using a dry multiplayer analytic slide method in a biochemistry auto analyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan). Plasma cortisol levels were analyzed using an immunoassay enzyme-linked immunosorbent assay (ELISA) kit (E08487f; Cusabio Biotech, Hubei, China). Plasma glucose levels were analyzed using a biochemistry auto analyzer (Fuji Dri-Chem 4000). Absorbance was read at 450 nm.

2.4. Comet assays

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells (Bajpayee et al., 2005). Liver cells (1×10^5 cells/mL) were examined using a CometAssay Reagent kit with single-cell gel electrophoresis assays (Trevigen Inc., USA), according to the method described by Singh et al., (1988), with some modifications. Cells were immobilized in agarose gels on CometAssay comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Next, slides were electrophoresed at 18 V for 30 min. The samples were stained with SYBR Green(Trevigen Inc.) for 30 min in the dark and then read using a fluorescence microscope (excitation filter 465–495 nm; Eclipse Ci, Nikon, Japan). At least 100 cells from each slide were analyzed. For quantification of comet assay results, I analyzed the tail length (distance of DNA migration from the head), percentage of DNA in tail (tail intensity/total intensity in tail), and tail moment (amount of DNA damage, product of tail length and percentage of DNA in tail) using comet assay IV image analysis software (version 4.3.2; Perceptive Instruments Ltd., UK).

2.5. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to compare differences in the data ($P < 0.05$). The values are expressed as means ± standard errors (SEs).

3. Results

3.1. Changes in HSP70 mRNA expression

HSP70 mRNA expression was increased significantly as the temperature increased in all experiment groups (Fig. 9). Additionally, the activity and expression of HSP70 in fish irradiated with green LED light were significantly lower than those in the control groups, although there were no significant differences in green LED irradiation groups exposed to 0.25 and 0.5 W/m^2 light. However, in groups irradiated with red LED light, HSP70 activity and expression were significantly increased as the light intensity increased.

3.2. Plasma AspAT/AlaAT levels

Plasma AspAT/AlaAT levels in all experiment groups were increased at high water temperatures. Moreover, plasma AspAT/AlaAT levels were significantly lower in fish exposed to green light irradiation compared with that in the control group. In contrast, plasma AspAT and AlaAT levels were significantly increased in fish exposed to red light irradiation compared with that in fish exposed to control light (Fig. 10).

3.3. Plasma cortisol and glucose levels

Plasma cortisol and glucose levels in all experiment groups were increased at high water temperatures (Fig. 11). Additionally, plasma cortisol and glucose levels were significantly lower in groups irradiated with green LED light than those in the control group, although there were no significant differences between the groups of green light at different intensities. Additionally, plasma cortisol and glucose levels were significantly higher in groups irradiated with red LED light than those in the control group.

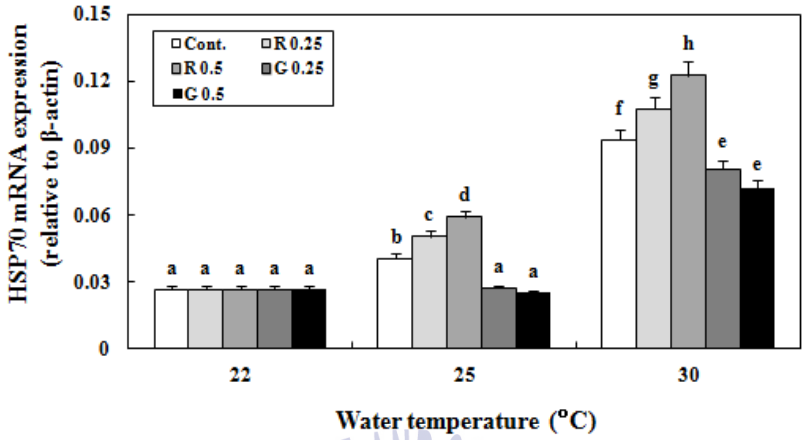


Fig. 9. Changes in HSP70 mRNA expression in rock bream. Results are shown for different water temperatures, wavelengths of green (G) and red light (R) at two light intensities (0.25 and 0.5 W/m^2), and the white fluorescent bulb (Cont.). Values with numbers are significantly different at different temperatures with in the same LED spectrum ($P < 0.05$). Lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All values are means ± SEs ($n = 5$).

