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Effects of melatonin and green-wavelength LED light on the physiological stress and immunity of goldfish, *Carassius auratus*, exposed to high water temperature

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Abstract This study investigated the effects of increasing water temperature (22–30 °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, we 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 activities, TR mRNA 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.

Keywords Cortisol · Goldfish · Green-wavelength LED · Melatonin · Thermal stress · Thyroid hormone

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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 and Pankhurst 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 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).

In this study, we exposed goldfish, *C. 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, we 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. We also measured the changes in immunity in goldfish by measuring lysozyme mRNA expression and plasma IgM levels and protein expression.

Materials and methods

Experimental fish and sampling (in vivo)

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 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.

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).

Total RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction

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 $[\Delta\Delta Ct = 2^{-(\Delta Ctsample\ -\ \Delta Ctinternal\ control)}]$. 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.

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 180 V,

Table 1 Primers used for qPCR amplification

Genes	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′

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; pH 7.4) for 45 min, and then washed in TBS. The membranes were incubated with IgM (dilution 1:5000; C-57070; LSBio, Seattle, WA, USA) antibodies, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (dilution, 1:4000; Bio-Rad) for 60 min. β-Tubulin (dilution, 1:5000; 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).

In vitro brain cell culture

The brain cells 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.

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).

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 mean ± standard error (SE). Tukey's post hoc test was used to assess statistically significant differences for the different temperatures and treatments.

Results

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 injection (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.

Expression of TR mRNAs in the brain

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 cells culture, TRα1, TRα2, and TRβ mRNA expression levels were similar to those of the in vivo groups (Fig. 3). The expression levels of TRα1, 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

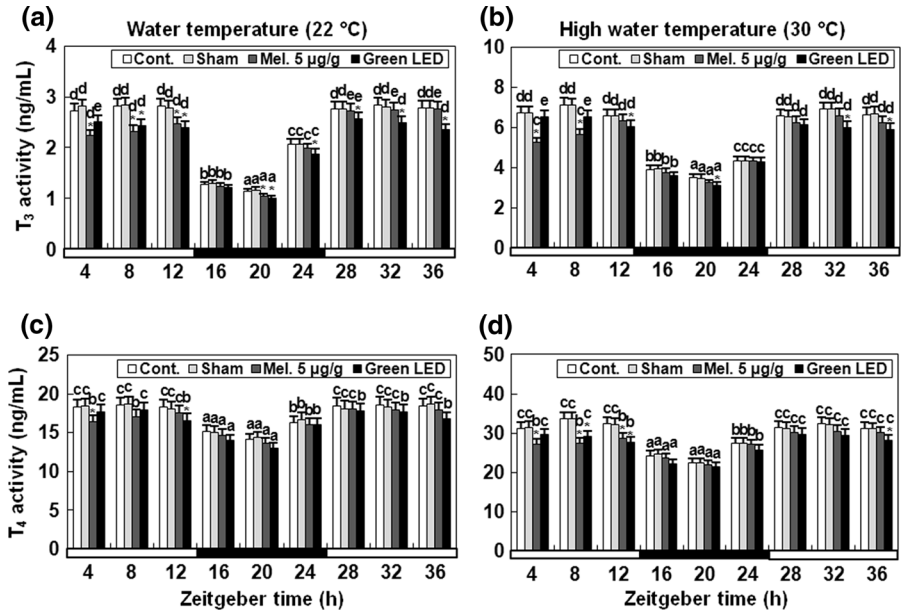

Fig. 1 Changes in plasma T_3 and T_4 levels during thermal change [water temperature (22 °C): **a**, **c**; high water temperature (30 °C): **b**, **d**] for each experimental group of goldfish [control, melatonin injection (5 μg/g of body mass), and green-wavelength LED treatment], measured using a 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 mean ± standard error ($n = 5$)

