Thesis for the Degree of Master of Science

Effect of Green-Wavelength LED Light on the Stress and Immunity in the Goldfish, *Carassius auratus* during Thermal Stress

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by

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List of Abbreviations

ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic acid
CAT	catalase
Ct	threshold cycle
ELISA	enzyme-linked immunosorbent assay
GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HPI	hypothalamic-pituitary-interrenal
HPT	hypothalamic-pituitary-thyroid
IgM	immunoglobulin M
LED	light-emitting diode
LPO	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
T_3	triiodothyronine
T_4	thyroxine
TBS	tris-buffered saline
TH	thyroid hormone
TR	thyroid hormone receptor
ZT	Zeitgeber time

고수온 환경에 노출된 금붕어, *Carassius auratus*의 스트레스 및 면역력 조절에 미치는 녹색 발광다이오드(LED) 파장의 효과

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요 약

본 연구는 고수온 환경에 금붕어, *Carassius auratus*를 노출시킨 후, 녹색 LED 파장의 조사만으로도 금붕어 체내에서 유발되는 스트레스를 감소시키고 면역력을 증강시킬 수 있는 지를 확인하기 위하여 수행되었다. 또한, 스트레스 감소 및 면역력 증강 효과를 갖고 있는 멜라토닌을 처리한 실험구와 녹색 LED 파장만을 조사한 실험구의 금붕어를 대상으로 다양한 생리학적 변화 및 차이를 비교/조사하였다.

1. 고수온 환경 변화에 노출된 금붕어의 항산화 조절 시스템에 미치는 녹색 LED 파장의 효과

고수온 환경에 노출된 금붕어에 멜라토닌 처리 및 녹색 LED 파장을 조사한 후, 금붕어의 항산화력 및 면역력 변화를 비교/확인하기 위하여 항산화효소인 superoxide dismutase (SOD)와 catalase (CAT) 활성, mRNA 및 단백질 발현량 변화를 관찰하였다. 또한, 금붕어 체내에서 산화스트레스 변동 수준을 확인하기 위하여 혈장 내 hydrogen peroxide (H_2O_2) 및 lipid peroxidation (LPO) 농도를 분석하였고, 고수온 환경으로부터 유도된 활성산소로 인한 세포 내 핵 DNA의 손상 정도는 comet assay 방법으로 관찰하였다.

그 결과, 고수온(30 ℃)에 노출된 금붕어에서는 SOD와 CAT 활성, mRNA 및 단백질 발현량이 유의적으로 증가하였지만, 멜라토닌 처리 및 녹색 LED 파장을 조사한 금붕어에서는 SOD와 CAT 활성, mRNA 및 단백질 발현량이 유의적으로 감소하는 경향이 관찰되었다. 또한, 고수온 환경으로부터 유도된 산화스트레스로 인하여 금붕어의 혈장 내 H_2O_2와 LPO 농도는 유의적으로 증가하였지만, 멜라토닌을 처리하거나 녹색 LED 파장을 조사한 금붕어에서는 혈장 내 H_2O_2와 LPO 농도가 유의적으로 감소하는 경향이 관찰되었다. 혈장 내 lysozyme의 활성 수준을 분석한 결과, 고수온 실험구의 금붕어에서는 lysozyme의 활성 수준이 유의적으로 감소하였으나, 멜라토닌을 처리한 실험구와 녹색 LED 파장을 조사한 실험구의 금붕어에서는 lysozyme의 활성 수준이 유의적으로 증가하였다. 또한, 고수온 스트레스로 인한 세포 내 핵 DNA의 손상 정도를 comet assay 방법으로 확인한 결과, 고수온 환경에 의해 유도된 산화스트레스는 금붕어의 간세포 내 핵 DNA를 손상시켰으나, 멜라토닌을 처리하거나 녹색 LED 파장을 조사함으로써 핵 DNA의 손상 정도가 감소된 것으로 나타났다.

따라서 고수온 환경에 노출로 인하여 금붕어 체내에서는 산화스트레스가 유도되었지만, 멜라토닌 처리 및 녹색 LED 파장의 조사는 고수온 환경에서 유도된 산화스트레스를 감소시키고 면역력을 증강시키는 효과가 있는 것으로 사료된다. 특히, 녹색 LED 파장의 조사만으로도 멜라토닌을 처리한 경우와 비슷한 효과를 보인 점으로 보아, 녹색 LED 파장의 조사만으로도 항산화력 및 면역력 증강 효과를 충분히 발휘할 수 있는 것으로 사료된다.

2. 고수온 환경 변화에 노출된 금붕어의 스트레스 및 면역력에 미치는 녹색 LED 파장의 효과

고수온 환경에 노출된 금붕어에 멜라토닌 처리 및 녹색 LED 파장을 조사한 후, 금붕어의 스트레스 및 면역력 변화를 비교/확인하기 위하여 갑상선호르몬인 triiodothyronine (T_3)과 thyroxine (T_4) 농도 및 갑상선호르몬 수용체(thyroid hormone receptor; TR) mRNA의 발현량 변화를 관찰하였다. 또한, 실험구별로 금붕어가 감지하는 스트레스 정도의 차이를 확인하기 위하여 혈장 내 cortisol과 glucose 농도를 분석하였고, 고수온 환경에 노출된 금붕어의 면역력 차이 여부는 lysozyme과 immunoglobulin M (IgM)의 변화를 통하여 분석하였다.

그 결과, 고수온(30 °C)에 노출된 금붕어의 혈장 내 T_3와 T_4 농도 및 TR mRNA의 발현량은 대조구(22 °C)의 금붕어에 비하여 유의적으로 증가하는 경향이 관찰되었다. 그러나 고수온 스트레스 환경에 노출되었음에도 불구하고 멜라토닌을 처리한 실험구 및 녹색 LED 파장을 조사한 실험구의 금붕어에서는 혈장 내 T_3와 T_4 농도 및 TR mRNA의 발현량이 유의적으로 감소하는 경향이 관찰되었다. 또한, 고수온 환경은 금붕어에게 스트레스 요인으로 작용하여 혈장 내 cortisol과 glucose 농도가 증가하는 경향이 관찰된 반면, 멜라토닌 처리 및 녹색 LED 파장을 조사한 실험구에서는 혈장 내 cortisol과 glucose 농도가 유의적으로 감소하는 경향이 관찰되었다. 특히, 멜라토닌을 처리한 실험구에서는 처리 시간이 경과될수록 인위적으로 주입된 멜라토닌의 약효(스트레스를 감소시키는 능력)는 감소된 반면, 녹색 LED 파장을 조사한 실험구에서는 실험기간 동안 지속적으로 스트레스가 감소하고 있음을 확인할 수 있었다. 고수온 스트레스 환경으로 인하여 면역 인자인 lysozyme mRNA 발현과 IgM 활성 및 단백질 발현은 유의적으로 감소하였지만, 멜라토닌을 처리하거나 녹색 LED 파장을 조사한 경우에는 lysozyme mRNA 발현과 IgM 활성 및 단백질 발현이 유의적으로 증가하는 경향이 관찰되었다.

따라서 고수온 환경에 금붕어를 노출시킨 경우, 금붕어 체내에서는 스트레스가 유도됨과 동시에 체내 면역력이 저하되었으나, 멜라토닌의 처리 및 녹색 LED 파장의 조사는 고수온 스트레스 환경으로부터 유도된 스트레스를 감소시키고 면역력을 증강시키는데 효과가 있는 것으로 사료되었다. 또한, 스트레스 감소 및 면역력 증강과 관련하여 금붕어에 인위적인 멜라토닌 처리는 처리 후 시간이 경과할수록 그 약효는 감소되었지만, 녹색 LED 파장의 조사는 멜라토닌 처리에 비하여 스트레스 감소 및 면역력 증강 효과도 탁월할 뿐만 아니라, 녹색 LED 파장에 노출되어 있는 한 지속적으로 그 효과를 유지시킬 수 있는 것으로 판단된다.

Chapter 1.

General Introduction

Water temperature is an important environmental factor, as it plays a key role in their physiological responses, metabolic rate, and distribution (Díaz et al., 2007). According to recent studies, due to global warming, water temperature in many oceans is expected to increase (Harvell et al., 2002; Noyes et al. 2009). The increasing water temperature can induce oxidative stress, and an increased reactive oxygen species (ROS) generation in fish (Cheng et al., 2015). ROS including superoxide (O_2^-), hydrogenperoxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$), can lead to the increase of the levels of lipid hydro eroxide (LPO) and negatively affect cell viability by causing membrane damage and enzyme activity (Roch, 1999; Nordberg et al., 2001). In addition, ROS can break the double strand of DNA, potentially leading to cell death, mutagenesis, and carcinogenesis (Pizarro et al., 2009).

The organisms have various antioxidant defense systems to protect themselves from oxidative stress (Bagnyukova et al., 2007). The defense system uses enzymes with antioxidant effects, such as, among others superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Freeman and Crapo, 1982; Rodriguez et al., 2004). The basic mechanisms of these antioxidant enzymes are as follows: SOD converts O_2^- into H_2O_2, and then CAT and GPX convert the produced toxic H_2O_2 into water and molecular oxygen (O_2), thus eliminating the toxic effects (Rama and Manjabhat, 2014).

Water temperature changes affect several hormones, including thyroid hormones (THs). THs play a crucial role in the growth and development of fish (Power et al., 2001). Two types of THs, triiodothyronine (T_3) and thyroxine (T_4), are released from the hypothalamic–pituitary–thyroid axis (HPT-axis; Peter, 2011) and their biological actions are mediated via the thyroid hormone receptors (TRs)—TRα and TRβ (Yaoita and Brown, 1990).

Cortisol is known as the stress hormone and is released from the hypothalamic–pituitary–interrenal axis (HPI axis). Cortisol secretion promotes gluconeogenesis and increases glucose levels (Begg et al., 2004).

The immune system is the physical defense against infection that acts by recognizing and attacking pathogens and helps to maintain homeostasis and health (Tort et al., 2004; Magnadóttir, 2006). Lysozyme is a particularly important indicator of the immune system function that helps to protect an organism against the invasion by pathogens and helps to mitigate the damage from stress (Saurabh et al., 2008; Shin et al., 2014). Humoral adaptive immunity in fish is mediated by immunoglobulins; the immunoglobulin M (IgM) class is the primary immune response in most teleost fish (Bag et al., 2009).

Melatonin is primarily secreted in the pineal organ during darkness and plays a crucial role in seasonal and circadian rhythms, physiology, and behavior in most vertebrates, including fish (Falcón et al., 2010). Several studies have demonstrated that melatonin is a strong antioxidant and a direct scavenger of internally generated ROS (Tan et al., 2000; Reiter et al., 2002).

As suggested in several recent studies, specific wavelengths of light can affect stress levels and physiological responses in fish (Shin et al., 2011; Kim et al., 2014). Light-emitting diodes (LEDs) manufactured to output specific wavelengths are effectively used in the fish farming industry (Villamizar et al., 2009; Choi et al., 2012). In particular, green-wavelength LEDs have been shown to inhibit stress in fish exposed tohigh water temperatures (Choi et al., 2014).

The present study was exposed goldfish, *Carassius auratus*, to high water temperatures and evaluated the effect of green-wavelength LED irradiation on their stress levels and immunity system. In addition, the effect of green-wavelength LED irradiation was compared to that of melatonin.

Chapter 2.

Effects of melatonin injection or green-wavelength LED light on the antioxidant system in the goldfish, *Carassius auratus* during thermal stress

Abstract

This study tested the mitigating effects of melatonin injections or irradiation from green-wavelength light-emitting diodes (LEDs) on goldfish, *Carassius auratus* exposed to thermal stress (high water temperature, 30°C). The effects of the two treatments were assessed by measuring the expression and activity levels of the antioxidant enzymes superoxide dismutase and catalase, plasma hydrogen peroxide, lipid hydroperoxide, and lysozyme. In addition, a comet assay was conducted to confirm that high water temperature damaged nuclear DNA. The expression and activity of the antioxidant enzymes, plasma hydrogen peroxide, and lipid hydroperoxide were significantly higher after exposure to high temperature and were significantly lower in fish that received melatonin or LED light than in those that received no mitigating treatment. Plasma lysozyme was significantly lower after exposure to high temperature and was significantly higher after exposure to melatonin or LED light. The comet assay revealed that thermal stress caused a great deal of damage to nuclear DNA; however, treatment with melatonin or green-wavelength LED light prevented a significant portion of this damage from occurring. These results indicate that, although high temperatures induce oxidative stress and reduce immune system strength in goldfish, both melatonin and green-wavelength LED light inhibit oxidative stress and boost the immune system. LED treatment increased the antioxidant and immune system activity more significantly than did melatonin treatment.

