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ORIGINAL PAPER

The effect of LED light spectra on antioxidant system by thermal stress in goldfish, *Carassius auratus*

Na Na Kim[1], Young Jae Choi[1], Hyun Suk Shin[1], Jee Rey Lim[1], Jeong Min Han[1], Ji Hyung Cho[1], Jehee Lee[2], Gyung-Suk Kil[3] & Cheol Young Choi[1]

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Abstract We investigated the effects of the different light emitting diodes (LEDs; red, green, blue, and mixed purple) on oxidative stress by measuring the expression and activities of antioxidant enzymes, plasma H_2O_2, lipid peroxidation (LPO), aspartate aminotransferase (AspAT), alanine aminotransferase (AlaAT) levels, and melatonin during thermal stress (low-water temperature, 22 ⟶ 14˚C; high-water temperature, 22 ⟶ 30˚C) on goldfish, *Carassius auratus*. The expression and activity of the antioxidant enzymes and levels of H_2O_2, LPO, AspAT/AlaAT, and melatonin were significantly higher after the fish were exposed to low-/high-water temperature environments. Furthermore, in green and blue lights, the expression of antioxidant enzymes and mRNA of heat shock protein 70, and the activity of the antioxidant enzymes and plasma H_2O_2, LPO, and melatonin levels were significantly lower than those under other light spectra. The results indicated that low-/high-water temperature conditions induced oxidative stress in goldfish and green and blue LEDs inhibit oxidative stress.

Keywords Antioxidant enzymes, Goldfish, Light emitting diodes, Thermal stress, Short wavelength light

Stress factors in fish can be divided into 2 groups: physical factors, such as salinity, temperature, culture density, temperature, dissolved oxygen, and chemical factors[1], such as sulfides, ammonia, and heavy metals[2]. Physical factors, such as water temperature changes, affect growth, reproduction, metabolism, osmoregulation, and immune function, thus causing negative effects under physiological conditions, such as a disturbance in growth and reproduction[3-5]. Additionally, changes in water temperature environment modify the heat shock proteins (HSPs) *in vivo*, and their function as chaperones to modulate the conformation of newly synthesized polypeptides appears to have been conserved throughout evolution[6,7].

Poikilothermic animals, including fish, have mechanisms that adapt the living body temperature to change in external temperature. However, acute changes of water temperature (low- and high-water temperature environment) combined with an increase in oxygen consumption to maintain[8,9] can cause stress in fish[10].

Generally, stress induced by changes in salinity has been associated with enhanced reactive oxygen species (ROS; such as superoxide; $O\cdot^{2-}$, hydrogen peroxide; H_2O_2, peroxyl radicals, hydroxyl radicals; OH^-), which may seriously affect immune function and lead to oxidative stress[2].

ROS overproduction by environmental stress factors can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage. ROS overproduction can also affect cell viability by causing membrane damage and enzyme inactivity and can then accelerate cell senescence and apoptosis[11,12]. Antioxidant functions may include ROS-scavenging or detoxifying enzymes[13-15] such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione (GSH) and proteins (e.g., metallothionein, MT). Antioxidant defense systems are observed in the livers and kidneys of marine organisms[15,16] and have the fol-

[1]Division of Marine Environment & BioScience, Korea Maritime and Ocean University, Busan 606-791, Korea
[2]Department of Marine Life Sciences, Jeju National University, Jeju Special Self-Governing Province 690-756, Korea
[3]Division of Electrical and Electronic Engineering, Korea Maritime and Ocean University, Busan 606-791, Korea
Correspondence and requests for materials should be addressed to C. Y. Choi (✉choic@hhu.ac.kr)

lowing antioxidant functions: as phase 1 enzymes, SOD and CAT directly scavenge ROS. SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2, and then H_2O_2, produced by SOD, is sequentially reduced to H_2O and O_2 by CAT and GPX. Finally, CAT and GPX is an oxidoreductase that breaks down two molecules of H_2O_2 into two molecules of H_2O and O_2, thereby counteracting the toxicity of H_2O_2[17]. Additionally, activity of antioxidant enzymes increase by thermal changes[8,18].

Furthermore, peroxiredoxins (PRDXs) are the most recently discovered family of antioxidant enzymes that catalyze the reduction of peroxides and alkyl peroxides using thioredoxin as the immediate reducing cofactor[19,20]. PRDX is also known as a thioredoxin-like super family and protects organisms from attack by ROS, such as $O\cdot^{2-}$, H_2O_2, and OH^-[21,22].

Melatonin is considered a broad-spectrum antioxidant that is more powerful than glutathione in neutralizing free radicals and is more effective than other antioxidants in protecting cell membranes[23].

Antioxidant enzymes affect antioxidant mechanisms and have an important role for acute changes in environmental temperature and will be able to provide the physiological stress response of fish[24,25].

Recently, studies of light effects have been performed by using light-emitting diodes (LEDs) rather than natural or metal halide lights[26,27]. LEDs, a relatively new lighting technology that remains under development, can be manufactured to output specific wavelengths[28]. Narrow bandwidth light, using such new technologies, could thus provide much more efficient lighting systems than those currently used in the fish farming industry because they can be tuned to a species-specific environmental sensitivity by emitting narrow bandwidths[29].

In a previous study reporting LED light effects on teleosts, Shin *et al*.[26] recently reported that green and blue LEDs, both of which have short wavelengths, increased antioxidant materials against oxidative stress in the yellowtail clownfish, *Amphiprion clarkii*. However, the red LED wavelength affects physiological function and induces oxidative stress in yellowtail clownfish[26]. However, investigations of the stress response of fish between light wavelengths and water temperature remain limited.

Therefore, we examined the possibility of controlling oxidative stress using a specific light source during fish starvation. We investigated the expression and activity changes of antioxidant enzymes (SOD, CAT, and GPX), PRDX, and HSP70 (70 kilodalton heat shock proteins) during thermal stress (low-/high-water temperature) under 4 wavelengths (red, green, blue, and mixed purple within a visible light) in goldfish, *Carassius auratus*. We also determined the oxidative stress in the fish by measuring changes in plasma H_2O_2 concentration and LPO levels, melatonin, and assessed liver damage by measuring aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) levels.

