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Effect of LED light spectra on starvation-induced oxidative stress in the cinnamon clownfish *Amphiprion melanopus*

Cheol Young Choi [a,*], Hyun Suk Shin [a], Young Jae Choi [a], Na Na Kim [a], Jehee Lee [b], Gyung-Suk Kil [c]

[a] Division of Marine Environment & BioScience, Korea Maritime University, Busan 606–791, Republic of Korea
[b] Department of Marine Life Sciences, Jeju National University, Jeju Special Self-Governing Province 690–756, Republic of Korea
[c] Division of Electrical and Electronic Engineering, Korea Maritime University, Busan 606–791, Republic of Korea

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ABSTRACT

The present study aimed to test starvation-induced oxidative stress in the cinnamon clownfish *Amphiprion melanopus* illuminated by light-emitting diodes (LEDs): red (peak at 630 nm), green (peak at 530 nm), and blue (peak at 450 nm) within a visible light. We investigated the oxidative stress induced by starvation for 12 days during illumination with 3 LED light spectra through measuring antioxidant enzyme (superoxide dismutase [SOD] and catalase [CAT]) mRNA expression and activity; CAT western blotting; and measuring lipid peroxidation [LPO]), plasma H_2O_2, lysozyme, glucose, alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), and melatonin levels. In green and blue lights, expression and activity of antioxidant enzyme mRNA were significantly lower than those of other light spectra, results that are in agreement with CAT protein expression level by western blot analysis. Also, in green and blue lights, plasma H_2O_2, lysozyme, glucose, AlaAT, AspAT, and melatonin levels were significantly lower than those in other light spectra. These results indicate that green and blue LEDs inhibit oxidative stress and enhance immune function in starved cinnamon clownfish.

1. Introduction

Starvation enhances aging, ischemia-reperfusion injury, and toxicity to chemicals and disease. These effects could be mainly attributed to the reactive oxygen species (ROS) generated by starvation that lead to oxidative stress (Robinson et al., 1997). Decreased feeding also depletes organ antioxidant stores and increases the generation of oxygen free radicals, particularly in the liver (Robinson et al., 1997).

ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$), are naturally produced during oxidative metabolism (Roch, 1999). ROS overproduction by environmental stress factors can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity, and can then accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003). Furthermore, ROS may seriously affect immune function by decreasing lysozyme activity and inducing oxidative stress (Fisher and Newell, 1986; Wang et al., 2008).

Therefore, complex antioxidant defense systems maintain homeostasis and protect aerobic organisms against ROS and the subsequent oxidative stress-induced damage. Antioxidants may include enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (McFarland et al., 1999). Antioxidant defense systems are found in the livers and kidneys of marine organisms (Basha Siraj and Rani Usha, 2003; Hansen et al., 2006).

Melatonin, a hormone that acts as a neuroendocrine messenger in the regulation of circadian rhythm, also has a number of other physiological functions, including clearing free radicals, improving immunity, and generally inhibiting the oxidation of biomolecules (Wu and Swaab, 2005). Melatonin is considered a broad-spectrum antioxidant that is more powerful than glutathione in neutralizing free radicals and more effective than other antioxidants in protecting cell membranes (Reiter et al., 1997).

Shin et al. (2011) recently reported that green and blue light-emitting diodes (LEDs), both of which have short wavelengths, increased antioxidant materials against oxidative stress in the yellowtail clownfish *Amphiprion clarkii*. The blue wavelength has also been shown to play a role in protecting against stress in the Nile tilapia *Oreochromis niloticus* (Volpato and Barreto, 2001). Meanwhile, the red LED wavelength affects physiological function and induces oxidative stress in yellowtail clownfish (Shin et al., 2011). LEDs, a relatively new lighting technology that remains under development, can be manufactured to output specific wavelengths (Migaud et al., 2007). Narrow bandwidth light using such new technologies could thus provide much more efficient lighting systems than those currently used in the fish farming industry since they can be tuned to a species environmental sensitivity by emitting narrow bandwidths (Villamizar et al., 2009).

* Corresponding author. Tel.: +82 51 410 4756; fax: +82 51 404 4750.
E-mail address: choic@hhu.ac.kr (C.Y. Choi).

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On the basis of these results, we hypothesized that LEDs could be applied to induce a photoenvironment among various inhabitation environment factors. It is known that the spectral composition of incident light changes differentially in underwater environments and that rapid attenuation occurs with increasing depth (Lythgoe, 1979). However, investigations on the stress response of fish to light wavelengths remain very limited (Head and Malison, 2000; Van der Salm et al., 2004; Shin et al., 2011).

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 the antioxidant enzymes SOD, CAT, and GPX, and the expression changes of the CAT protein using CAT western blotting during 12-day starvation under 3 wavelengths (red, green, and blue within a visible light) in cinnamon clownfish, a well-known high-value ornamental fish. We also determined the oxidative stress in the fish by measuring changes in plasma H_2O_2 concentration and LPO levels, examined immune function changes by measuring lysozyme activity, and assessed liver damage by measuring aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) levels.

2. Materials and methods

2.1. Experimental fish

Cinnamon clownfish ($n=200$; total length, 6.2 ± 0.5 cm; mass, 5.2 ± 1.1 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and maintained in eight 300-L circulation filter tanks before the experiments.