1. Introduction

Recent studies have examined the impact of global warming on the global ecosystem (Walther et al., 2002; Pandit et al., 2010; Foucreau et al., 2014). The Intergovernmental Panel on Climate Change (IPCC) reported that the global temperature has increased by an average 0.85°C (0.65°C–1.06°C) over the last 133 years, between 1880 and 2012 (IPCC, 2014). An increase in global temperature also increases water temperature, which, in turn, affects the physiology of fish, including their growth, propagation, metabolism, osmoregulation (Logue et al., 1995), and immune system function (Bowden, 2008). In addition, increasing water temperature could increase reactive oxygen species (ROS) generation, thereby inducing oxidative stress (Cheng et al., 2015). ROS, including superoxide (O_2^-), hydrogenperoxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$), lead to increased levels of lipid hydro eroxide (LPO) and negatively affect cell viability by causing membrane damage and enzymein activity (Nordberg et al., 2001; Roch, 1999). In addition, ROS can break the double strand of DNA, which may play a role in cell death, mutagenesis, and carcinogenesis (Pizarro et al., 2009). Organisms protect themselves from oxidative stress by operating an antioxidant defense system that reduces cell damage caused by ROS (Bagnyukova et al., 2007). The defense system uses enzymes that have antioxidant effects, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-*S*-transferase (GST) (Freeman and Crapo, 1982; Rodriguez et al., 2004). The first step of ROS elimination is performed by SOD, which converts intracellular O_2^- into H_2O_2 and molecular oxygen (O_2). Then, CAT breaks down H_2O_2, thus reducing its toxic effect (Rama and Manjabhat, 2014). Antioxidant activity is known to increase in the liver, kidneys, intestines, and gills, as well as increasing by small amounts in other fish tissues, due to exposure to stress (Basha and Rani, 2003; Huculeci et al., 2009).

In addition, water temperature may affect immune system function (Cnaani, 2006). The immune system is the physical defense against infection that acts by recognizing

and attacking pathogens and helping to maintain homeostasis and health (Tort et al., 2004; Magnadóttir, 2006). In particular, lysozyme is an important indicator of immune system function that helps to protect against invasion by pathogens and to mitigate the damage from stress (Saurabh et al., 2008; Shin et al., 2014).

Melatonin is a hormone that is primarily secreted by the pineal organ and that plays a crucial role in the regulation of the seasonal and circadian rhythms of physiology and behavior in most vertebrates, including fish. It is involved in feeding, reproduction, and stress responses (Falcón et al., 2010). Recently, several studies have shown that melatonin is a direct scavenger of free radicals and a highly effective antioxidant (Reiter et al., 2000; Tan et al., 2000). Melatonin also increased the expression and activity of antioxidant such as SOD, CAT, and GPX and removed internally generated ROS (Reiter et al., 2002). Therefore, it augments antioxidant defenses (Antolín et al., 1996).

Light-emitting diodes (LEDs) manufactured to output specific wavelengths are used effectively in the fish farming industry (Villamizar et al., 2009; Shin et al., 2011; Choi et al., 2012). Red wavelengths of LED light negatively affected the physiological function of fish and induced oxidative stress, while green wavelengths increased the antioxidant capacity of the fish, in part by increasing the production of antioxidants (Shin et al., 2011). Another study suggested that it may be possible to control oxidative stress in fish via treatment with specific-wavelength LEDs, because green-wavelength LEDs effectively inhibit thermally induced oxidative stress and help protect fish from the effects of harmful ROS (Kim et al., 2014).

Although separate studies have reported that green wavelength LEDs inhibit oxidative stress and that melatonin, which is a strong antioxidant, scavenges free radicals (Choi et al., 2012; Sreejith et al., 2007), to date no studies have compared these two treatments on the antioxidant capacity of fish. The present study examined the antioxidant and immune system activity of goldfish, *Carassius auratus* that were exposed to high levels of thermal stress. The levels and expression of antioxidant enzymes and immune response were compared for melatonin treatment and irradiation with green-wavelength LED.

The present study evaluated mRNA expression, concentrations, and activity of the antioxidant enzymes SOD and CAT, as well as the changes in oxidative stress in goldfish by measuring plasma H_2O_2 and LPO concentration. The present study also confirmed via a comet assay that high thermal conditions induced free radicals that damaged the nuclear DNA.

2. Materials and methods

2.1. Experimental fish and melatonin injections

Goldfish (n = 480, length 12.0 ± 0.4 cm; mass 15.1 ± 0.5 g) were purchased from a commercial fish farm (Busan, Korea) and maintained in eight 100 L circulation filter tanks prior to the experiments, which were conducted in a laboratory. During the experiments, the fish were randomly divided into three groups: a control, a group that received melatonin injections, and a group that was exposed to green-wavelength LED light. The control and melatonin injection groups were exposed to light produced by a white fluorescent bulb, while the green-wavelength LED group was exposed to light produced by a green wavelength (530 nm) LED (Daesin LED Co., Kyunggi, Korea). The light sources were placed 50 cm above the water's surface, and the irradiance at the water's surface was maintained at approximately 4.5 μmol/m^2/s. The photoperiod consisted of a 12 h light (L):12 h dark (D) cycle, intended to match natural conditions; lights were turned on at 07:00 and turned off at 19:00. The goldfish were reared using an automatic temperature regulation system (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatize to the conditions for 24 h. The water temperature was then increased from 22°C to 30°C in daily increments of 2°C. The fish received commercial feed once per day until the day prior to sampling. Sampling was performed at the experimental temperatures (22 and 30°C) and the experiments were started at 07:00. We sampled at the following Zeitgeber time (ZT) intervals: ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24.

The fish were anesthetized with tricaine methanesulfonate (MS-222; Sigma, USA) prior to receiving the melatonin injection. The melatonin (Sigma, USA) was dissolved in 0.9% physiological saline, and each fish was injected with either 5 or 10 μg melatonin/g body mass, in a volume of 10 μL/g body mass. After injection, fish were reared at the temperatures of 22°C or 30°C during the experimental period for 24 h. All fish were anesthetized using tricaine methanesulfonate (Sigma) and 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*, 5 min) and stored at -80 °C until analysis.

2.2. Total RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted from each sample using TRI Reagent® (Molecular Research Center, Inc., USA), according to the manufacturer's instructions. Then, a 2 μg sample of total RNA was reverse transcribed in a total reaction volume of 20 μL, using an oligo-$(dT)_{15}$ anchor and M-MLV reverse transcriptase (Promega,USA), according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in PCR and real-time qPCR analysis.

The qPCR analysis was conducted to determine the relative expression levels of the antioxidant enzymes SOD and CAT using total RNA extracted from the livers of goldfish. The primers for qPCR were designed using known goldfish sequences (Table 1). I conducted qPCR amplification 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), according to the manufacturer's instructions. β -actin was also amplified as a control for each sample, and all data were expressed as the change with respect to the corresponding calculated β-actin threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was 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) of each sample and the internal control (β-actin) were calculated [$\Delta\Delta Ct = 2^{\wedge}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. After PCR was completed, the qPCR data from three replicate samples were analyzed using Bio-Rad to estimate the transcript copy numbers of each sample.

Table 1. Primers used for qPCR amplification

Genes (accession no.)	Primer	DNA sequences
SOD (**JQ776518**)	Forward	5′-ACA ACC CTC ATA ATC AAA CTC A-3′
	Reverse	5′-GCA ACA CCA TCT TTA TCA GC-3′
CAT (**JQ776513**)	Forward	5′-ATC TTA CAG GAA ACA ACA CCC-3′
	Reverse	5′-CGA TTC AGG ACG CAA ACT-3′
β-actin (**AB039726**)	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG -3′

2.3. SOD and CAT activity analysis

The liver tissues were homogenized in 1× PBS. The homogenates were centrifuged at 5,000 ×*g* for 5 min at 4 °C. The supernatant was used for the analyses. SOD and CAT activity were determined using the fish SOD ELISA kit (CSB-E15929fh, Cusabio Biotech Co., Ltd., China) and the fish catalase ELISA kit (CSB-E15928fh, Cusabio Biotech Co., Ltd., China).

2.4. Western blot analysis

The total protein isolated from the livers of goldfish 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 30 μg protein was loaded per lane onto Mini-PROTEAN®TGX™Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used as a reference. Samples were electrophoresed at 180V, and the gels were immediately transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Next, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min, after which they were washed in TBS. Membranes were incubated with SOD (1:2,000 dilution, NBP1-47443, Novus Biologicals, USA) and CAT (1:2,000 dilution, sc-58332, Santa Cruz Biotechnology, USA) antibodies, followed by incubation with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (SOD and CAT dilution 1:2,000, Bio-Rad) for 60 min. β -Tubulin (dilution 1:5,000, ab6046, Abcam, UK) was used as the internal control. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and 30 s of exposure with a Molecular Imager® from ChemiDoc™ XRS+ Systems (Bio-Rad, Hercules, CA, USA). The membrane images were scanned using a high-resolution scanner and the band density was estimated using Image Lab™ Software, version 3.0 (Bio-Rad).

2.5. *In vitro* culture of liver cells

The goldfish liver cells culture was performed using both enzymatic and mechanical procedures. The liver tissue was quickly removed and placed in 3 mL ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride or magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; Gibco-BRL, Rockville, MD, USA). The isolated liver tissues were then transferred to 6 mL fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the liver tissues were chopped into small pieces using scissors. The liver cells and the minced liver tissue were transferred to a flask and incubated for 10 min at room temperature with slow stirring. The mixture of dispersed liver cells and tissues was filtered, and the culture medium (medium 199, Invitrogen, USA) was added. The cell suspension was centrifuged at 800 × *g* for 10 min, and the cells were then resuspended in fresh culture medium. The liver cells (1.2×10^6 cells 800 μL/well) were placed in a 24 well tissue culture plate.

For the experiments, the fish were randomly divided into three groups: control, melatonin treatment (0.1 and 1.0 μg/μL), and green-wavelength LED treatment. The control and melatonin treatment groups were exposed to light produced by a white fluorescent bulb, and the green-wavelength LED group was exposed to light produced by a green (530 nm) LED (Daesin LED Co. Kyunggi, Korea). The light sources were placed 50 cm above the water surface, and the irradiance at the surface was maintained at approximately 4.5 μmol/m^2/s. The photoperiod consisted of a 12 h light (L):12 h dark (D) cycle with the photophase lasting from 07:00 to 19:00 (lights on at 07:00, and lights off at 19:00) and sampled at ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24.

2.6. Plasma parameter analysis

Plasma samples were separated by centrifugation (4°C, 10,000 ×*g*, 5 min), and analyzed by examining the H_2O_2, LPO, and lysozyme levels.

H_2O_2 concentrations were measured using a modified version of the methods described by Nourooz-Zadeh et al. (1994), and a PeroxiDetect kit (Sigma). Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. The concentrations are expressed as nmol peroxide/mL.

LPO was quantified by measuring the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs), and hydroperoxides, according to the manufacturer's instructions (Fish Lipid Peroxide ELISA Kit, MyBioSource Inc., San Diego, CA, USA). The absorbance was read at 450 nm using a plate reader. LPO was expressed as nmol/mL.

Lysozyme levels were determined via a turbidimetric assay performed according to the method described by Ellis (1990). Briefly, 50 µL test plasma was added to 50 µL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.2). The absorbance at 450 nm was measured using a spectrophotometer, and levels were expressed as ng/mL.

2.7. Comet assay

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells (Bajpayee et al., 2005). The liver cells (1×10^5 cells/mL) of gold fish were examined using a CometAssay® Reagent kit for single-cell gel electrophoresis assay (Trevigen Inc., USA), according to the method described by Singh et al. (1988), with some modifications. Cells were immobilized in an agarose gel on CometAssay® comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Next, slides were electrophoresed at 15 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 C*i*, Nikon, Japan). At least 100 cells from each slide were analyzed at ZT8 (15:00). For a quantification analysis of the comet assay, I analyzed the tail length (distance of DNA migration from 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).