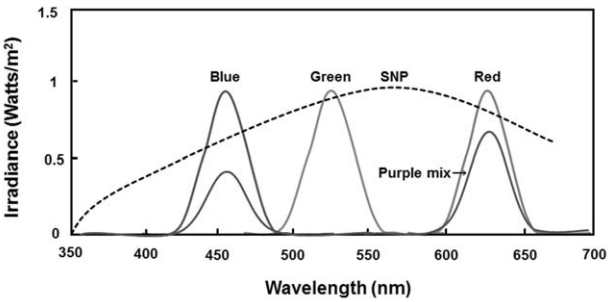

Figure 1. Spectral profiles of red, green, blue, and purple mix (mixed blue [450 nm] and red [630 nm]) lightemitting diodes (LEDs) used in this study. The filled square-dotted line Spectral profile of white fluorescent light (SNP, simulated natural photoperiod). Reprinted from ref. Shin *et al*.[26], with permission from Comparative Biochemistry and Physiology, Part-A.

Results

The expression and activity of antioxidant enzymes (SOD, CAT, and GPX) in the liver

This study investigated the levels of mRNA and antioxidant enzyme activity (SOD, CAT, and GPX) during temperature changes under different LED light spectra using QPCR (Figures 2, 3). The antioxidant enzyme mRNA and activity levels in the high-water temperature group of SNP and red light spectra groups were significantly higher than the low-water temperature group. In short, the mRNA expression and activity of SOD and CAT in SNP and red LED were significantly increased higher in high-water temperature group than low-water temperature group. Additionally, the mRNA expression and activity of GPX was lower increase levels than SOD and CAT. Particularly, the levels in the red LED group were significantly higher than those of the other LED spectra groups, but the levels in the green and blue LED groups were significantly lower than the other groups. Furthermore, the levels in the purple mix LED, which mixed red and blue lights, were significantly lower than the red LED group.

The expression of PRDX and HSP70 mRNA in the liver

The antioxidant enzyme, PRDX mRNA expression levels in all experimental groups were significantly increased by temperature change stress, the levels in

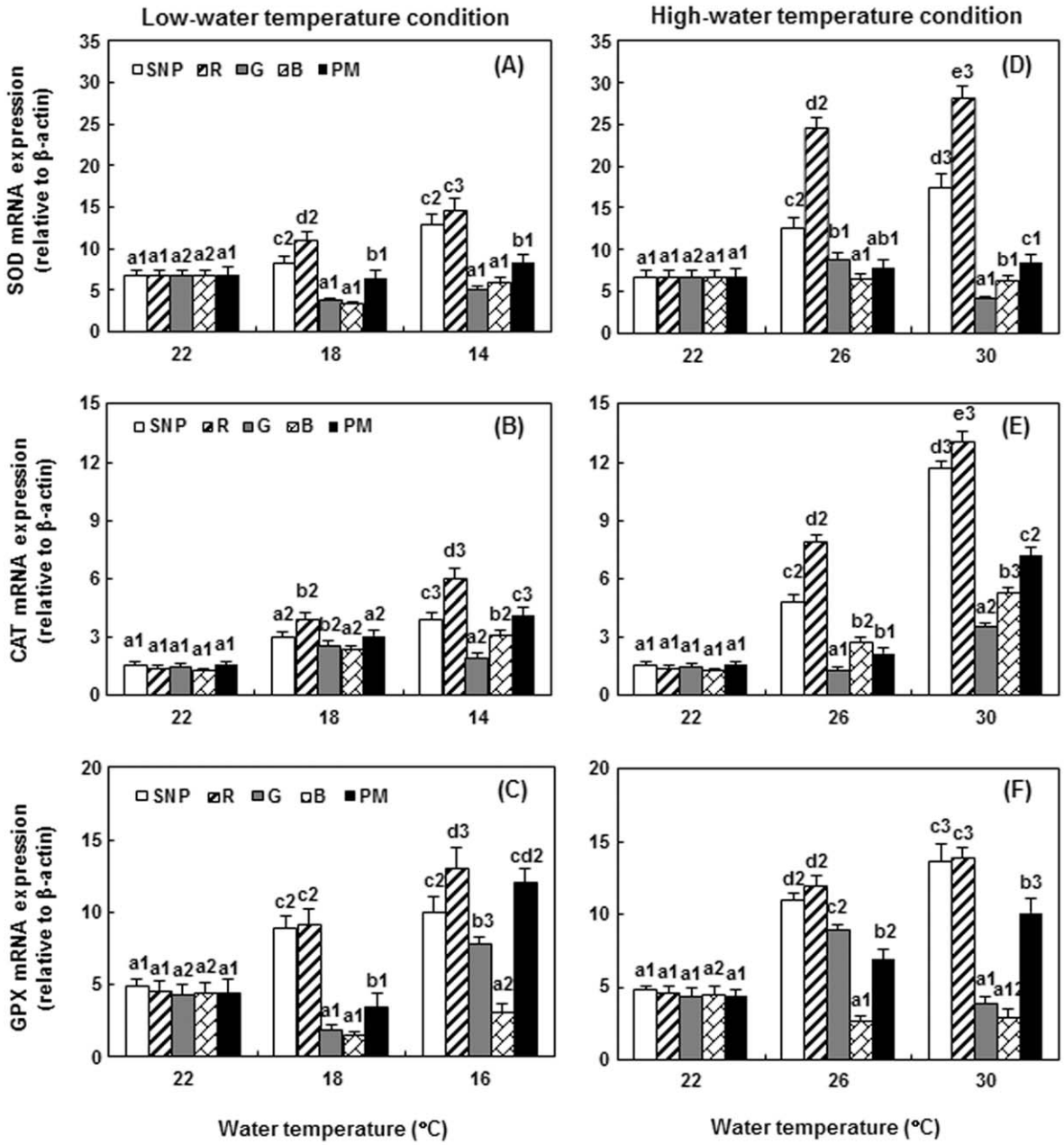

Figure 2. Changes in the expression levels of SOD (A, D), CAT (B, E), and GPX (C, F) mRNA in the liver during thermal changes (low-water temperature-A, B, C; high-water temperature-D, E, F) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Total liver RNA (2.5 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. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

the green and blue LED groups were significantly lower than the other groups. However, the levels in the red LED groups were significantly higher than the other groups. Especially, the PRDX mRNA in the high-water temperature group of SNP groups were significantly higher than the low-water temperature group, and in red LED group, expression of PRDX mRNA was increased by thermal changes (Figure 4).