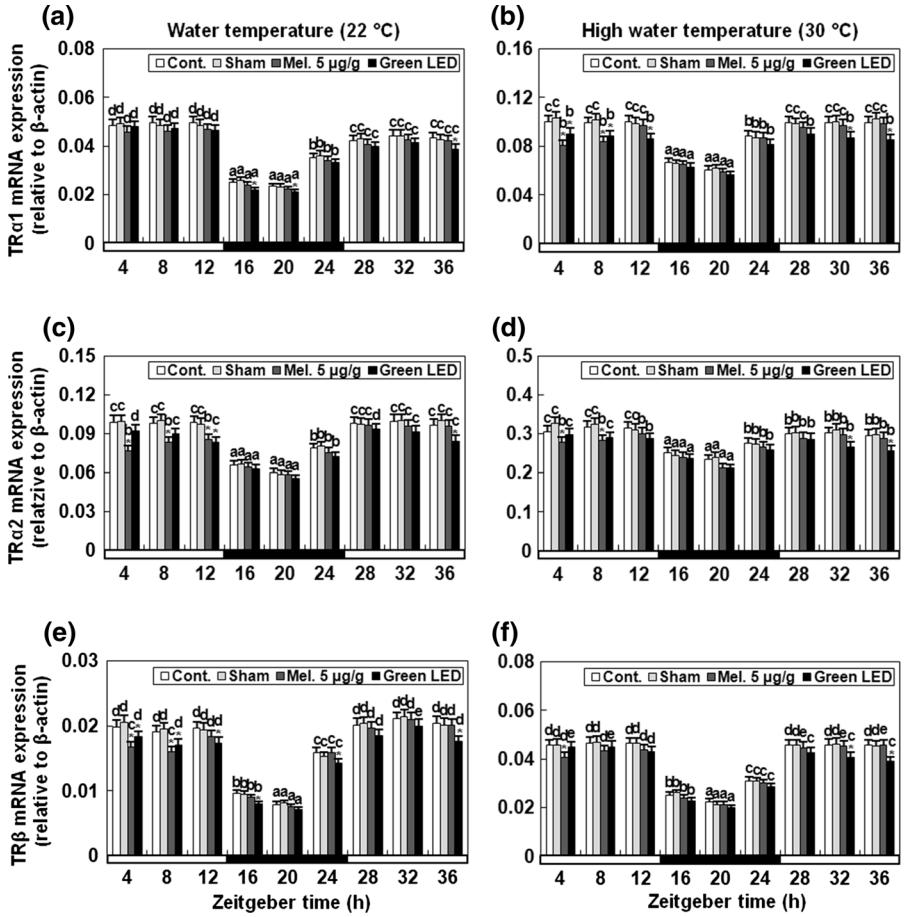

Fig. 2 Changes in TR (TRα1, TRα2, and TRβ) mRNA expression levels in the brain during thermal change [water temperature (22 °C): **a**, **c**, **e**; high water temperature (30 °C): **b**, **d**, **f**] for each experimental group of goldfish [control, melatonin injection (5 μg/g of body mass), and green-wavelength LED treatment], 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 treatment ($P < 0.05$). The *asterisk* indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are mean ± standard error ($n = 5$)

green-wavelength LED treatment groups showed significantly decreased TRα1, TRα2, and TRβ mRNA expression levels.

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 ($P > 0.05$).