2.8. Statistical analysis

All 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 differences in the data ($P < 0.05$). The values are expressed as the mean ± standard error (SE).

3. Results

3.1. Expression and activity of antioxidant enzymes SOD and CAT

The present study investigated changes in the activity and mRNA levels of the antioxidant enzymes SOD and CAT in liver tissues of goldfish in response to high water temperature. Antioxidant mRNA levels and activity were significantly higher in fish that experienced high-water temperature (30°C) water than in the groups maintained at 22°C (Figs. 1 and 2). In addition, values were significantly lower during the scotophase than during the photophase. Levels were significantly lower in the groups that received melatonin, and were significantly lower in the group that received 10 μg melatonin/g than in the group that received 5 μg/g. The fish that were exposed to green-wavelength LED treatment also showed significant decreases in the activity and mRNA levels of SOD and CAT. In particular, at ZT12, the levels in the green-wavelength LED treatment group were significantly lower than those observed in the melatonin injection groups.

In vitro experiments that used liver cells culture revealed that SOD and CAT mRNA expression levels were significantly higher at 30°C than at 22°C. In addition, expression levels during the scotophase were significantly lower than those during the photophase. SOD and CAT mRNA expression levels fell significantly in both the melatonin treatment and the green LED spectra groups (Fig. 3). The mRNA expression levels in the 1 μg/μL melatonin treatment group were significantly lower than those in the 0.1 μg/μL melatonin treatment group. Meanwhile, mRNA expression levels in the green-wavelength LED treatment group were similar to those seen in the melatonin treatment groups.

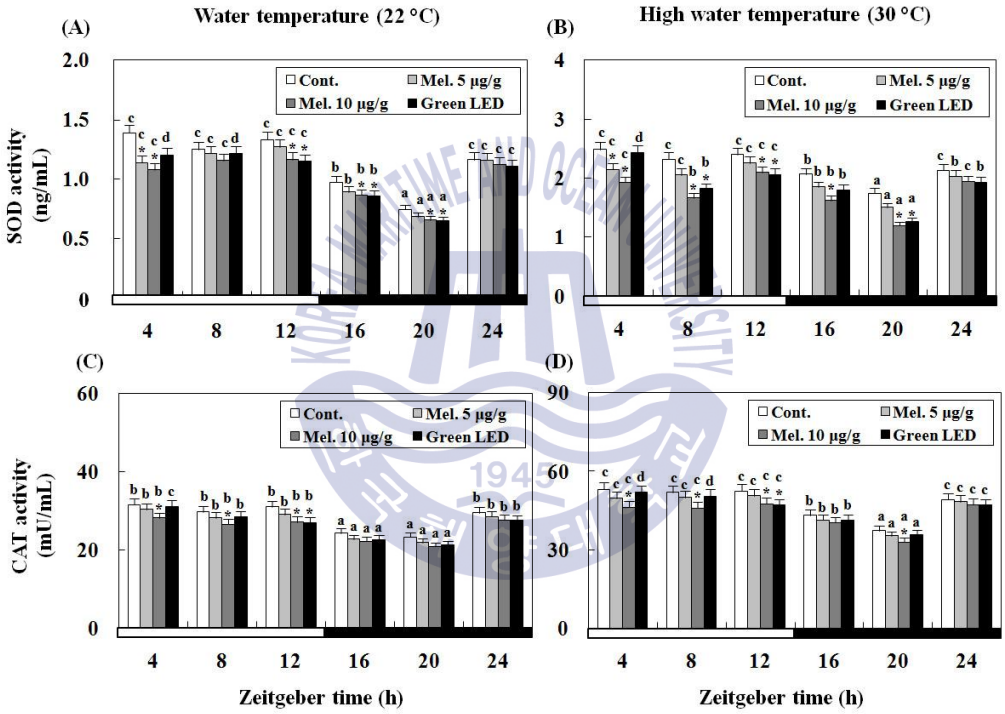

Fig. 1. Changes in SOD activity during thermal changes at 22°C (A) and 30°C (B), and CAT activity during thermal changes at 22°C (C) and 30°C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

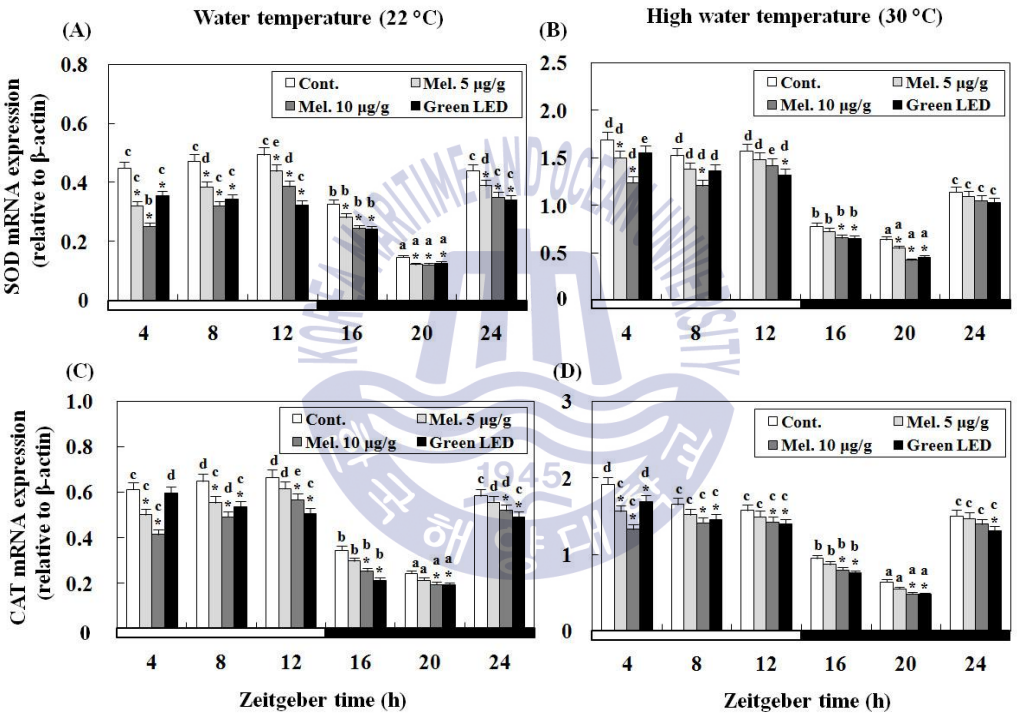

Fig. 2. Changes in SOD mRNA expression during thermal changes at 22°C (A) and 30°C (B), and CAT mRNA expression during thermal changes at 22°C (C) and 30°C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass) and green-wavelength LED light, as measured by quantitative real-time PCR (qPCR). 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. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

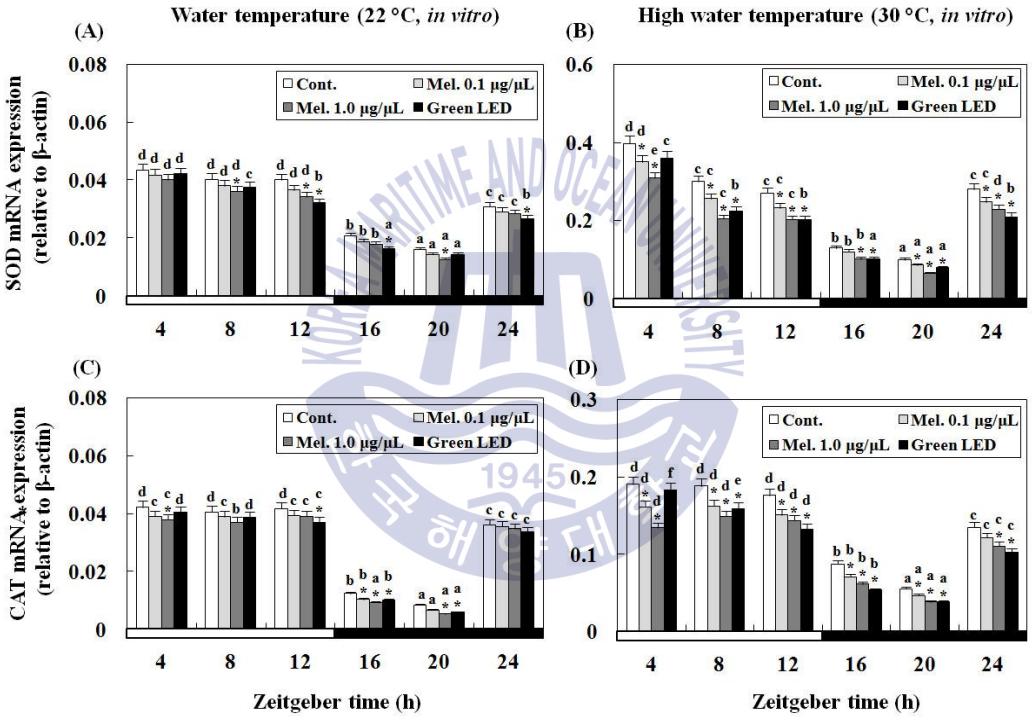

Fig. 3. Changes in SOD mRNA expression during thermal changes at 22°C (A) and 30°C (B), CAT mRNA expression during thermal changes at 22°C (C) and 30°C (D) of each experimental groups in goldfish (*in vitro*): control, melatonin treatment (0.1 and 1.0 μg/μL) and green-wavelength LED light, as measured by quantitative real-time PCR (qPCR). Results are expressed as normalized fold expression levels, relative to the β-actin levels in the same sample. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

3.2. Western blotting of antioxidant enzymes SOD and CAT

SOD and CAT protein expression levels were significantly higher in the 30°C treatment group than in the 22°C treatment group (Fig. 4A and B). In addition, expression levels during the scotophase were significantly lower than those during the photophase. SOD and CAT protein expression in both the melatonin injection and green-wavelength LED groups significantly decreased relative to the control (Fig. 4).

3.3. Plasma H_2O_2 and LPO concentrations

The plasma H_2O_2 concentrations observed in the 30°C groups were significantly higher than in the 22°C groups, although they were significantly lower during the scotophase than during the photophase (Fig. 5A and B). Plasma H_2O_2 concentrations in the melatonin injection and green-wavelength LED groups were also significantly decreased.

The variation in the plasma LPO concentrations was similar to that of the variation in the plasma H_2O_2concentration(Fig. 5C and D). The plasma LPO concentrations of the 30 °C treatment group were significantly lower than those of the 22°C group. Concentrations were significantly lower during the scotophase than during the photophase. In particular, the plasma LPO concentrations at ZT12 were significantly lower in the group irradiated by green-wavelength LEDs than in the melatonin injection groups.

3.4. Plasma lysozyme levels

The plasma lysozyme levels of the 30°C group were significantly lower than those of the 22°C group (Fig. 6). However, the levels were significantly higher in the melatonin injection and green-wavelength LED groups than in the control. In particular, after ZT12, the plasma lysozyme levels were significantly higher in the group exposed to light from green-wavelength LED light than in melatonin injection-treated groups.