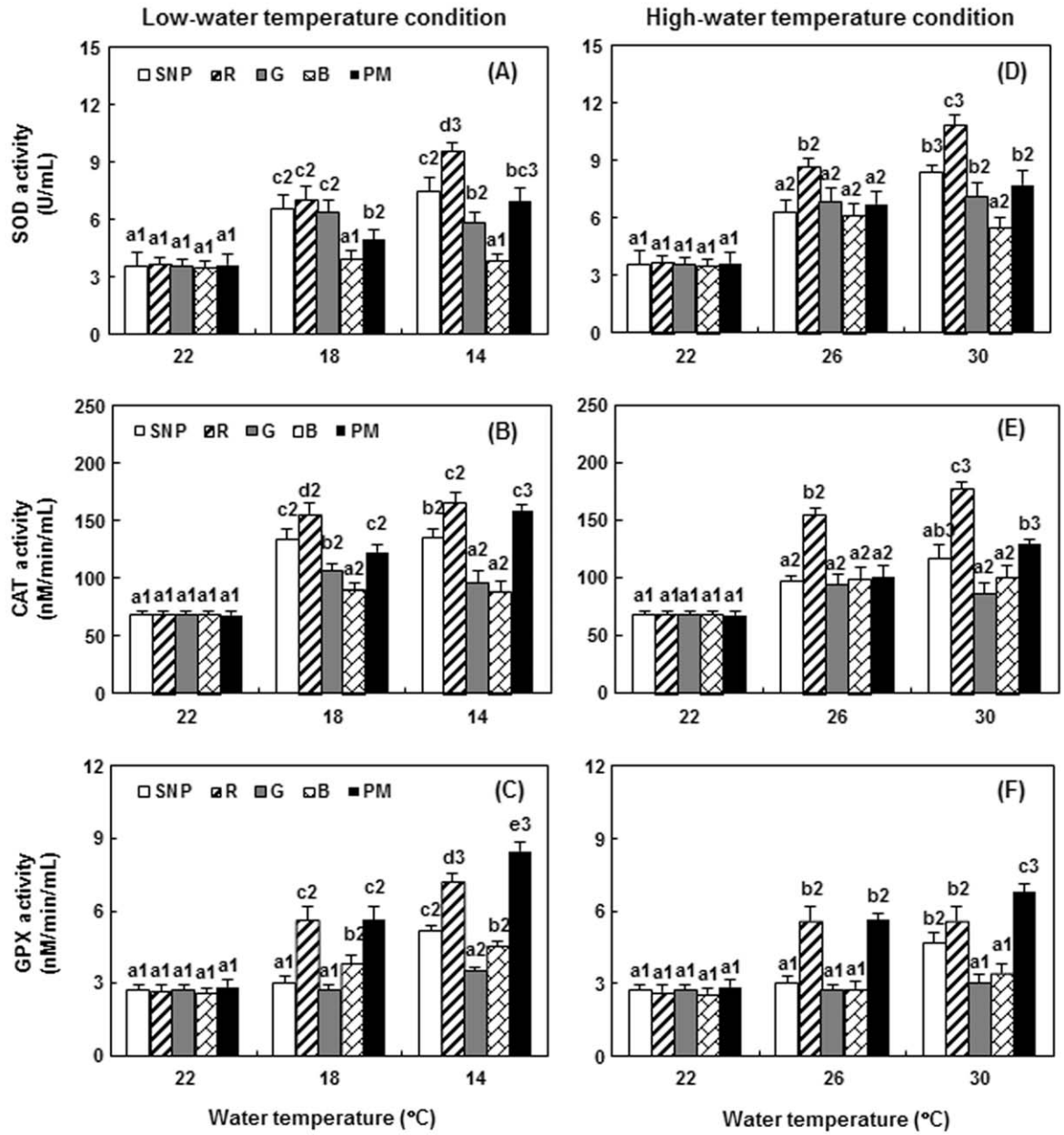

Figure 3. Changes in the activity of SOD (A, D), CAT (B, E), and GPX (C, F) mRNA in the liver during thermal changes (low-water temperature-A, B, C; high-water temperature-D, E, F) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod (SNP), as measured by microplate reader. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

HSP70 mRNA expression levels in the high-water temperature group in SNP and red LED spectra groups were significantly higher than the low-water temperature group, and the levels in the green and blue LED groups were significantly lower. Especially, the levels in the red LED groups were significantly higher than the other groups (Figure 5).

Plasma H_2O_2 and LPO levels

This study investigated the LPO and plasma H_2O_2 levels during temperature changes among different

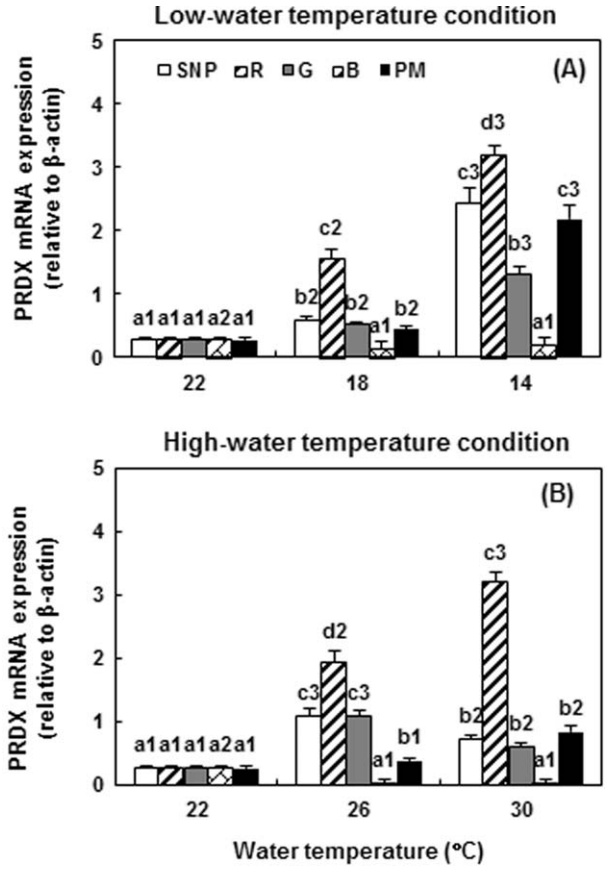

Figure 4. Changes in the PRDX mRNA expression in the liver during thermal changes (low-water temperature-A; high-water temperature-B) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