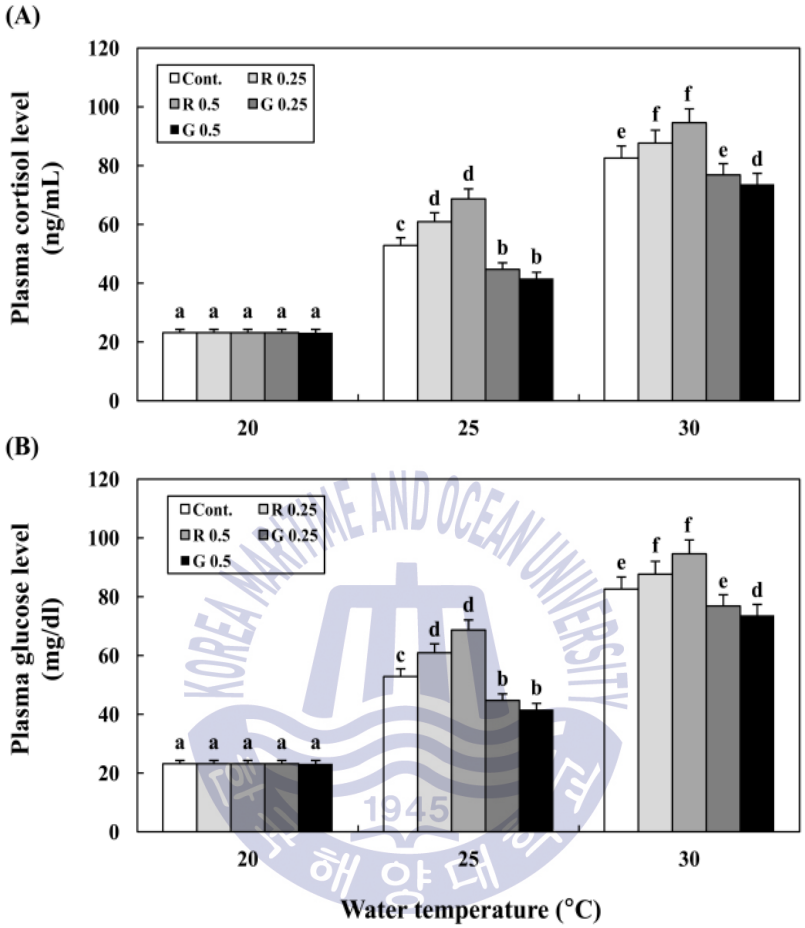


Fig. 10. The levels of cortisol (A) and glucose (B) in rock bream livers. Results are shown for different water temperatures, wavelengths of green (G) and red light (R) at two light intensities (0.25 and 0.5 W/m^2), and the white fluorescent bulb (Cont.). Values with numbers are significantly different at different temperatures with in the same LED spectrum ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All values are means ± SEs ($n = 5$).