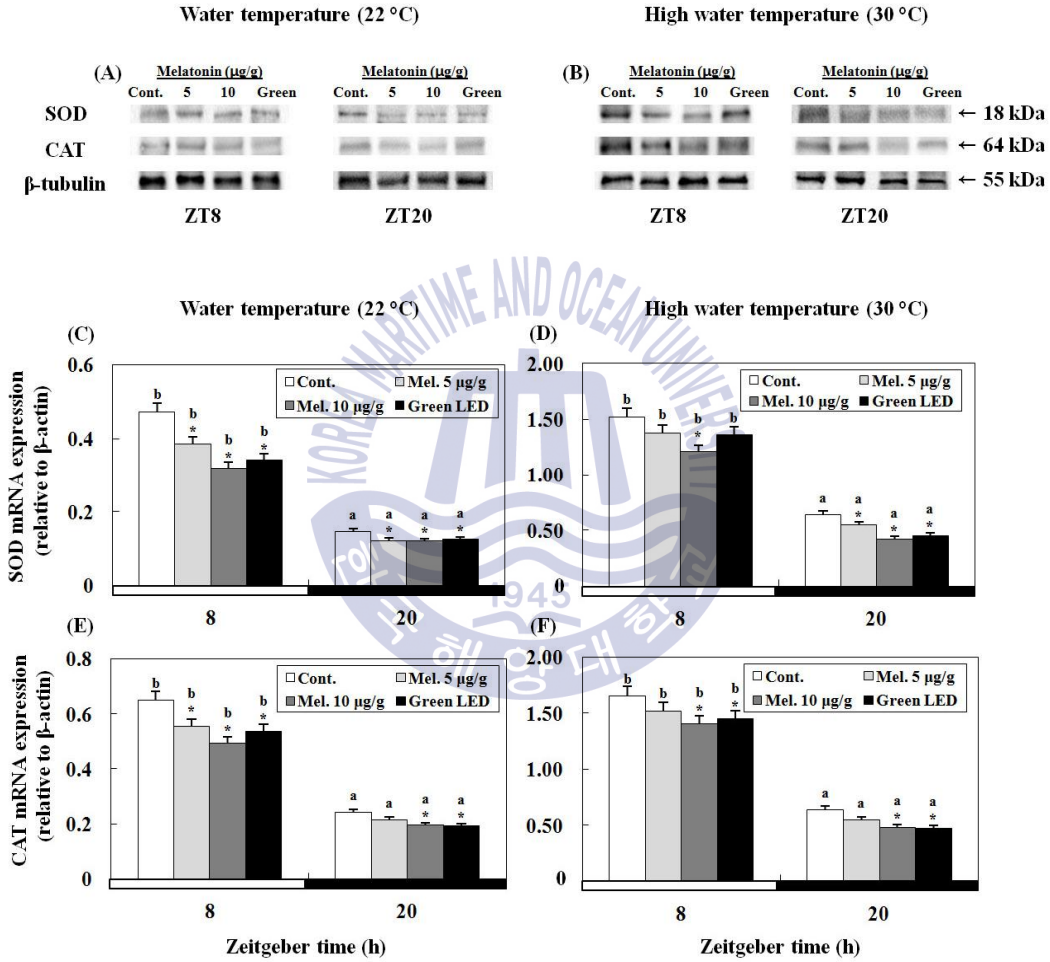

Fig. 4. Changes in SOD and CAT protein expression during thermal changes at 22°C (A) and 30°C (B), and SOD and CAT mRNA expression during thermal changes at 22°C (C and E) and 30°C (D and F), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light. Western blots of the expression of antioxidant enzymes [SOD (18 kDa) and CAT (64 kDa)] in the liver of goldfish after thermal change, and β-tubulin (55 kDa) was used as an internal control. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

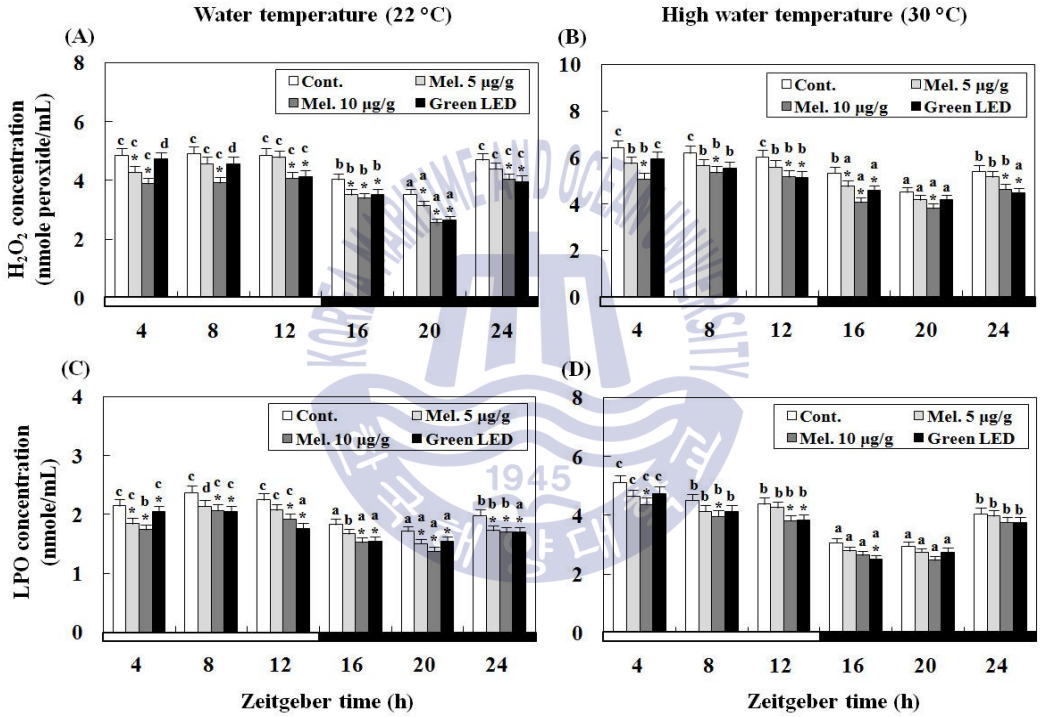

Fig. 5. Changes in plasma H_2O_2 levels during thermal changes at 22°C (A) and 30°C (B), and plasma LPO levels during thermal changes at 22°C (C) and 30°C (D), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

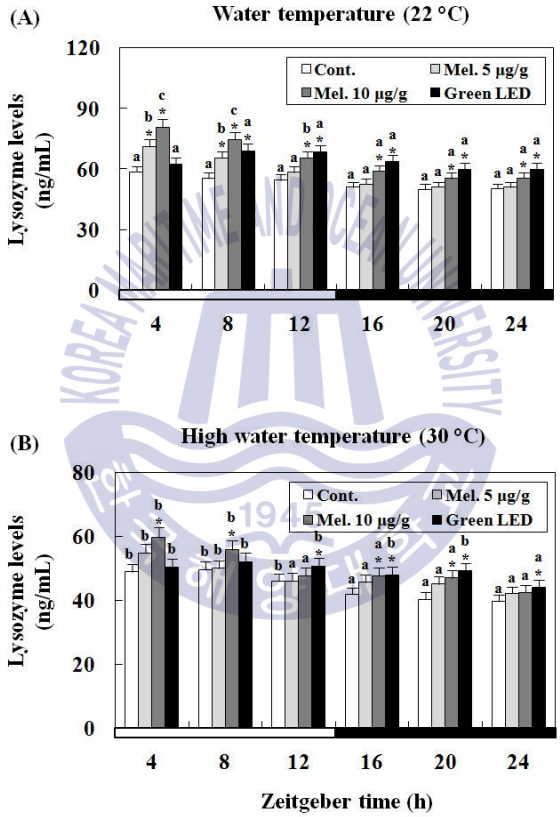

Fig. 6. Changes in plasma lysozyme levels during thermal changes at 22°C (A) and 30°C (B), in each experimental goldfish group: control, melatonin injection (5 and 10 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are displayed as the means ± SE ($n = 5$).

3.5. Comet assay

A total of 100 cells were randomly chosen for analysis at ZT8, using a fluorescence microscope (Fig. 7), 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 22°C, the liver cells possessed normal nuclear DNA, but at 30°C, cells with damaged nuclear DNA were visible in all groups (Fig. 7A). The samples exhibited significantly higher tail length, percentage DNA in tail, and tail moment at 30°C than at 22°C (Fig. 7B). 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 moment. However, the groups that received melatonin injection or were exposed to green-wavelength LED light exhibited significantly decreased tail length, percentage DNA in the tail, and tail moment, indicating that they sustained a low level of damage to their nuclear DNA. Therefore, even after exposure to 30°C, the nuclear DNA in these individuals was only slightly damaged and could be repaired.

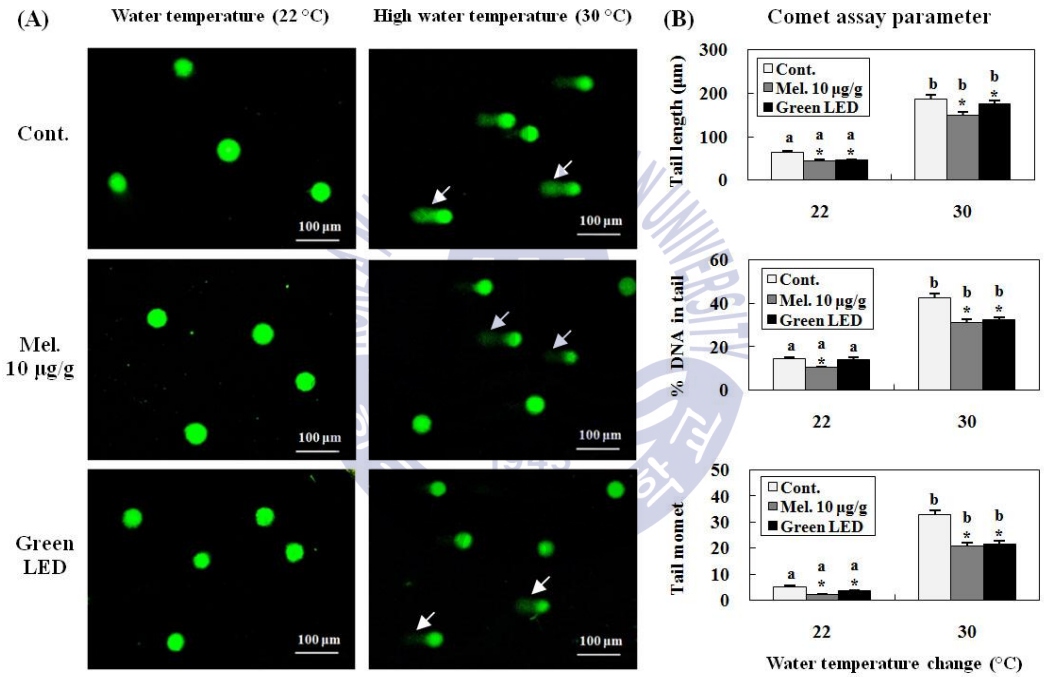

Fig. 7. Comet assay images (A), comet assay parameter for tail length, percentage DNA in tail, and tail moment (B) during thermal change at ZT8 (15:00), in each experimental goldfish group: control, 10 μg/g body mass of melatonin injection, and green-wavelength LED light, as measured by fluorescence microscopy. White arrows indicate the damaged nuclear DNA (DNA breaks) of liver cells, which are stained with SYBR-green. Scale bars = 100 μm. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatment ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$).

4. Discussion

In order to confirm that melatonin and green-wavelength LED light increase antioxidant production and promote immune function, and to compare the responses to these two treatments, this study exposed goldfish to thermal stress, analyzed the resulting changes in antioxidant enzyme and protein expression, and tracked changes in oxidative stress over time. Changes in the activity, mRNA, and protein expression of SOD and CAT indicate responses to high levels of environmental stress.

These changes were analyzed in order to confirm the effects of thermal stress on the antioxidant enzymes in the goldfish liver tissue. Experimental groups kept at 30°C demonstrated significantly higher activity, mRNA, and protein expression levels for SOD and CAT, compared with groups maintained at 22°C. However, melatonin-injected groups showed a significant decrease in the activity, mRNA, and protein expression levels for SOD and CAT relative to that of the control. In particular, groups that received 10 μg melatonin/g body mass showed significantly decreased activity, mRNA, and protein expression levels for SOD and CAT, compared with groups that received 5 μg melatonin/g body mass. The results for groups that were exposed to green-wavelength LED light were similar to those of the melatonin-injection groups.

This study demonstrated that activity levels of the antioxidant enzymes SOD and CAT were affected by thermal stress and were significantly affected during photophase on ZT8. In particular, during the photophase, activity levels in the green-wavelength LED treatment group were significantly lower on ZT12 than in the melatonin-injected groups. The results of the experiment using cultured liver cells treated with melatonin were similar to those of the *in vivo* experiment.