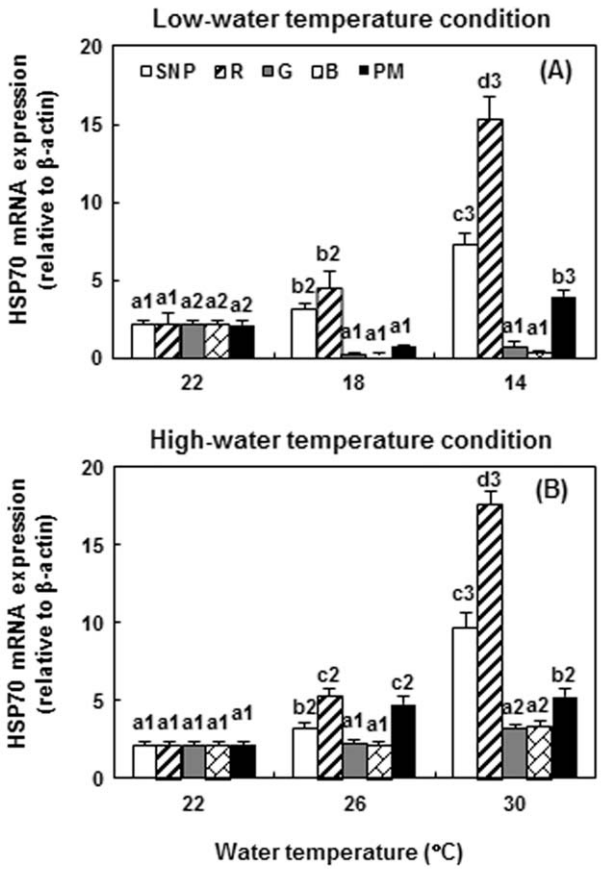

Figure 5. Changes in the HSP70 mRNA expression in the liver during thermal changes (low-water temperature-A; high-water temperature-B) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

LED light spectra using a plate reader (Figure 6).

The plasma H_2O_2 levels in the fluorescence bulb and red LED groups were significantly increased during temperature changes, but the levels in green and blue LED groups were significantly increased and then decreased, and the levels were lower than the other groups (Figure 6A, C). Additionally, the levels in the purple mix LED were similar to the green and blue LED groups. Furthermore, the levels of LPO in the muscle were significantly increased during temperature changes, but the levels in the green and blue LED groups were significantly lower than the other groups (Figure 6B, D).

Plasma melatonin concentration

The plasma melatonin levels in the control group were 12.5 ± 0.6 pg/mL, and the levels in the red LED groups during temperature changes significantly increased to 52.5 ± 2.6 pg/mL (Figure 7A, 14°C) and then decreased to 70.3 ± 2.1 pg/mL (Figure 7B, 30°C) (Figure 7). However, the levels in the green LED (25.6 ± 2.2 pg/mL, 14°C; 32.8 ± 2.8 pg/mL, 30°C) and blue LED (24.8 ± 1.8 pg/mL, 14°C; 27.7 ± 1.7 pg/mL, 30°C) groups decreased.

Plasma AspAT and AlaAT levels

The AspAT levels in the control group were 625.0 ±

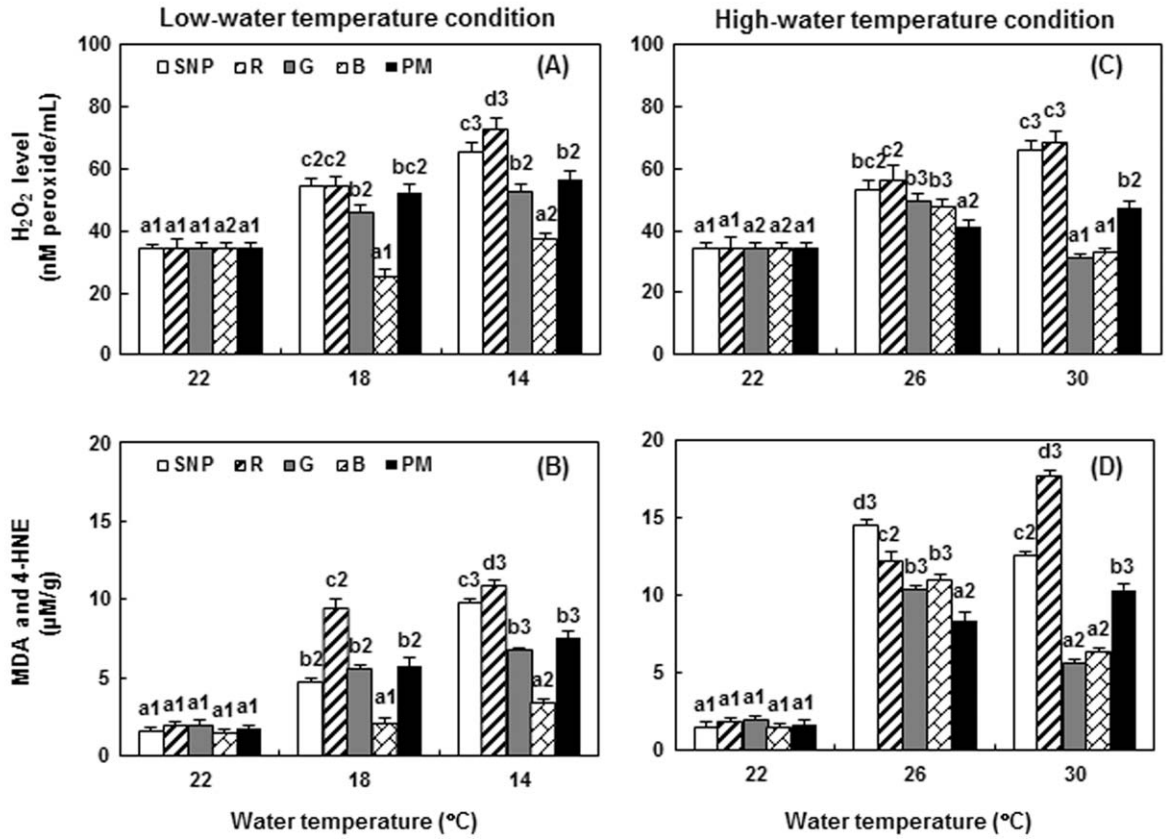

Figure 6. Plasma H_2O_2 concentrations (A, C) and LPO levels (B, D) during thermal changes (low-water temperature-A, B; high-water temperature-C, D) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod, as measured by microplate reader. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

45.5 U/L, and the levels in the red LED groups during temperature changes significantly increased to 1,572.8 ± 94.9 U/L (18°C) and then decreased to 2,490 ± 97.9 U/L (26°C) (Figure 8A, C). However, the levels in the green (880.7 ± 36.2 IU/L, 18°C; 390.7 ± 70.2 IU/L, 26 °C) and blue LED groups (390.7 ± 26.2 IU/L, 18°C; 520.7 ± 93.2 IU/L, 26°C) significantly decreased to the levels of the control groups. The levels in the purple LED group, which mixed red and blue LED lights, were significantly lower than red LED groups.