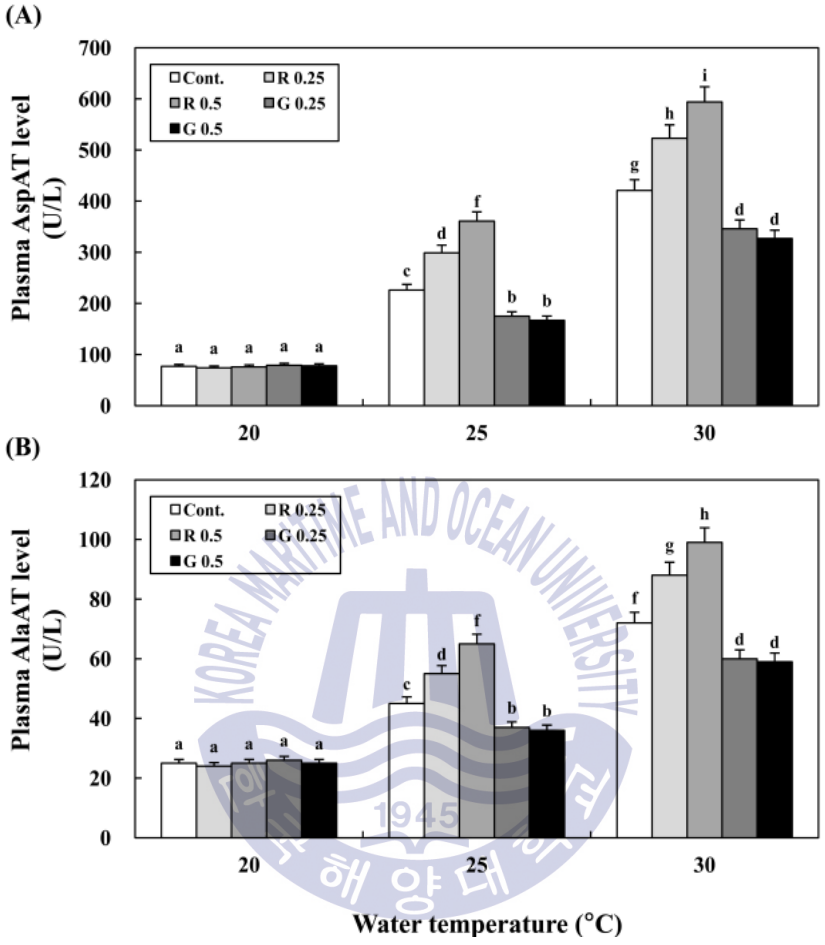


Fig. 11. The activities of plasma AspAT (A) and AlaAT (B) in rock bream. Results are shown for different water temperatures, wavelengths of green (G) and red light (R) at two light intensities (0.25 and 0.5 W/m^2), and the white fluorescent bulb (Cont.). Values with numbers are significantly different at different temperatures with in the same LED spectrum ($P < 0.05$). The lowercase letters indicate significant differences between different LED spectra within the same temperature ($P < 0.05$). All values are means ± SEs ($n = 5$).

3.4. Comet assays

A total of 100 cells were randomly chosen for analysis using a fluorescence microscope (Fig. 12), and the DNA damage response was determined by measuring the fraction of cells that had nuclear DNA comet tails (a phenomenon indicative of DNA breaks). At 20°C, the liver cells possessed normal nuclear DNA, but at 30°C, cells with damaged nuclear DNA were visible in all groups (Fig. 12A). The groups at 30°C exhibited significantly higher tail length, percentage DNA in tail, and tail moment than did the groups at 20°C (Fig. 5B). The cells of the control groups at 30°C sustained high levels of damage to their nuclear DNA and exhibited long tails, high percentages of DNA in the tail, and high tail moments. However, the high temperature group exposed to green-wavelength LED light exhibited significantly decreased tail length, percentage DNA in the tail, and tail moments, indicating that they sustained lower levels of damage to their nuclear DNA.

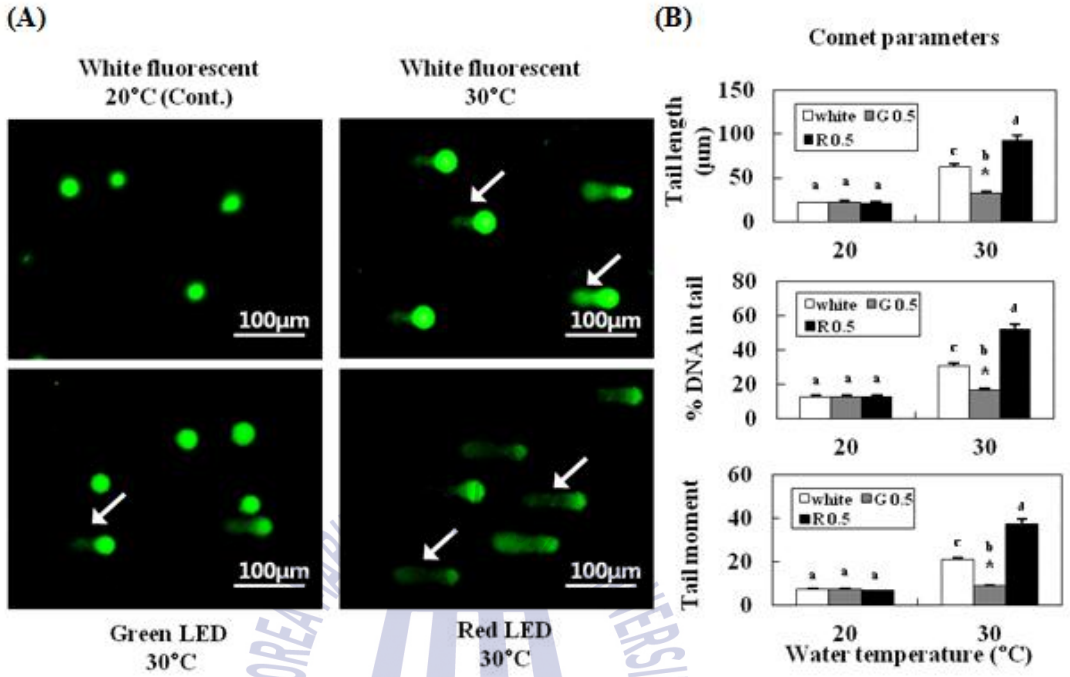


Fig. 12. Comet assay images (A) and comet assay parameters (B; tail length, percentage DNA in tail, and tail moment) during thermal changes using a white fluorescent bulb (Cont.), green light (G), and red light (R) at 0.5 W/m^2 light intensity. White arrows indicate the damaged nuclear DNA (DNA breaks) of liver cells, which were stained with SYBR-green. Scale bars = 100 μm. Lowercase letters with different characters are significantly different between different LEDs within the same temperature ($P < 0.05$). The asterisk (*) indicate the lowest value between different LEDs within the same temperature. All values are represented as means ± SEs ($n = 5$).