This results of this study are in agreement with those of Shin et al. (2011), who reported that yellowtail clownfish, *Amphiprion clarkii* injected with melatonin exhibited reduced expression levels of antioxidant enzymes; therefore, they concluded that melatonin acts as a strong antioxidant by scavenging ROS. In

addition, the antioxidant enzyme activities of liver cells in climbing perch, *Anabas testudineus* were significantly lower in melatonin-treated groups than in untreated groups, under a 24-h photoperiod (Sreejith et al., 2007). Thus, melatonin was determined to be a strong antioxidant that effectively protected the cell membrane and scavenged for internally generated free radicals (Reiter et al., 1997). Therefore, the results of this study are similar to those of previous studies, which reported that green-wavelength LED light can effectively decrease oxidative stress and that melatonin, which is mainly secreted during the scotophase, acts as a powerful antioxidant.

In order to confirm that oxidative stress was experienced by goldfish subjected to high temperatures, we analyzed the plasma H_2O_2 levels and the LPO concentration of the fish. Groups exposed to 30°C showed exhibited significantly higher plasma H_2O_2 levels and LPO concentrations than did groups maintained at 22°C. However, H_2O_2 levels and LPO concentrations were significantly lower in melatonin-injected groups. Fish that were exposed to green-wavelength LED treatment exhibited results similar to those of melatonin-injected fish.

The results of this study are similar to those of previous study in which Nakano et al. (2014) reported that oxidative stress, thus the level of plasma LPO, was increased when coho salmon, *Oncorhynchus kisutch* were exposed to thermal stress. According to Choi et al. (2012), the results showed that oxidative stress was induced in starved cinnamon clownfish, *Amphiprion melanopus*; nevertheless, in the present study, the groups exposed to green-wavelength LED light exhibited significantly reduced levels of plasma LPO and lower H_2O_2 concentrations. Gülcin et al. (2009) reported that after rainbow trout, *Oncorhynchus mykiss* received a melatonin injection, their LPO levels decreased significantly over time. Thus, melatonin plays a role in reducing LPO levels. The present study confirmed the reports of previous studies that green-wavelength LED light is effective at preventing cell damage due to thermal stress and can protect fish in the absence of melatonin treatments.

In addition, this study investigated the activity level of plasma lysozyme to

determine the effect of heat stress on the immune systems of goldfish, as lysozyme content is an indicator of non-specific immune function. The groups that experienced 30°C exhibited significantly decreased lysozyme activity compared with the 22°C groups, whereas groups that received melatonin injections or green-wavelength LED treatment exhibited significantly increased lysozyme activity. In particular, after ZT12, green-wavelength LED groups exhibited higher lysozyme activity than the melatonin-injected groups.

Wang et al. (2008) reported that acute changes in salinity caused internal oxidative stress and reduced lysozyme activity and thus directly decreased the efficacy of the immune system in sea cucumber, *Apostichopus japonicus*. In addition, Choi et al. (2012) reported that starvation induces oxidative stress, but treatment with green-wavelength LED light decreased oxidative stress and increased immune function in cinnamon clownfish. The direct effect of melatonin on lysozyme activity remains unclear. However, Esteban et al. (2006) reported that lysozyme activity in the circadian rhythm was significantly higher during the scotophase than during the photophase in sea bass, *Dicentrarchus labrax* L.; thus, melatonin, a hormone secreted during the scotophase, may affect lysozyme activity. In addition, Cuesta et al. (2008) reported that in gilthead sea bream, *Sparus aurata* L., the injection of melatonin causes immune-relevant genes and activity to be upregulated; thus, melatonin increased immune function. Therefore, our work confirmed that of previous studies that both green-wavelength LED and melatonin treatment increased both lysozyme activity and immune capacity.

A comet assay at ZT8 was used to investigate oxidative nuclear DNA damage. The comet assay clearly identified damage to nuclear DNA due to increased free radicals caused by stress. Goldfish cells possess normal nuclear DNA at 22°C, whereas cells with damaged nuclear DNA were observed in all groups at 30°C. In addition, *in vitro* analysis using liver cells showed that the groups at 30°C exhibited significantly higher tail length, percentage DNA in the tail, and tail moment than did those at 22°C. However, cells from fish treated with melatonin injections or green-wavelength LED light showed significantly decreased tail length,

percentage DNA in tail, and tail moment. They appeared to incur hardly any damage compared with the fish in the high-temperature groups.

Anitha et al. (2000) reported that nuclear DNA was damaged by the increased water temperatures when goldfish were exposed to water temperatures of 34°C, 36°C, and 38°C. In addition, Villarini et al. (1998) reported that high temperatures induced oxidative stress and damaged the DNA of rainbow trout, *O. mykiss* erythrocytes, but antioxidant treatment reduced the level of damage to the nuclear DNA.

The results of the present study are similar to those of previous studies that showed oxidative stress is induced through exposure to high temperatures, in that thermal stress damaged the nuclear DNA of goldfish liver cells, but the results showed that treatment with melatonin or green-wavelength LED light played a role in protecting and repairing damaged nuclear DNA.

In conclusion, the results of the present study indicate that acute changes in water temperature induce oxidative stress in goldfish and reduce their liver cells' antioxidant capacity and immune function; however, melatonin injection and exposure to green-wavelength LED light effectively controlled (or reduced) the oxidative stress induced by thermal stress. In other words, these treatments can increase the antioxidant capacity and boost the immune system of fish. In particular, the effect of exposure to green-wavelength LED light was comparable to that of treatment with 10 μg melatonin/g body mass; therefore, irradiation with green-wavelength LED light should be considered a potential replacement for melatonin treatment.

Chapter 3.

Effects of melatonin injection and green-wavelength LED light on the physiological stress and immunity of goldfish, *Carassius auratus* during thermal stress

Abstract

This study investigated the effects of increasing water temperature (22°C to 30°C) on the physiological stress response and immunity of goldfish, *Carassius auratus*, and the ability of green light emitting diode (LED) irradiation or melatonin injections to mitigate this temperature-induced stress. To evaluate the effects of either green-wavelength LED light or melatonin on stress in goldfish, this study measured plasma triiodothyronine (T_3), thyroxine (T_4), and thyroid hormone receptor (TR) mRNA expression; plasma cortisol and glucose; and immunoglobulin M (IgM) and lysozyme mRNA expression. The thyroid hormone (TH) activities, TR mRNAs expression, and plasma cortisol and glucose were higher in goldfish exposed to high temperature water, but were lower after exposure to melatonin or green-wavelength LED light. Lysozyme mRNA expression and plasma IgM activity and protein expression were lower after exposure to high water temperatures and higher after melatonin or green-wavelength LED light treatments. Therefore, high water temperature induced stress and decreased immunity; however, green-wavelength LED light and melatonin treatments mitigated the effects of stress and enhanced immunity. The benefits of melatonin decreased with time, whereas those of green-wavelength LED treatment did not.

1. Introduction

Water temperature is an important factor in the survival of fishes since it plays a key role in their physiological responses, metabolic rate, and distribution (Díaz et al., 2007). Global atmospheric temperatures are expected to rise because of climate change—a serious, worldwide threat. Therefore, an increase in water temperature in many oceans has been anticipated (Harvell et al., 2002; Noyes et al., 2009). Changes in water temperature have been shown to cause stress in marine organisms (Malev et al., 2010; Ming et al., 2012), which results in the activation of their sympathetic chromaffin system, and then leads to increased levels of catecholamines (primarily adrenaline) and the release of the stress hormone cortisol by the hypothalamic–pituitary–interrenal axis (HPI axis). The secretion of cortisol promotes gluconeogenesis and increases glucose levels (Begg et al., 2004). Ming et al., (2012) reported that serum cortisol and glucose levels tended to increase under high temperature stress in Wuchang bream, *Megalobrama amblycephala*. Severe or prolonged exposure to stress might affect the growth, immune function, and reproduction of organisms (Sumpter, 1991; Barton et al., 2002). In particular, in fish, stress inhibits the immune system functions, leading to diseases and other adverse health effects (Karvonen et al., 2010). The immune system plays an important role in protecting fish from diseases via non-specific mechanisms, including activation of lysozymes and anti-disease factors, as well as immunoglobulins (Ingram, 1980; Ellis, 2001). Humoral adaptive immunity in fish is mediated by immunoglobulins, and the immunoglobulin M (IgM) class is the primary immune response in most teleost fish (Bag et al., 2009). Lysozyme, another immune indicator, is one of the most studied innate immunity responses in fish (Tort et al., 2003).

Thyroid hormones (THs) play a crucial role in the growth and development of fish (Power et al., 2001) and regulate the metabolism of fish exposed to environmental changes, including alterations in water temperature (Matty, 1985). Two types of THs—triiodothyronine (T_3) and thyroxine(T_4)—are released from the

hypothalamic–pituitary–thyroid axis (HPT-axis; Peter 2011), and their biological actions are mediated via the thyroid hormone receptors (TRs)—TRα and TRβ (Yaoita and Brown, 1990). THs are affected by many factors, including gender, nutritional status, season, and physiological condition (Rolland, 2000; Schnitzler et al., 2012). Water temperature changes also affect thyroid hormones, and Abbas et al., (2012) reported that, in the white grouper, *Epinephelus aeneus,* T_4 increased as the water temperature rose.

Water temperature as an environmental factor also affects the synthesis and release of melatonin, which is produced by the pineal organ. Max and Menaker (1992) reported that plasma melatonin levels increased with increasing temperature in rainbow trout, *Salmo gairdneri*. Melatonin is a hormone primarily secreted in association with day–night cycles and plays a crucial role in seasonal and circadian rhythms, physiology, and behavior in most vertebrates, including fish (Falcón et al., 2010). In addition, melatonin reduces the levels of cortisol, a stress response indicator; increases antioxidant enzyme expression; and decreases oxidative stress; therefore, it plays an important role in reducing internal stress (Özturk et al., 2000; Herrero et al., 2007).

Recent studies have suggested that particular wavelengths of light can affect stress levels and physiological responses in fish (Shin et al., 2011; Kim et al., 2014). Light emitting diodes (LEDs), which can be manufactured to output specific wavelengths, have lower power requirements and a longer life span than fluorescent bulbs and can improve the efficiency of lighting systems. Fish farming lighting systems that use specific-wavelength LEDs have been reported to reduce the stress generated in fish and can be a valuable tool for the fish farming industry (Villamizar et al., 2009; Choi et al., 2012). In particular, green-wavelength LEDs have been shown to inhibit stress in fish exposed to high water temperatures (Choi et al., 2014).

This study exposed goldfish, *Carassius auratus,* to high water temperatures and evaluated the effect of green-wavelength LED irradiation on their stress levels and immunity system. The stress reduction and immunity enhancing effect of

green-wavelength LED irradiation were comparable to those of melatonin that has an anti-stress function. Further, this study was measured the changes in plasma T_3 and T_4 levels, TRs mRNA expression, and plasma cortisol and glucose levels, in order to evaluate the difference in stress levels among the treatment groups. Finally, this study was evaluated the changes in immunity in goldfish by measuring lysozyme mRNA expression and plasma immunoglobulin M (IgM) levels and protein expression.

2. Materials and methods

2.1. Experimental fish and sampling

Goldfish (n = 575; length, 13.2 ± 0.6 cm; mass, 15.1 ± 0.7 g) were purchased from a commercial fish farm (Busan, Korea) and maintained in eight 100 L circulation filter tanks before the experiments, which were conducted in a laboratory. The fish were exposed to a simulated natural photoperiod. For the experiments, the fish were randomly divided into four groups: a control, sham (injection of saline solution), melatonin injection treatment, and green-wavelength LED treatment. The control, sham, and melatonin injection groups were exposed to light produced by a white fluorescent bulb, whereas the green-wavelength LED group was exposed to light produced by a green (530 nm wavelength) LED (Daesin LED Co., Kyunggi, Korea). The light sources were placed 50 cm above the surface of the water, and the irradiance at the surface of the water was maintained at approximately 4.5 μmol/m^2/s. 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 and were turned off at 19:00 h).

The goldfish were reared using automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and were allowed to acclimatize to the conditions for 24 h. The water temperature was then increased from 22°C to 30°C at daily increments of 2°C. The fish received commercial feed once per day until the day before sampling. The sampled fish were maintained at the experimental temperature (22 and 30°C), and the experiments were started at 07:00 h. The fish were sampled at the following Zeitgeber time (ZT; the time imposed by light–dark cycles) intervals: ZT4, ZT8, ZT12, ZT16, ZT20, ZT24, ZT28, ZT32, and ZT36. All fish were anesthetized using tricaine methanesulfonate (Sigma, USA), and blood and tissue samples were collected at the same time. Blood was collected from the caudal vein by using heparin-coated 1 mL syringes. Plasma samples were separated by centrifuging the blood samples at 10,000 × g for 5 min (4°C) and stored at -80°C until analysis. Tissues were collected, immediately frozen in liquid

nitrogen, and stored at -80°C until total RNA was extracted for analysis.