The AlaAT levels in the control group were 18.0 ± 2.5 U/L, and the levels in the red LED groups during temperature changes significantly increased to 38.8 ± 1.9 U/L (18°C) and 39 ± 2.9 U/L (26°C), respectively, and then decreased (Figure 8B, D). However, the AlaAT levels in the green (12.7 ± 1.2 IU/L, 18°C; 12.8 ± 1.0 IU/L, 26°C) and blue LED groups (14.5 ± 2.2 IU/L, 18°C; 14.7 ± 1.7 IU/L, 30°C) significantly decreased to the levels of the control groups.

Discussion

The present study aimed to test the effects of thermal (low-water temperature, 22 ⟶ 14°C; high-water temperature, 22 ⟶ 30°C) and oxidative stress on goldfish, as produced by different LED spectra (red, green, blue, and mixed purple), to investigate antioxidant response mechanisms. We investigated the effects of different LEDs on oxidative stress by measuring the expression and activities of antioxidant enzymes (SOD, CAT, GPX, and PRDX), an index of oxidative stress (H_2O_2 and LPO), AspAT, AlaAT, and a well-known antioxidant material, melatonin, to investigate the oxidative stress and immune function induced by low-/high-water temperature environments to investigate possibly controlling oxidative stress using specific LED light sources and molecular physiological effects.

Initially, we observed that antioxidant enzymes (SOD, CAT, and GPX) mRNA expression and activity signi-

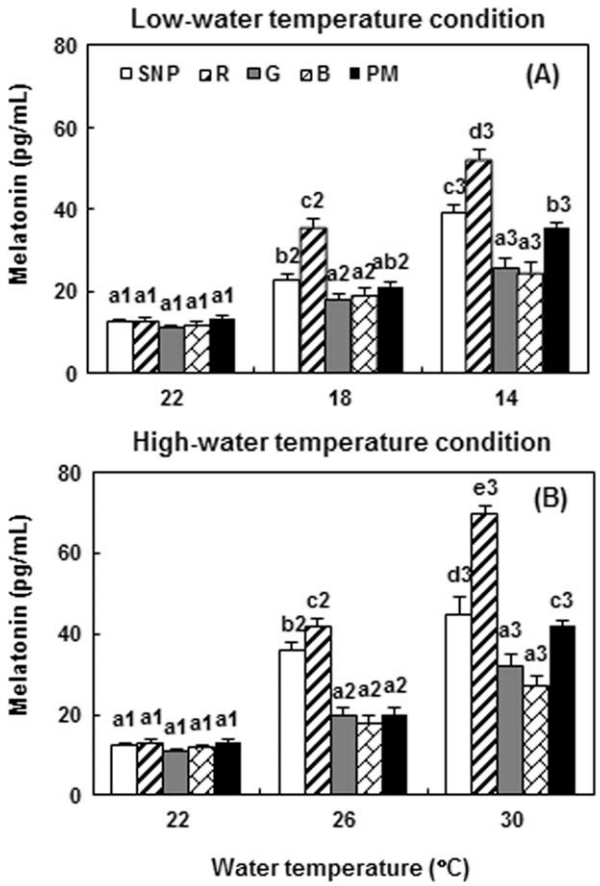

Figure 7. Plasma melatonin levels during thermal changes (low-water temperature-A; high-water temperature-B) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod, as measured by microplate reader. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means $\pm$SE (n=5).

ficantly increased in all experimental groups during thermal changes (Figures 2, 3) and significantly increased in the high-water temperature environment than the low-water temperature environment (Figures 2, 3). Additionally, we observed that the mRNA expression and activity of antioxidant enzymes was significantly higher under the red and mixed purple LED spectrum than for the other spectra. Thus, the result of this study was suggested that oxidative stress of goldfish was detected the temperature higher high-water temperature than low-water temperature, and green and blue LEDs reduced the oxidative stress than other LEDs.

Thermal stress accelerates the oxidation of polyamine in cells to generate ROS; thus, oxidative stress induced by ROS may be closely connected to the antioxidant response[10,30].

Additionally, mRNA expression PRDXs, more recently discovered-antioxidant enzymes, increased during thermal stress and decreased under short wavelength green and blue LEDs (Figure 4). PRDX enhances activity of natural killer cells and immune ability[31,32], and a previous study reported that PRDX increased during common aquaculture stressors, such as nutrient deficient diets, high-rearing density, and chronic exposure to *Enteromyxum leei* in the gilthead sea bream, *Sparus aurata*[33].

Choi *et al.*[27] reported that green and blue LEDs inhibit oxidative stress and enhance immune function in starved cinnamon clownfish, *A. melanopus*. In yellowtail clownfish, green and blue LEDs, both of which have short wavelengths, decreased activity and expression of antioxidant enzymes; therefore, light wavelength affected oxidative stress[26]. Regarding these results, LPO and plasma H_2O_2 concentrations increased according to increased thermal stress, but those in the green and blue LED groups were relatively lower than those in the other spectrum groups (Figure 6). These results indicated that lipid peroxidation material increased and then damaged tissues by acute thermal stress-induced ROS accumulation, whereas the short wavelength green and blue LEDs inhibited the acute thermal change-induced oxidative stress.