4. Discussion

In this study, I investigated the effects of specific wavelengths (green, 520 nm; red, 630 nm) and intensities (0.25 and 0.5 W/m^2) of light on the water temperature stress response of rock bream. The results showed that HSP70 mRNA levels increased as the water temperature increased. Moreover, HSP70 mRNA levels were significantly lower upon exposure to the green LED compared with that in the control group but significantly increased following exposure to red LED light. Thus, our findings provided important insights into the effects of environmental factors on stress responses in fish.

In a similar study, Kim et al., (2014) observed that as the temperature increased, the expression of HSP70 mRNA increased. Our results, which are consistent with the previous findings, suggested that HSP70 mRNA expression was significantly increased when rock bream was exposed to a high temperature environment and that green LED light effectively reduced the expression of HSP70 mRNA, regardless of the intensity of light. However, red LED light was found to increase the expression of HSP70 mRNA.

In this study, I also investigated changes in cortisol and glucose concentrations, which are controlled by the synthesis and release of HPI axis activity, in order to investigate the stress response induced by the high temperature environment. Our results showed that cortisol and glucose concentrations differed according to light source, with significant increases upon exposure to green LED light compared with that in the control group. However, cortisol and glucose concentrations were decreased following exposure to red light. In a similar study, Ming et al., (2012) showed that when wuchang bream *Megalobrama amblycephala* are exposed to a high temperature environment (34°C), the cortisol and glucose concentrations are increased significantly as the water temperature increases. Additionally, Kim et al., (2014) showed that when goldfish are exposed to high temperatures, cortisol and

glucose concentrations are increased significantly as water temperature increases. Moreover, cortisol and glucose concentrations in fish exposed to the green LED light are significantly lower when compared with those in groups exposed to other types of light (fluorescent bulbs and green, blue, red, and purple LEDs). Consistent with these findings, our results showed that the cortisol and glucose concentration were significantly increased when rock bream were exposed to a high-temperature environment, although the green LED light effectively reduced the stress generated in the fish body.

Notably, I found that AspAT and AlaAT concentrations were increased as the water temperature increased. Additionally, regardless of light intensity, AspAT and AlaAT concentrations were significantly lower following exposure to the green LED light compared with that in the control group; in contrast, AspAT and AlaAT concentrations increased with the intensity of red light. In a similar study, Ciereszko et al., (1998) reported that when rainbow trout are exposed to high temperatures (up to 40°C), AspAT and AlaAT concentrations are increased significantly as the water temperature increases. Moreover, Kim et al., (2014) reported that when goldfish are exposed to high temperatures, AspAT and AlaAT concentrations are increased significantly as the water temperature increases. Our results also showed that the green LED light reduced oxidative stress and decreased AspAT and AlaAT concentrations in the plasma, even for the lower light intensity (0.25 W/m^2).

In this study, I performed comet assays to measure the degree of nuclear DNA damage in the liver cells of rock bream following exposure to high water temperatures. Notably, our results showed that high water temperatures caused nuclear DNA damage in liver cells. However, exposure to green LED light significantly decreased nuclear DNA damage in rock bream liver cells. In a similar study, Jung et al., (2016) reported nuclear DNA damage in liver cells of goldfish

exposed to high temperatures. Additionally, Kim et al., (2016) reported that when flatfish are exposed to high temperatures (25 and 30°C), the nuclear DNA damage in cells is increased significantly as the water temperature increases. Taken together, these findings suggested that green LED light effectively reduced nuclear DNA damage in liver cells and repressed cellular damage caused by high water temperatures.

In conclusion, the results of this study suggested that the green LED light reduced environmental stress and nuclear DNA damage in rock bream and effectively mitigated water temperature stress, even when used at a low intensity (0.25 W/m^2). In contrast, the red LED light caused stress and nuclear DNA damage, with increased alterations observed as the intensity of light increased. Further studies are required to assess the physiological effects of light wavelength and intensity in fish of various species. Our results may have applications in other basic studies of the effects of light on stress responses in fish.

Chapter 4.