Expression of lysozyme mRNA in the liver

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

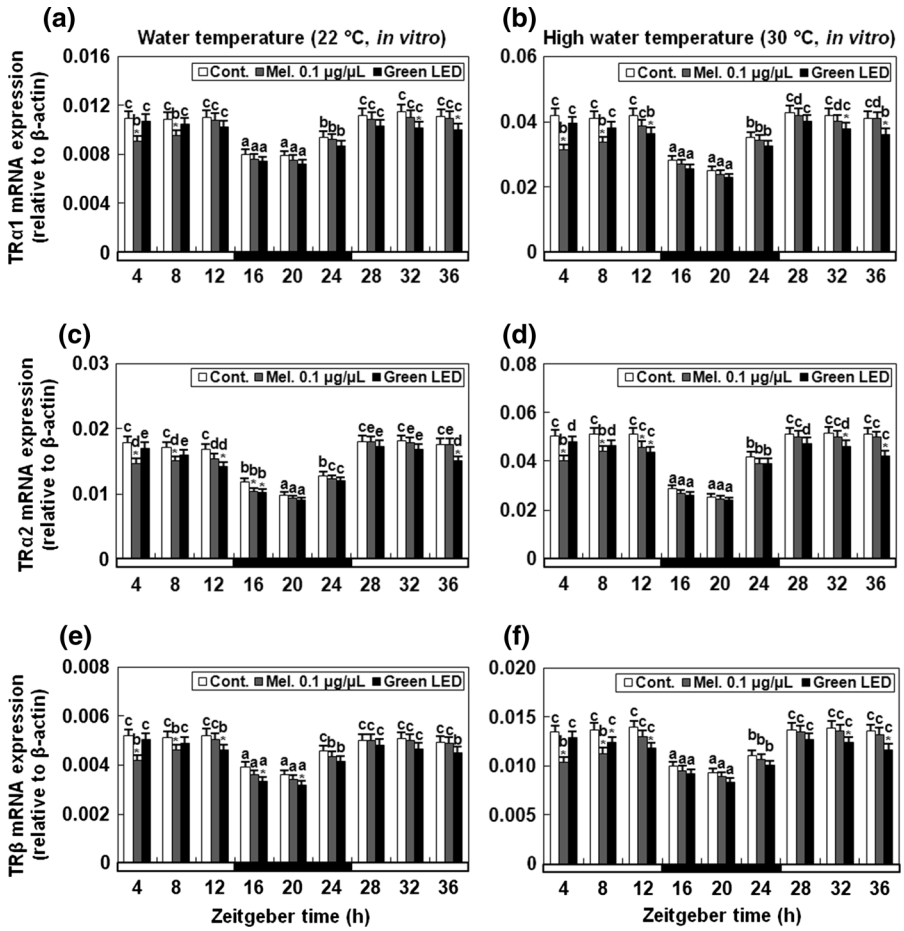

Fig. 3 Changes in TR (TRα1, TRα2, and TRβ) expression levels in the brain (in vitro) during thermal change [water temperature (22 °C): **a**, **c**, **e**; high water temperature (30 °C): **b**, **d**, **f**] for each experimental group of goldfish [control, melatonin treatment (0.1 μg/μL), and green-wavelength LED treatment], 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 treatment ($P < 0.05$). The *asterisk* indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are mean ± standard error ($n = 5$)

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.

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, 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, d). In particular, over time, the IgM expression levels in the green-wavelength LED groups remained significantly lower than those in the melatonin injection groups.

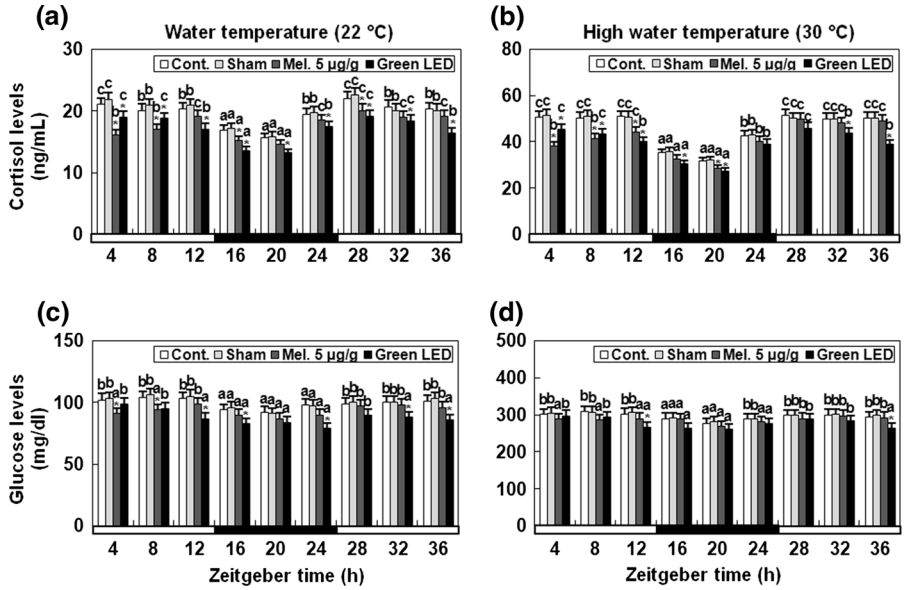

Fig. 4 Changes in plasma cortisol and glucose levels during thermal change [water temperature (22 °C): **a**, **c**; high water temperature (30 °C): **b**, **d**] for each experimental group of goldfish [control, melatonin injection (5 μg/g of body mass), and green-wavelength LED treatment], measured 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 mean ± standard error ($n = 5$)

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 injection (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 mRNA expression levels. Further, the melatonin injection 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 and 30 °C groups during the entire experimental period than the control group. The irradiated green-wavelength LED groups exposed to either 22 or 30 °C water showed slightly higher plasma T_3 and T_4 levels than the melatonin injection (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