When oxidative stress is induced in fish, antioxidant compounds and enzymes are secreted[16]. Among these antioxidant compounds, melatonin, which is a known strong antioxidant within the antioxidant defense system, plays a role in clearing free radicals and is thought to directly detoxify free radicals and effectively protect the cell membrane against oxidative damage[23]. In this study, we observed lower melatonin levels in the green and blue LED groups than in the other groups during thermal stress (Figure 7). This result was consistent with the ROS result that showed lower H_2O_2 concentrations in the green and blue LED groups; thus, melatonin levels, which can reduce ROS, were low. However, melatonin levels in the fluorescence bulb and red LED groups were significantly high, thus suggesting that in the presence of a high level of ROS, melatonin was secreted to clear ROS. A previous study reported that in yellowtail clownfish[26] and cinnamon clownfish[27], lower melatonin levels in green and blue LED groups than in the other groups. Additionally, ROS was eliminated by melatonin in yellowtail clownfish under red, green, and blue LEDs, and melatonin levels in the green and blue LED groups were significantly lower than those in the other groups. Therefore, this result and that reported by Shin *et al.*[26] suggested that the short wavelength green and blue LED groups in-

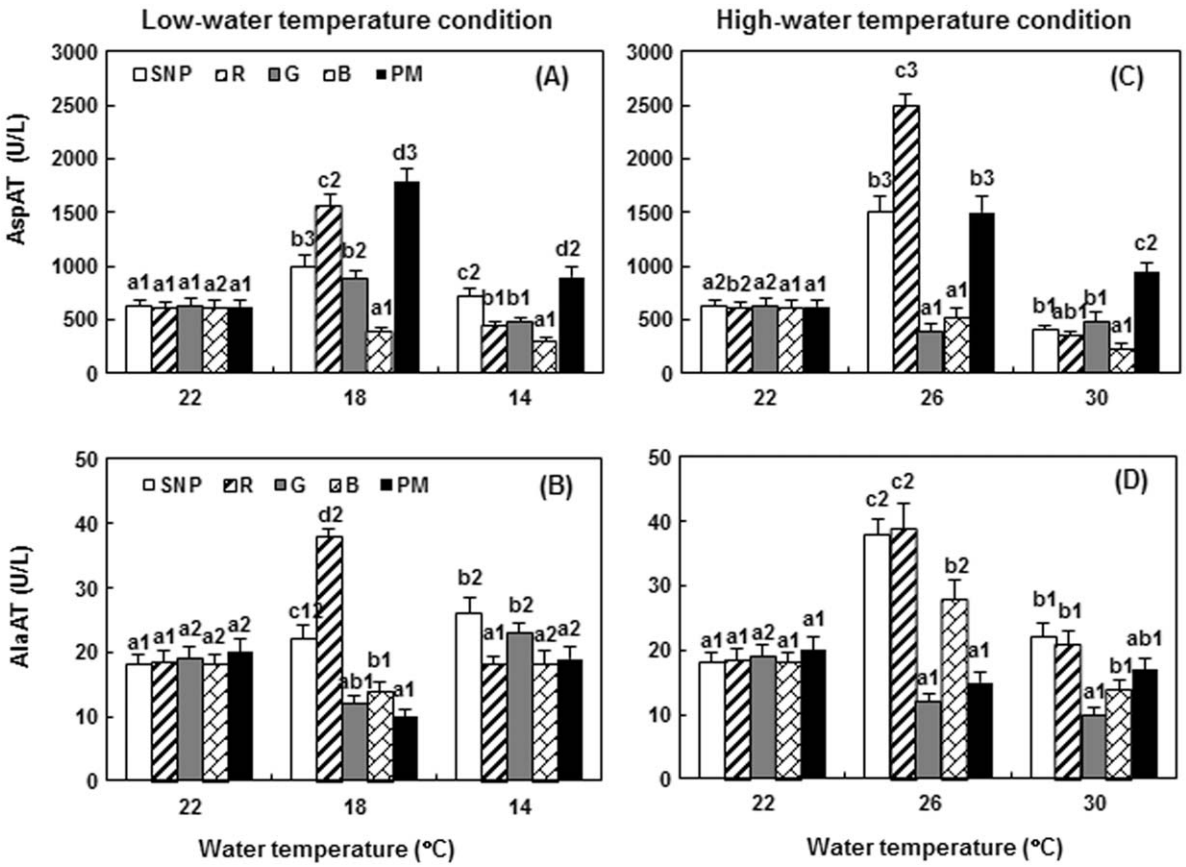

Figure 8. Plasma AspAT (A, C) and AlaAT levels (B, D) during thermal changes (low-water temperature-A, B; high-water temperature-C, D) of goldfish under lighting conditions using red (R), green (G), blue (B), and purple mix (PM) light-emitting diodes and a simulated natural photoperiod, as measured by microplate reader. Values with numbers are significantly different the temperature within the same LED spectra ($P<0.05$). The lowercase letters indicates significant differences between different LED spectra within the same temperature ($P<0.05$). All values are means ± SE ($n=5$).

duced relatively less oxidative stress.

HSP70 is detected during oxidative stress with environmental water temperature changes in teleosts, and the expression of HSP70 mRNA was higher in high-water temperatures and the red LED group than in low-water temperatures and the other LED groups (Figure 5). It is reported that HSP70 is associated with water temperature to be particularly sensitive to environmental stress factors[34], and their universality, extraordinary conservation in structure, and consistency with which they are induced by a broad spectrum of stressors have made HSPs good candidates for bio-monitoring of the environment[6,7]. This result is consistent with a previous study: the expression of HSP70 mRNA was higher in 30˚C of a high-water temperature environment group than 20˚C of the control group in the black porgy *Acanthopagrus schlegeli*[5]. Furthermore, the HSP70 mRNA expression level was lower for the green and blue LED groups based on the results of this study, and we considered that oxidative stress was produced by acute temperature changes; thus, the wavelengths of green and blue LEDs can effectively control oxidative stress.

Additionally, acute environmental water temperature changes induce stress in fish, and the primary stress responses induce secondary responses that affect energy requirements, such as increases in plasma glucose levels[35]. AspAT and AlaAT, well-known liver damage indicators, are also increased by stress[36]. In this study, plasma AspAT and AlaAT levels were significantly higher in high-water temperatures than low-water temperatures, and the green and blue LED groups were significantly lower than those in the other spectrum groups during thermal stress (Figure 8). This result suggested that oxidative stress was induced by environmental water temperature changes and increased concentrations of AspAT and AlaAT lowered liver function. In particular, plasma AspAT and AlaAT levels

were decreased in 30°C of high-water temperatures, this result was similar to the previous study, Pérez-Casanova *et al*.[37]. The previous study was reported that when 10°C acclimated Atlantic cod, *Gadus morhua* were exposed to graded thermal changes (10-20°C), secondary stress responses such as cortisol and glucose were elevated significantly at 16°C but returned to control levels thereafter[37]. Pérez-Casanova *et al*.[37] was suggested that 16°C is stressful for Atlantic cod, and that this effect is independent of the rate of temperature increase and thermal challenges is likely due to a negative-feedback effect of elevated cortisol on the hypothalamus-pituitary-interrenal (HPI) axis. Additionally, blue and green LED wavelengths reduced plasma AspAT and AlaAT levels. Volpato & Barreto[38] reported that cortisol levels were significantly lower in the blue illumination group, when *O. niloticus* were placed into high-density tanks under green, blue, and white spectra for 48 h, and the cortisol levels under the blue spectrum were significantly lower than those in the other groups. This result indicates that hormone changes by light spectrum affect the pituitary-adrenal axis and that the light spectra affect the biological system[38].