2.2. Melatonin injections

The fish in the melatonin treatment group were anesthetized with tricaine methanesulfonate (MS-222; Sigma, USA) before the injection of melatonin. Melatonin (Sigma, USA) was dissolved in 0.9% physiological saline, and each fish was injected with a concentration of melatonin (5 μg/g of body mass) at a volume of 10 μL/g body mass. After injection, the fish were reared at experimental water temperature. The sham group was injected with an equal volume of 0.9% physiological saline (10 μL/g of body mass).

2.3. Total RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted from the tissues by using TRI Reagent® (Molecular Research Center Inc., USA), according to the manufacturer's instructions. Next, 2 μg of total RNA was reverse transcribed in a total reaction volume of 20 μL by using an oligo-$(dT)_{15}$ anchor and M-MLV reverse transcriptase (Promega, USA), according to the manufacturer's instructions. The resulting cDNA was stored at 4°C until needed for polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR).

The qPCR was used to determine the relative expression levels of mRNA of the thyroid hormone receptors, TRα and TRβ, by using the total RNA extracted from the brains of goldfish. The primers for qPCR were designed using known goldfish sequences (Table 1). The qPCR amplification was conducted 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), according to the manufacturer's instructions. β-actin was amplified as a control for each sample, and all data were expressed as the change with respect to the corresponding calculated β-actin threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence

signal crossed a threshold at the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) of each sample and the internal controls (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge}-(\Delta Ct_{sample}- \Delta Ct_{internal\ control})$]. After PCR, the QPCR data from three replicate samples were analyzed using a Bio-Rad program to estimate the transcript copy numbers of each sample.

2.4. Western blot analysis

The total protein isolated from the livers of goldfish 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 30 μg protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used as are ference. Samples were electrophoresed at 180 V, and the gels were immediately transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min by using the Trans-Blot® Turbo™ Transfer System. Subsequently, the membranes were blocked with 5% milk in Tris-buffered saline (TBS; pH7.4) for 45 min, and then washed in TBS. The membranes were incubated with IgM (dilution 1:5,000; C-57070; LSBio, Seattle, WA, USA) antibodies, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (dilution, 1:4,000; Bio-Rad) for 60 min. β-tubulin (dilution, 1:5,000; ab6046; Abcam, UK) was used as the internal control. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) and 30 s of exposure with a Molecular Imager® from ChemiDoc™ XRS+ Systems (Bio-Rad, Hercules, CA, USA). The membrane images were scanned using high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0; Bio-Rad).

Table 2. Primers used for qPCR amplification

Genes (accession no.)	Primer	DNA sequences
TRα1 (**AY973629**)	Forward	5′-TCG AGA AGT GTC AGG AGA T-3′
	Reverse	5′-GCC AGA AGT GTG AGA TGT T-3′
TRα2 (**DQ172902**)	Forward	5′-TAG GAG TGG TGT CGG ATG-3′
	Reverse	5′-CTG GGT GTT GAT AAT ACC TGA G-3′
TRβ (**AY973630**)	Forward	5′-CGC ACA ATT CAG AAG AAC TTG-3′
	Reverse	5′-CAC TCT TGG CAC TGG TTG-3′
Lysozyme (**KJ703111**)	Forward	5′-CTG TTG TTG TCT TGT GTC TGA-3′
	Reverse	5′-AGT CCC TCT CGC TTG AAG-3′
β-actin (**AB039726**)	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

2.5. *In vitro* culture of brain cells

The brain cell culture in goldfish was performed using both enzymatic and mechanical procedures. The brain tissue was rapidly removed and placed in 3 mL of ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone; Gibco-BRL, Rockville, MD, USA). The isolated brain tissues were transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma, USA). The connective tissues and other impurities were removed, and the brain tissue was cut into small pieces by using scissors. The brain cells and minced brain tissue were transferred to a flask and slowly stirred in an incubator for 10 min at room temperature. The mixture of dispersed brain cells and tissues was filtered, and the culture medium (neurobasal medium, without l-glutamine, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, and 1% fetal bovine serum; Gibco-BRL, USA) was added. The cell suspension was centrifuged at 800 × *g* for 10 min, and then the cells were resuspended in fresh culture medium. The brain cells (1.2×10^6 cells at 800 μL/well) were applied to a 24 well tissue culture plate. For the experiments, the fish were randomly divided into experimental groups: control, melatonin treatment (0.1 μg/μL), and green-wavelength LED (530 nm) treatment. The photoperiod and sampling times were the same as mentioned above.

2.6. Plasma parameter analysis

Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, for 5 min). Plasma T_3, T_4, cortisol, and IgM levels were analyzed using immunoassay by using an ELISA kit (T_3, CSB-E08488f; T_4, CSB-E08489f; cortisol, CSB-E08487f; IgM, CSB-E12045fh; Cusabio Biotech, Hubei, China). The levels of plasma glucose were measured using the dry multiplayer analytic slide method by using a biochemistry autoanalyzer (FUJI DRI-CHEM 4000i; Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data were analyzed using 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 ($P < 0.05$). Values are expressed as means ± standard error (SE). Tukey's *post hoc* test was used to assess statistically significant differences for the different temperatures and treatments.

3. Results

3.1. Plasma T_3 and T_4 levels

This study investigated plasma T_3 and T_4 levels in response to water temperature changes. No significant differences were noted between the sham group and controls ($P > 0.05$). Plasma T_3 and T_4 activities were significantly higher (by approximately 1.6- to 3.1-fold) in the high water temperature (30°C) groups than in the 22°C groups ($P < 0.05$; Fig. 1). The activities of T_3 and T_4 in the scotophase groups were significantly lower than those in the photophase groups ($P < 0.05$). However, their activities were significantly lower in the melatonin injected (5 μg/g of body mass) and green-wavelength LED groups than in the control and sham groups (~1.3-fold, $P < 0.05$). In particular, over time, the T_3 and T_4 levels in the green-wavelength LED groups showed a decreasing trend compared with those of the melatonin injection groups.

3.2. Expression of TRs mRNA

The expression of TRα1, TRα2, and TRβ mRNA in the 30°C groups was significantly higher than that of the 22°C groups ($P < 0.05$; Fig. 2). In addition, the expression of TR mRNAs in the scotophase groups was significantly lower than that of the photophase groups ($P < 0.05$). The melatonin injection and green-wavelength LED groups had significantly lower TRα1, TRα2, and TRβ mRNA expression levels.

In the *in vitro* brain culture, TRα1, TRα2, and TRβ mRNA expression levels were similar to those of the *in vivo* groups (Fig. 3). The expression levels of TRα 1, TRα2, and TRβ mRNA in the 30°C group were significantly higher than those in the 22°C group, and the levels in the scotophase groups were significantly lower than those in the photophase groups. In addition, the melatonin (0.1 μg/μL) and green-wavelength LED treatment groups showed significantly decreased TRα1, TRα 2, and TRβ mRNA expression levels.

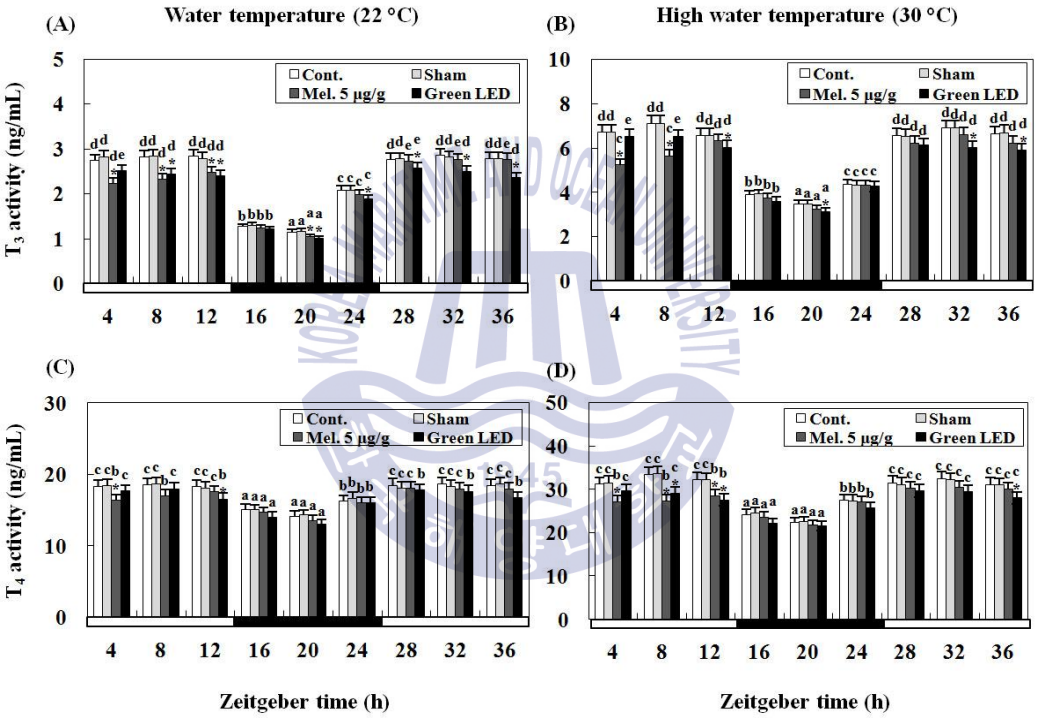

Fig. 8. Changes in plasma T_3 and T_4 levels during thermal change at 22°C (A and C) and 30°C (B and D), in each experimental goldfish group: control, sham, melatonin injection (5 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

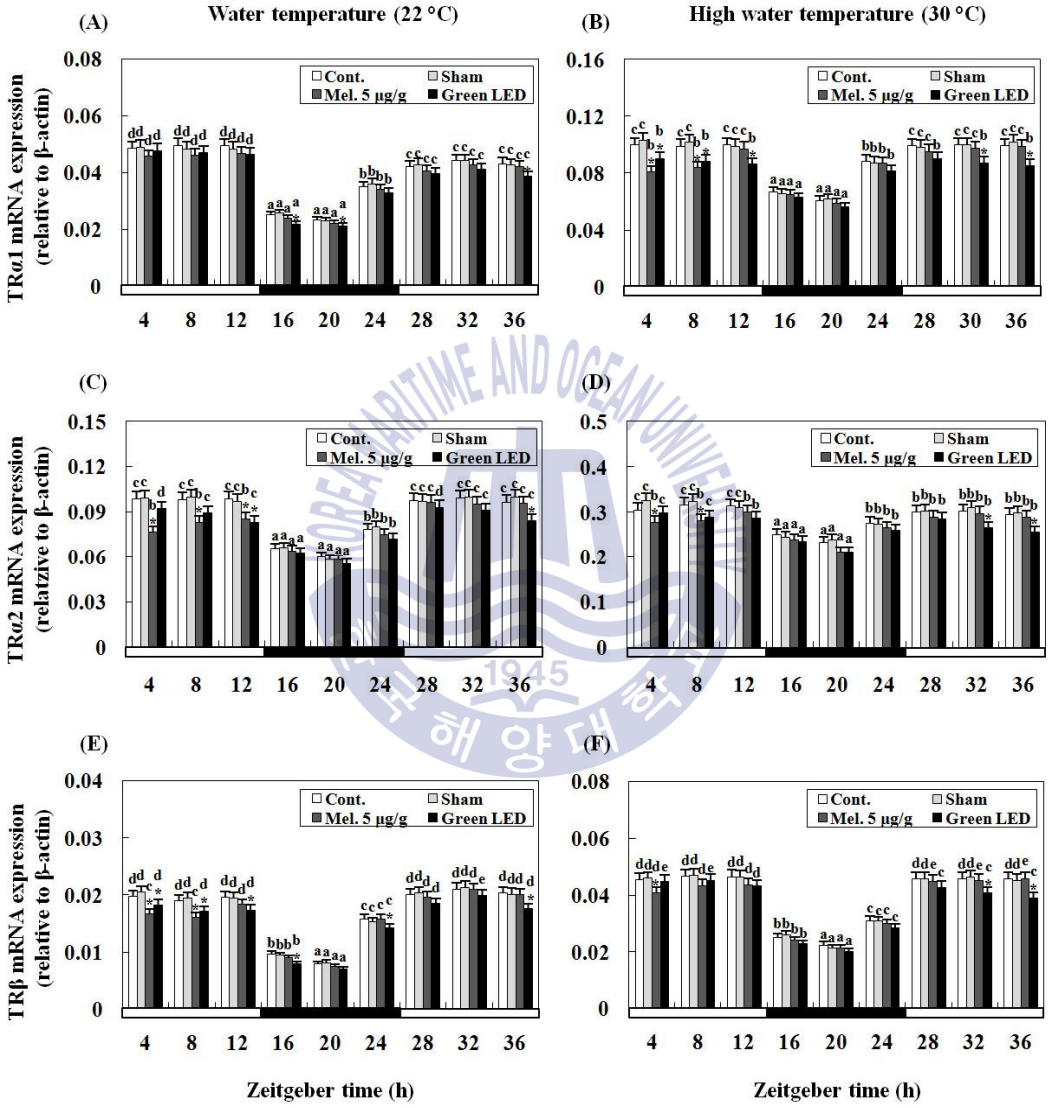

Fig. 9. Changes in TR (TRα1, TRα2, and TRβ) mRNA expression levels in the brain during thermal change at 22°C (A, C, and E) and 30°C (B, D, and F), in each experimental goldfish group: control, sham, melatonin injection (5 μg/g of body mass), and green-wavelength LED light, as measured using quantitative real-time polymerase chain reaction. Total brain 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. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