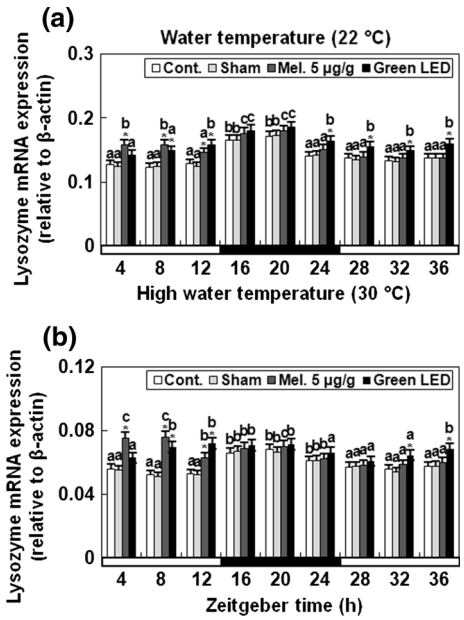

Fig. 5 Changes in lysozyme mRNA expression levels in the liver during thermal change [water temperature (22 °C): **a**; high water temperature (30 °C): **b**] for each experimental group of goldfish [control, melatonin injection (5 μg/g of body mass), and green-wavelength LED treatment], measured using 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 treatment ($P < 0.05$). The *asterisk* indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are mean ± standard error ($n = 5$)

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 Özturk 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 injection and green-wavelength LED-irradiated groups showed significantly decreased plasma cortisol and glucose levels. In addition, the melatonin injection 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

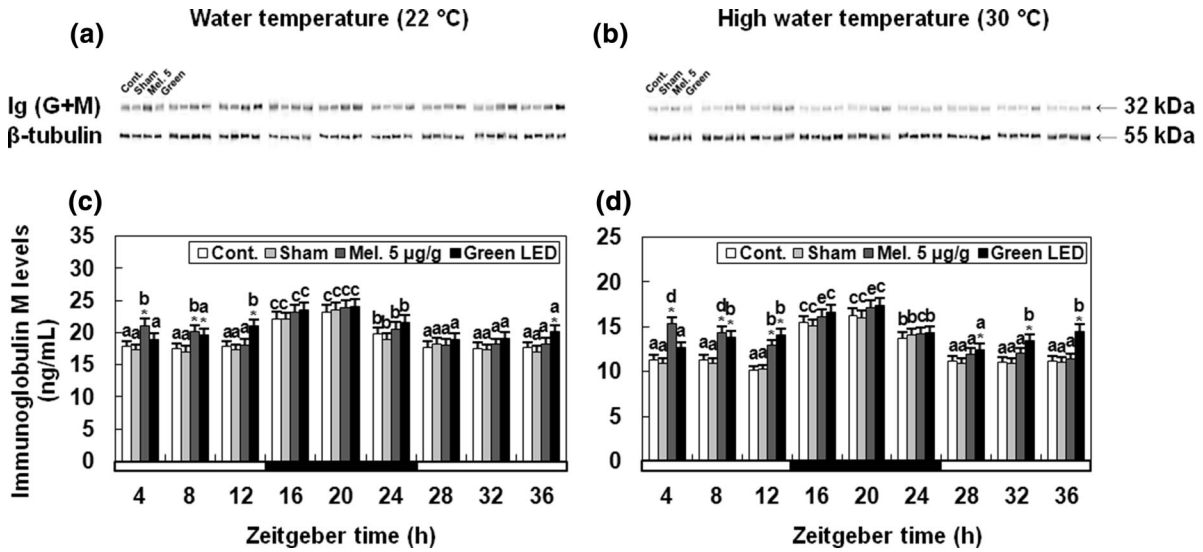

Fig. 6 Changes in IgM protein expression and activity during thermal change [water temperature (22 °C): **a**, **c**; high water temperature (30 °C): **b**, **d**] for each experimental group of goldfish [control, melatonin injection (5 μg/g of body mass), and green-wavelength LED treatment]. Western 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 treatment ($P < 0.05$). The *asterisk* indicates significant differences between different experimental groups within the same temperature treatment ($P < 0.05$). All values are mean ± standard error ($n = 5$)

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, our findings 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 our 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.

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