General Discussion

The present study was exposed rock bream *Oplegnathus fasciatus* to high water temperatures and evaluated the effect of green-wavelength LED irradiation on stress, immunity, apoptosis and nuclear DNA damage.

I. To examine changes in oxidative stress in rock bream in response to the treatment, I evaluated the mRNA expression and activity of the antioxidant enzymes SOD, CAT, and GPX and plasma H_2O_2 and melatonin and caspase-3 concentration. Furthermore, the damage to the cell due to the induction of free radicals by highly thermal conditions was confirmed by using a TUNEL assay. Additionally, I established that the expression and activity of SOD, CAT, and GPX were significantly higher after exposure to high temperatures and significantly lower after exposure to the green-wavelength LED light. The plasma H_2O_2 concentrations and caspase-3 mRNA expression followed the pattern outlined above. Plasma melatonin was significantly lower after exposure to high temperatures and significantly higher after exposure to the green-wavelength LED light. This study observed damaged cell by using the TUNEL assay and found that cell damage incurred a great deal of damage as a result of exposure to a high temperatures; however, the treatment with the green-wavelength LED light significantly prevented the occurrence of such damage. These results indicate that acute changes in water temperature induce oxidative stress and reduce the cells' antioxidant capacity and immunity. However, exposure to the green-wavelength LED light effectively controlled (or reduced) the oxidative stress induced by high water temperatures. Therefore, I have determined that irradiation with the green-wavelength LED light will have sufficient antioxidant and immunity-enhancing effects.

II. To evaluate the effects of green-wavelength LED light on thermal stress in rock bream, I measured by using stress indicators; HSP70 mRNA expression, and plasma cortisol, glucose, aspartate aminotransferase (AspAT), and alanine aminotransferase (AlaAT) levels. This study also confirmed the changes in DNA damage in rock bream by comet assays. The result suggest that the HSP70 mRNA expression significantly increased in response to thermal stress; however, their levels significantly decreased after the green-wavelength LED irradiation. In addition, while high water temperature acted as an stress factor and led to an increase in plasma cortisol, glucose, AspAT, and AlaAT levels, the green-wavelength LED irradiation decreased these levels. While the DNA damage was significantly higher after exposure to high temperatures, they were significantly lower after exposure to the green-wavelength LED light treatment. Taken together, these findings suggest that physiological ability of rock bream reduces after stress caused by the exposure to high water temperature; however, the green-wavelength LED irradiation can both reduce the physiological stress and DNA Damage of rock bream.

In conclusion, the present study showed that high water temperature can lead to oxidative stress in goldfish due to the production of reactive oxygen species (ROS); however, the present study observed that the green-wavelength LED irradiation have positive stress-reducing and immunity-enhancing effects. Furthermore, the effect of green-wavelength LED gradually declined apoptosis and DNA Damage. Therefore, it can be concluded that the green-wavelength LED light irradiation will have sufficient physiological stress-reducing and immunity-enhancing effects.

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먼저, 학부시절부터 전공적인 지식을 가르쳐 주시고 부족한 저에게 많은 조언, 관심, 칭찬을 해준 지도교수님이신 최철영 교수님께 진심으로 감사를 드립니다. 그리고 대학원 과정 동안 제게 전공분야에 대하여 폭 넓게 배움의 기회를 주신 박인석 교수님, 조성환 교수님, 그리고 바쁘신 와중에도 논문 심사를 맡아주신 롯데아쿠아리움 고정락 관장님, 한국해양과학기술원 최영웅 박사님께도 감사의 말씀을 드립니다. 연구실 생활에 잘 적응할 수 있게 도와주시고, 실험과 논문 작성을 하면서 큰 힘이 되어주신 지용이형, 진아누나 그리고 실험실의 활력소이신 태호형에게 감사의 뜻을 전합니다. 그리고 학부 때부터 함께 고생한 한석이, 든든하게 도와주는 희린이, 성학이, 선우, 의정이, 수민이, 정현이 및 논문조인 미진이, 지연이, 태은이 3학년 학부생들에게 모두에게 고마움을 전합니다. 항상 응원의 말로 힘을 전해주는 상현, 현준, 지훈, 혜련, 다정, 설혜, 그리고 마지막으로 뒤에서 큰 힘이 되는 우리 가족인 아버지, 어머니 그리고 동생 종혁이에게 정말 감사하고 사랑합니다.

논문이 완성되기까지 도와주신 모든 분들께 진심어린 감사의 말씀을 올리며, 많이 부족한 현재 모습이지만 앞으로 보다 노력하는 모습으로 부족한 부분을 하나씩 하나씩 채워가고 보완하는 그런 사람이 되겠습니다. 감사합니다.

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