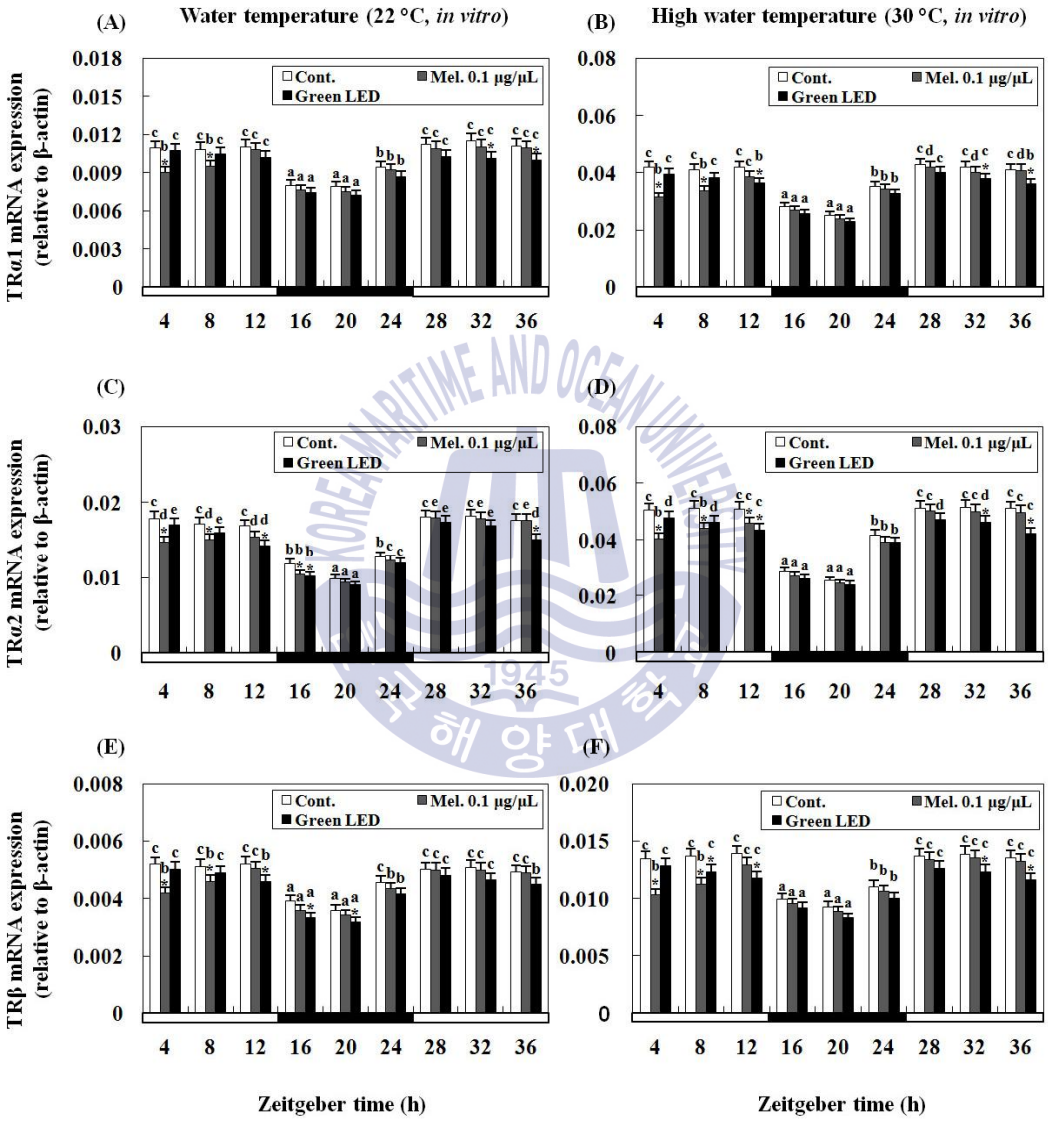

Fig. 10. Changes in TR (TRα1, TRα2, and TRβ) expression levels in the brain (*in vitro*) during thermal change at 22°C (A, C, and E) and 30°C (B, D, and F), in each experimental goldfish group: control, melatonin treatment (0.1 μg/μL), and green-wavelength LED light, as measured using quantitative real-time polymerase chain reaction. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

3.3. Plasma cortisol and glucose levels

The plasma levels of cortisol and glucose in response to water temperature changes were analyzed. The plasma cortisol and glucose levels of the 30°C groups were significantly higher (by approximately 2.0- to 3.5-fold, $P < 0.05$) than those of the 22°C groups (Fig. 4). The scotophase groups had significantly lower cortisol and glucose plasma levels than those of the photophase groups. Plasma cortisol and glucose levels were significantly decreased (~1.3-fold, $P < 0.05$) in the melatonin injection and green-wavelength LED groups. No significant differences were noted between the sham group and intact controls ($P > 0.05$).

3.4. Expression of lysozyme mRNA

Lysozyme mRNA expression levels were analyzed using the liver tissue samples. In the high-water temperature (30°C) groups, lysozyme mRNA expression was significantly lower than that of the 22°C groups ($P < 0.05$; Fig. 5). However, lysozyme mRNA expression in the scotophase groups was significantly higher than that in the photophase groups. The melatonin injection groups and green-wavelength LED groups also had significantly increased lysozyme mRNA expression levels.

3.5. The activity and western blot of IgM

The protein expression level of immunoglobulin M (IgM) was investigated using liver tissues. The IgM expression levels were significantly lower in the 30°C groups than in the 22°C groups ($P < 0.05$; Fig. 6A and B). The protein expression levels in the melatonin injection and green-wavelength LED groups were significantly increased. In addition, the plasma IgM activity in the 30°C groups was significantly lower than that in the 22°C groups; however, the melatonin injection and green-wavelength LED groups showed significantly increased plasma IgM activity (Fig. 6C and D). In particular, over time, the IgM expression levels in the green-wavelength LED groups remained significantly lower than those in the melatonin-injected groups.

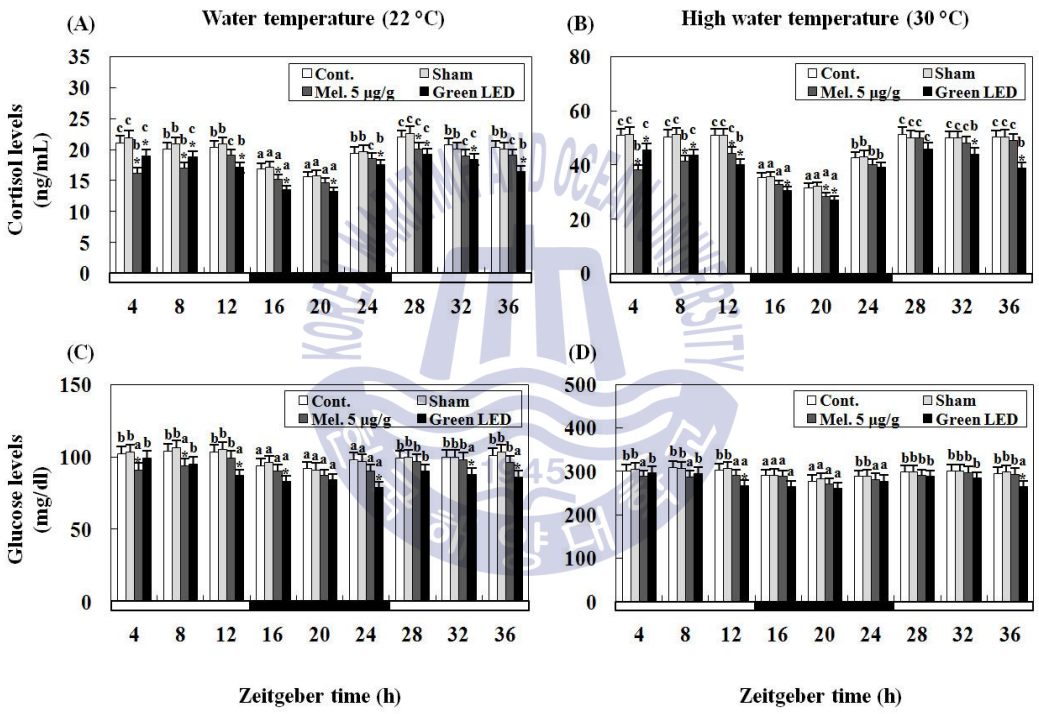

Fig. 11. Changes in plasma cortisol and glucose levels during thermal change at 22°C (A, C, and E) and 30°C (B, D, and F), in each experimental goldfish group: control, sham, melatonin injection (5 μg/g of body mass), and green-wavelength LED light, as measured using a microplate reader. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

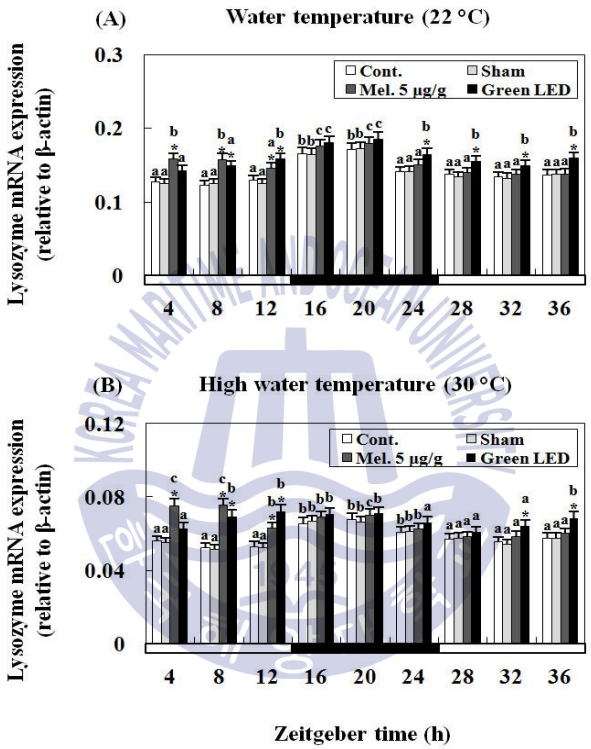

Fig. 12. Changes in lysozyme mRNA expression levels in the liver during thermal change at 22°C (A) and 30°C (B), in each experimental goldfish group: control, sham, melatonin injection (5 μg/g of body mass), and green-wavelength LED light, as measured using quantitative real-time polymerase chain reaction. 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. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