In conclusion, we hypothesized that goldfish would detect higher thermal stress in high-water temperatures than low-water temperatures, and short wavelength green and blue LEDs would inhibit thermal-induced oxidative stress through antioxidant enzymes, ROS and LPO, and play a role in protecting against harmful ROS in goldfish. Green and blue wavelengths within a visible light also effectively enhance immune function. Additional studies are necessary to identify the mechanism of protective capacity using photoreceptors and various stress factors in fish related to different light spectra at various molecular levels.

Materials & Methods

Experimental fish

Goldfish (length, 11.0 ± 0.2 cm; BW, 12.1 ± 0.6 g) were purchased from the commercial aquarium (Busan, Korea) and were allowed to acclimate for 2 weeks in ten 100-L circulation filter tanks in the laboratory. The fish were exposed to a simulated natural photoperiod (SNP). A white fluorescent bulb (27 W) was used for the control group, and light intensity near the water surface of the tanks was approximately 0.96 W/m^2. For the experimental groups, the fish were exposed to either red (630 nm), green (530 nm), blue (peak at 450 nm), or a purple mix (mixed 450 nm and 630 nm) LED (Daesin LED Co. Kyunggi, Korea) (Figure 1). The LEDs were located 50 cm above the surface of water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2. The water temperature and photoperiod were 22 ± 1°C and a 12 h light : 12 h dark period (lights on at 07 : 00 h and lights off 19:00 h), respectively.

Temperature change

The goldfish were reared in freshwater in the ten circulating filter tanks (100-L) with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimate to the conditions for 24 h. The water temperature was then decreased from 22°C to 14°C in daily increments of 2°C. Additionally, water temperature was then increased from 22°C to 30°C in daily increments of 2°C. No fish died during the water temperature changes. During the experimental period, the salinity and photoperiod were maintained at 35 psu and 12 h light : 12 h dark period, respectively.

Sampling

Five fish from each group (low-water temperature, 22, 18, and 14°C; high-water temperature, 22, 26, and 30°C) were randomly selected for tissue and blood collection and anesthetized with tricaine methanesulfonate (200 mg/L, MS-222; Sigma, St. Louis, MO, USA); blood was obtained from the caudal vasculature using a 3-mL heparinized syringe. After centrifugation (10,000 × g, 4°C, 5 min), the plasma was stored at −80°C before analysis, and the fish were killed by a spinal transection to collect the liver.

Quantitative real-time PCR (QPCR)

QPCR was conducted to determine the relative expression of antioxidant enzymes (SOD, CAT, GPX, and PRDX) and HSP70 mRNA by using the total RNA extracted from the liver of goldfish. Primers for QPCR were designed with reference to the known sequences of goldfish, which are shown in Table 1. QPCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ^{TM} SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The experiments were performed in triplicate to ensure consistency. β-actin was used as an internal control, and all data were expressed as the change with respect to the corresponding calculated threshold cycle for β-actin (ΔCt). The calibrated ΔCt value (ΔCt) for each sample and internal control (β-actin) was calculated as $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

SOD, CAT, and GPX activity analysis

The liver tissues were homogenized in ice-cold 0.1 M

Table 1. Primers used for QPCR amplification.

Genes	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′
GPX (JQ776515)	Forward	5′-CGG TGA ACA GGA ATG ACA-3′
	Reverse	5′-GTG AGG AAC CTT CTG CTG-3′
PRDX (KF040053)	Forward	5′-CCT CCG CTC CAT ATC TCA-3′
	Reverse	5′-CCT CAG AAT GCC CTT GTC-3′
HSP70 (DQ872648)	Forward	5′-TCA CCA TCA CCA ATG ACA AG-3′
	Reverse	5′-GGC GTA AGA CTC CAG AGA-3′
β-actin (AB039726)	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

PBS (pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was removed, and the remaining pellet was used for the analyses. SOD, CAT, and GPX activities were determined using commercial kits supplied by Cayman Chemical (Ann Arbor, MI, USA). Each assay was performed in duplicate, and the enzyme units were recorded as U/mL, nM/min/mL, and nM/min/mL (SOD, CAT, and GPX; respectively).

H_2O_2 and LPO assay

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh *et al.*[39] and a Peroxid Detect 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 nM/mL.

LPO was quantified by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs) hydroperoxides[40] and was measured according to the manufacturer's instructions (Lipid Hydroperoxide Assay Kit, Cayman Chemical). The absorbance was read at 500 nm using a plate reader. LPO was expressed as nM of MDA and 4-HNE/g protein.

Melatonin assay

To determine the melatonin concentration in the plasma, the immunoenzymoassay method was used with a commercial ELISA kit (IBL, Nunc, GmbH, Germany). Absorbance was read at 405 nm.

Plasma parameter analysis

The plasma AspAT, and AlaAT levels were measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-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 means ± standard error (SE).

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References

1. Beckmann, R. P., Mizzen, L. E. & Welch, W. J. Interaction of HSP70 with newly synthesized proteins: implications for protein folding and assembly. *Science* **248**: 850-854 (1990).
2. Yang, J. H. & Yeo, I.-K. Physiological studies on acute water-temperature stress of olive flounder (*Paralichthys olivaceus*). *Korean J Ichthyol* **16**:19-26.
3. Chang, Y. J., Hur, J. W., Lim, H. K. & Lee, J. K. Stress in olive flounder (*Paralichthys olivaceus*) and fat cod (*Hexagrammos otakii*) by the sudden drop and rise of water temperature. *J Korean Fish Soc* **34**:91-97 (2001).
4. Bowden, T. J. Modulation of the immune system of fish by their environment. *Fish Shellfish Immunol* **25**: 373-383 (2008).
5. Choi, C. Y., Min, B. H., Kim, N. N., Cho, S. H. & Chang, Y. J. Expression of HSP90, HSP70 mRNA and change of plasma cortisol and glucose during water temperature rising in freshwater adapted black porgy, *Acanthopagrus schlegeli*. *J Aquaculture* **19**:315-322 (2006).
6. Welch, W. J. The role of heat-shock proteins as mole-