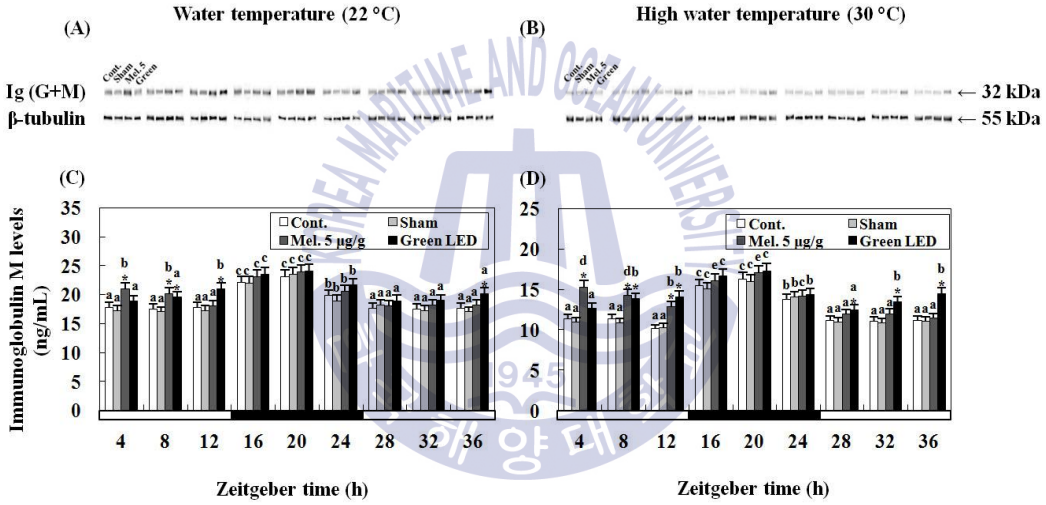

Fig. 13. Changes in IgM protein expression and activity during thermal change at 22°C (A and C) and 30°C (B and D), in each experimental goldfish group: control, sham, melatonin injection (5 μg/g of body mass), and green-wavelength LED light. Western blot of the antioxidant enzymes of IgM (32 kDa) protein expression in the liver of goldfish after thermal change; β-tubulin (55 kDa) was used as the internal control. The white bar represents the photophase, and the black bar represents the scotophase. The lowercase letters represent significant differences within the same experimental groups and within the same temperature treatments ($P < 0.05$). The asterisk (*) indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are means ± SE ($n = 5$).

4. Discussion

The physiological effects (i.e., stress reduction and immune-enhancement) of green-wavelength LEDs were confirmed by comparing the responses of goldfish under thermal stress to two types of treatment: irradiation and immune-enhancing melatonin. Further, stress regulation and immunity in each experimental group were investigated by measuring plasma T_3 and T_4 activities and TR mRNA expression levels during the experimental period. In addition, stress response indicators (cortisol and glucose) and immunity indicators (lysozyme and immunoglobulin) were compared.

Changes in plasma T_3 and T_4 levels, as well as alterations in the expression of thyroid hormone receptor (TRα1, TRα2, and TRβ) mRNA were observed. No significant differences were observed between the sham group and controls. The high water temperature (30°C) groups showed significantly higher plasma T_3 and T_4 concentrations and expression of TR mRNAs than the 22°C groups. However, at high water temperatures, the melatonin injected (5 μg/g of body mass) groups and irradiated green-wavelength LED groups showed significantly decreased plasma T_3 and T_4 levels, and TR mRNAs expression levels. Further, the melatonin-injected groups showed a reduced artificial melatonin effect (medicinal effect for reducing stress) over time. In contrast, the green-wavelength LED irradiated groups had lower plasma T_3 and T_4 levels in both the 22°C and 30°C groups during the entire experimental period than the control group. The irradiated green-wavelength LED groups exposed to either 22°C or 30°C water showed slightly higher plasma T_3 and T_4 levels than the melatonin injected (5 μg/g of body mass) groups from the start of the experiment to 8 h. However, they showed lower plasma T_3 and T_4 levels than the melatonin groups from 12 h until the end of the experiment.

The results of the experiment performed using cultured brain cells treated with melatonin or green-wavelength LED irritation were similar to those of experiments conducted *in vivo* and *in vitro*.

The results of this study are similar to those of a previous one suggesting that

plasma T_3 and T_4 levels in yellowfin seabream, *Acanthopagrus latus*, increased with rising water temperatures, and the highest plasma T_3 and T_4 levels were noted in August, the month in which seasonal water temperatures peaked (Salamat et al. 2012). Min et al., (2015) reported that plasma T_3 concentrations in starry flounder, *Platichthys stellatus* were significantly higher in the group exposed to 24°C than in the group exposed to 15°C; thus, T_3 concentrations were expected to increase due to temperature stress. In addition, our findings are consistent with those of Öztürk et al. (2000), who reported that rats injected with melatonin showed decreased levels of plasma T_3 and T_4 and thyroid stimulating hormone (TSH), indicating that melatonin enhanced antioxidant and free radical scavenging capacity. In addition, Kim et al. (2014) showed that green-wavelength LED light effectively controlled oxidative stress and reduced free radicals in goldfish exposed to thermal stress.

In this study, the stress caused by high temperature environment increased the plasma T_3 and T_4 levels and expression of TR mRNAs of goldfish. However, melatonin decreased the levels of plasma T_3 and T_4 and TR mRNA expression that had increased by temperature stress. Further, the effect of green-wavelength LED light was similar to that of melatonin supplementation and decreased the levels of plasma T_3 and T_4 and the expression of TR mRNAs.

In this study, plasma cortisol and glucose levels were analyzed to determine the degree of stress in goldfish exposed to a high temperature environment. The groups exposed to a high water temperature (30°C) showed significantly higher plasma cortisol and glucose levels than those of the groups maintained at 22°C. However, the melatonin-injected and green-wavelength LED irradiated groups showed significantly decreased plasma cortisol and glucose levels. In addition, the melatonin-injected groups showed a reduced melatonin effect (medicinal effect for reducing stress) that caused a decrease in the effects of stress relief with time, whereas the green-wavelength LED irradiated groups showed an effective stress reduction (as shown by lower cortisol and glucose concentrations) throughout the experimental period of 36 h compared to that in the control. In particular, the green-wavelength LED groups showed lower plasma cortisol and glucose levels than

those of the melatonin injection groups starting at 12 h after the start to the end of the experiment, suggesting the continuation of the effect of stress relief of green-wavelength LED light.

Herrero et al. (2007) reported that increased plasma melatonin levels after supplementation of diet with melatonin reduced the cortisol levels in European sea bass, *Dicentrarchus labrax*. Further, Azpeleta et al. (2007) induced acute stress in goldfish by exposing them to air and found that this stress caused increased plasma cortisol levels; however, the melatonin injection groups had decreased levels of plasma cortisol, indicating that melatonin can play an anti-stress role. Choi et al. (2012) reported that, in starved cinnamon clownfish, *Amphiprion melanopus,* the levels of plasma glucose, alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) increased because of oxidative stress, but the green-wavelength LED groups showed significantly decreased plasma glucose, AlaAT, and AspAT levels, suggesting that green-wavelength LEDs can reduce oxidative stress caused by starvation. The results of these studies support our findings that irradiation by green wavelength effectively decreased plasma cortisol and glucose levels, relieving the adverse effects of high water temperature stress. Melatonin was thought to reduce cortisol secreted by the HPI axis and play a role in alleviating stress; green wavelength LED might also have a similar action mechanism, although this needs to be confirmed by conducting further studies.

The effects of artificial melatonin injection and green-wavelength LED irradiation on the immunity of goldfish exposed to high water temperatures were confirmed by investigating lysozyme mRNA expression and immunoglobulin M (IgM) protein expression and activity. The groups exposed to high water temperature (30°C) showed significantly lower lysozyme mRNA expression than those exposed to 22°C. However, melatonin injection or green-wavelength LED irradiation treatments significantly increased lysozyme mRNA expression and IgM protein expression and activity. These findings are similar to those of Cuesta et al. (2008), who injected melatonin into gilthead seabream, *Sparus aurata* L. and found an increase in the immune-relevant gene expressions, such as interleukin-1β, as well as non-specific

immune responses, including phagocytes (cells that protect the body by ingesting bacteria), and melatonin, which enhances the immune responses. In addition, Shin et al. (2014) reported that intact and ophthalmectomized cinnamon clownfish, *Amphiprion clarkii* exposed to green-wavelength LED showed higher expression of IgM mRNA and protein and lysozyme activity than those of the control groups, suggesting that green-wavelength LEDs can enhance the immune function. Therefore, the results of the present study are consistent with those of previous studies, suggesting that green-wavelength LED and melatonin treatment increase the immune capacity of fish exposed to temperature stress.

In conclusion, the present study showed that immunity in fish is reduced during the times of stress, but melatonin injection and green-wavelength LED irradiation can both enhance the immunity of goldfish. In the present study, melatonin treatment and green-wavelength LED irradiation were found to reduce stress and increase immunity in goldfish exposed to high water temperatures. In addition, in the initial 8 h of the experiment, artificial melatonin injection (5 μg/g of body mass) enhanced immunity more than that after treatment with green-wavelength LED irradiation, but over the course of the experiment, the LED irradiation produced prolonged stress reduction and immunity enhancement effect compared to that after melatonin treatment. The results of this study suggest that melatonin treatment or green-wavelength irradiation might be useful to reduce stress and enhance immunity in aquatic species.

Chapter 4.

General Discussion

The present study was exposed goldfish, *Carassius auratus*, to high water temperatures and evaluated the effect of green-wavelength LED irradiation on stress and immunity, as well as examined the effect of melatonin injection.

I. To examine changes in oxidative stress in goldfish in response to the treatment, I evaluated the mRNA expression and activity of the antioxidant enzymes SOD and CAT, and plasma H_2O_2 and LPO concentration. Furthermore, the damage to the nuclear DNA due to the induction of free radicals by highly thermal conditions was confirmed by using a comet assay. Additionally, I established that the expression and activity of SOD and CAT were significantly higher after exposure to high temperatures and significantly lower after exposure to melatonin or the green-wavelength LED light. The plasma H_2O_2 and LPO concentrations followed the pattern outlined above. Plasma lysozyme was significantly lower after exposure to high temperatures and significantly higher after exposure to melatonin or the green-wavelength LED light. This study observed damaged DNA by using the comet assay and found that nuclear DNA incurred a great deal of damage as a result of exposure to a high temperatures; however, the treatment with melatonin or 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, melatonin injection and exposure to the green-wavelength LED light effectively controlled (or reduced) the oxidative stress induced by high water temperatures. In particular, the exposure to the green-wavelength LED light had an effect similar to that of melatonin treatment. Therefore, I have determined that irradiation with the green-wavelength LED light will have sufficient antioxidant- and immunity-enhancing effects, and thus, can be considered a potential replacement for melatonin treatment.

II. To evaluate the effects of green-wavelength LED light and melatonin on stress in goldfish, I measured by using stress indicators; plasma T_3 and T_4 levels, thyroid hormone receptor (TRs; TRα1, TRα2 and TRβ) mRNA expression, and plasma cortisol and glucose levels. This study also confirmed the changes in immunity in goldfish by measuring lysozyme mRNA expression and plasma IgM levels and protein expression. The result suggest that the plasma T_3 and T_4 levels, and TRs mRNA expression levels significantly increased in response to thermal stress; however, their levels significantly decreased after melatonin treatment and the green-wavelength LED irradiation. In addition, while high water temperature acted as an stress factor and led to an increase in plasma cortisol and glucose levels, melatonin treatment and the green-wavelength LED irradiation decreased these levels. Furthermore, the artificial melatonin effect (medicinal effect for reducing stress) in the melatonin injection groups gradually declined over time. While the lysozyme mRNA expression and plasma immunoglobulin M (IgM) levels were significantly lower after exposure to high temperatures, they were significantly higher after exposure to melatonin or the green-wavelength LED light treatment. Taken together, these findings suggest that immunity of goldfish reduces after stress caused by the exposure to high water temperature; however, melatonin treatment and the green-wavelength LED irradiation can both reduce the stress and enhance the immunity of goldfish.

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 melatonin injection and the green-wavelength LED irradiation have positive stress-reducing and immunity-enhancing effects. Furthermore, the effect of injecting melatonin gradually declined over time. Therefore, it can be concluded that the green-wavelength LED light irradiation will have sufficient stress-reducing and immunity-enhancing effects.

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