cular chaperones. *Curr Opin Cell Biol* **3**:1033-1038 (1991).
7. Young, D., Roman, E., Moreno, C., Brien, R. O. & Born, W. Molecular chaperones and the immune response. *Philos Trans R Soc London* **339**:B363-367 (1993).
8. Parihar, M. S., Dubey, A. K., Faveri, T. & Prakash, P. Changes in lipid peroxidation, superoxide dismutase activity, ascorbic acid and phospholipids content in liver of freshwater catfish *Heteropneustes fossilis* exposed to elevated temperature. *J Therm Biol* **21**:323-330 (1996).
9. Hwang, D. F. & Lin, T. K. Effect of temperature on dietary vitamin C requirement and lipid in common carp. *Comp Biochem Physiol* **131**:1-7 (2002).
10. Abele, D., Burlando, B., Viarengo, A. & Pörtner, H. O. Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal linpet *Nacella conxcinna*. *Comp Biochem Physiol* **120**:B425-435 (1998).
11. Pandey, S. *et al*. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (BI. & Schn.). *Sci Total Environ* **309**:105-115 (2003).
12. George, S. *et al*. A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platicthys flesus*) subjected to estuarine pollution. *Mar Environ Res* **58**:571-575 (2004).
13. Chan, H. M. & Cherian, M. G. Protective roles of metallothionein and glutathione in hepatotoxicity of cadmium. *Toxicology* **72**:281-290 (1992).
14. Klaassen, C. D. & Liu, J. Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. *Environ Health Perspect* **106**:297-300 (1998).
15. Hansen, B. H., Romma, S., Garmo, O. A., Olsvik, P. A. & Andersen, R. A. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. *Comp Biochem Physiol* **143**:C263-274 (2006).
16. Basha, S. P. & Rani, U. A. 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). *Ecotoxicol Environ Saf* **56**:218-221 (2003).
17. Shull, S. *et al*. Differential regulation of Antioxidant enzymes in response to oxidants. *J Biol Chem* **226**: 24398-24403 (1991).
18. Parihar, M. S., Javeri, T., Hemnani, T., Dubey, A. K. & Prekash, P. Responses of superoxide dismutase, glutathione peroxidaseand reduced glutathione antioxidant defenses in gills ofthe freshwater catfish (*Heteropneustes fossilis*) to short-term elevated temperature. *J Therm Biol* **22**:151-156 (1997).
19. Hofmann, B., Hecht, H. J. & Flohé, L. Peroxiredoxins. *Biol Chem* **383**:347-364 (2002).
20. Wood, Z. A., Schröder, E., Harris, J. R. & Poole, L. B. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* **28**:32-40 (2003).
21. Aguirre, J., Momberg, M. R., Hewitt, D. & Hansberg, W. Reactive oxygen species and development in microbial eukaryotes. *Trends Microbiol* **13**:111-118 (2009).
22. Mu, C. *et al*. Molecular cloning and characterization of peroxiredoxin 6 from Chinese mitten crab *Eriocheir sinensis*. *Fish Shellfish Immunol* **26**:821-827 (2009).
23. Reiter, R. J., Carneiro, R. C. & Oh, C. S. Melatonin in relation to cellular antioxidative defense mechanisms. *Horm Metab Res* **29**:363-372 (1997).
24. Chen, X. *et al*. A strategy for the ubiquitous overexpression of human catalase and CuZn superoxide dismutase genes in transgenic mice. *Mech Ageing Dev* **124**:219-227 (2003).
25. Heise, K., Puntarulo, S., Nikinmaa, M., Abele, D. & Portner, H. O. Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus*. L. *J Exp Biol* **209**:353-363 (2006).
26. Shin, H. S., Lee, J. & Choi, C. Y. Effects of LED light spectra on oxidative stress and the protective role of melatonin in relaation to the daily rhythm of the yellowtail clownfish, *Amphiprion clarkia*. *Comp Biochem Physiol* **160**:A221-228 (2011).
27. Choi, C. Y. *et al*. Effects of LED light spectra on starvation-induced oxidative stress in the cinnamon clownfish, *Amphprion melanopus*. *Comp Biochem Physiol* **163**:A357-363 (2012).
28. Migaud, H., Cowan, M., Taylor, J. & Ferguson, H. W. The effect of spectral composition and light intensity on melatonin, stress and retinal damage in post-smolt Atlantic salmon, *Salmo salar*. *Aquaculture* **270**:390-404 (2007).
29. Villamizar, N., García-Alcazar, A. & Sánchez-Vázquez, F. J. Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **292**: 80-86 (2009).
30. Liu, X. L. *et al*. Gene expression of different wheat genotypes during attack by virulent and avirulent Hessian fly (*Mayetiola destructor*) larvae. *J Chem Ecol* **33**: 2171-2194 (2007).
31. Sauri, H., Ashjian, P. H., Kim, A. T. & Shau, H. Recombinant natural killer enhancing factor augments natural killer cytotoxicity. *J Leukocyte Biol* **59**:925-931 (1996).
32. Utke, K. *et al*. Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. *Fish Shellfish Immunol* **22**:182-196 (2007).
33. Pérez-Sánchez, J., Bermejo-Nogales, A., Calduch-Giner, J. A., Kaushik, S. & Sitjà-Bobadilla, A. Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream *Sparus aurata* insights from fish exposed to dietary, pathogen and confinement stressors. *Fish Shellfish Immunol* **31**:294-302 (2011).
34. Feder, M. E. & Hofmann, G. E. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Phys* **61**: 243-282 (1999).

35. Carmichael, G. J., Tomasso, J. R., Simco, B. A. & Davis, K. B. Confinement and water quality-induced stress in largemouth bass. *Trans Am Fish Soc* **113**:767-777 (1984).
36. Pan, C. H., Chien, Y. H. & Hunter, B. The resistance to amminia stress of *Penaeus monodon* Fabricius juvenile fed diets supplemented with astaxanthin. *J Exp Mar Biol Ecol* **297**:107-118 (2003).
37. Pérez-Casanova, J. C. *et al*. The immune and stress responses of Atlantic cod to long-term increases in water temperature. *Fish Shellfish Immunol* **24**:600-609 (2008).
38. Volpato, G. L. & Barreto, R. E. Environmental blue light prevents stress in the fish Nile tilapia. *Braz J Med Biol Res* **34**:1041-1045 (2001).
39. Nouroozzadeh, J., Tajaddinisarmadi, J. & Wolff, S. P. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* **220**: 403-409 (1994).
40. Esterbauer, H., Schaur, R. J. & Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11**:81-128 (1991).