The fish were exposed to a simulated natural photoperiod (SNP). A white fluorescent bulb (27 W) was used for the control group, and the light intensity near each tank's water surface was approximately 0.96 W/m^2. The water temperature and photoperiod were 27 ± 1 °C and a 12-h light:12-h dark period (light on, 07:00 h; light off, 19:00 h), respectively. The control group was given white fluorescent light and fed a commercial feed twice daily (09:00 h and 17:00 h). The experimental groups were exposed to blue (peak at 450 nm), green (peak at 530 nm), or red (peak at 630 nm) within a visible light LEDs (Daesin LED Co., Kyunggi, Korea) during the starvation period (12 days). The LEDs were positioned 50 cm above the water surface, and the irradiance at the water surface was maintained at approximately 0.96 W/m^2. Blood was collected from the caudal vein using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000 g, 5 min) and stored at −80 °C. The fish were euthanized by spinal transection at 3-day intervals (days 3, 6, 9, and 12 of the starvation period) and the livers were collected under dim light.

2.2. Sampling

The livers were collected from 5 fish in each group [Fed-SNP, Starved-group (SNP, Red, Green, and Blue LED)] at 0, 3, 6, 9, and 12 days. Immediately after collection, the livers were frozen in liquid nitrogen and stored at −80 °C until total RNA extraction was performed. Blood was taken from the caudal vasculature using a 1-mL syringe coated with heparin. After centrifugation (4 °C, 10,000 g, 5 min), the plasma was stored at −80 °C before analysis.

2.3. Quantitative polymerase chain reaction (QPCR)

QPCR was conducted to determine the relative expression of SOD (accession no. JQ906787), CAT (JQ906788), and GPX (GU799604) mRNA using total RNA extracted from the cinnamon clownfish livers. The primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows: SOD forward primer (5′-GTT GCC AAG ATA GAC ATC AC-3′), SOD reverse primer (5′-TTA GAC TCT CCT CGT TGC-3′), CAT forward primer (5′-GCA ACT ACC AGC GTG ATG-3′), CAT reverse primer (5′-CAG ACA CCT TGA ACT TGG A-3′), GPX forward primer (5′-CAG GAG AAC GGC AAG AAT-3′), GPX reverse primer (5′-TTC CAT TCA CAT CCA CCT T-3′), β-actin forward primer (5′-GCA AGA GAG GTA TCC TGA CC-3′), and β-actin reverse primer (5′-CTC AGC TCG TTG TAG AAG G-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was performed as follows: 95 °C for 5 min, followed by 35 cycles each of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with β-actin calculated threshold cycle (Ct) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.4. Western blot analysis

Total protein isolated from livers of cinnamon clownfish during starvation was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM ethylenediaminetetraacetic acid [EDTA], 0.55 mM ethyleneglycoltetraacetic acid, 0.1% sodium dodecyl sulfate, 0.15 mg/mL phenylmethylsulfonyl fluoride, and 0.15 mg/mL leupeptin], sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded in each lane of a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A protein ladder (Fermentas, Glen Burnie, MD, USA) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye front ran off of the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min followed by washing in TBS. Membranes were incubated with catalase (sc-58332, 1/2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody (dilution 1:2000; Bio-Rad) for 60 min. The internal control was β-tubulin (dilution 1:5000, ab6046; Abcam, Cambridge, UK), followed by HRP-conjugated anti-rabbit IgG secondary antibody (1:5000; Bio-Rad) for 60 min. Bands were detected using standard and more sensitive electrochemiluminescence (ECL) systems (ECL Advance; GE Life Sciences, Uppsala, Sweden) and exposure to autoradiography-sensitive film for 2 min.

2.5. SOD, CAT, and GPX activity analysis

Liver tissues were homogenized in ice-cold 0.1 M phosphate buffered saline (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 (EC 1.15.1.1), CAT (EC 1.11.1.6), and GPX (EC 1.11.1.9) activities were determined using commercial kits supplied by Cayman Chemical (Ann Arbor, MI, USA). Each assay was performed in three repetitions using five fish, respectively, the enzyme units were recorded in U/mL, and the CAT activity was expressed in nmol/min/mL.

Cu/Zn-SOD activity was assessed using a tetrazolium salt for detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of Cu/Zn-SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Absorbance was read at 450 nm. Each assay was performed in duplicate and the enzymes were recorded as U/mL. The CAT activity assay was based on reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald®) as the chromogen (Wheeler et al., 1990). Purpald® specifically forms a bicyclic heterocycle with aldehydes; upon oxidation, the

solution changes from colorless to purple. Absorbance was read at 540 nm. Each assay was performed in duplicate, and CAT activity was expressed in nmol/min/mL. GPX activity was measured indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPX, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. GPX activity was expressed in nmol/min/mL.

2.6. H_2O_2 assay

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect Kit (Sigma, USA). A total 20 μL of cinnamon clownfish plasma was added to each well of flat-bottom 96-well plates. Plates were left at room temperature for 20 min to allow the plasma to settle and adhere to the plate. A working color reagent was prepared by mixing 100 mL of distilled water containing 100 mM sorbitol and 125 μM xylenol orange (Sigma) with 1 mL of 25 mM ferrous ammonium sulfate prepared in 2.5 M sulfuric acid (Sigma). Two hundred microliters of this reagent was then added to each well and allowed to incubate at room temperature for 1 h. Absorbance was read at 560 nm, and the concentration of H_2O_2 was interpolated from a standard curve. Concentrations are expressed as nM/mL.

2.7. LPO assay

LPO was quantified by measuring the amounts of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acid hydroperoxides (Esterbauer et al., 1991), using a Lipid Hydroperoxide Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Tissue (1 g) was homogenized in 10 mL of high-performance liquid chromatography-grade water. A total of 500 μL of the cytosolic fraction of the homogenate was added to a glass tube, and 500 μL of chloroform, 450 μL of chloroform–methanol, and 50 μL of ferric thiocyanide solution (FTS); reagent 1 and FTS reagent 2 mixtures (Cayman Chemical) were added to the glass tube and mixed. This sample was incubated for 5 min at room temperature. Samples (300 μL per well) were added to flat-bottom 96-well plates. The absorbance was read at 500 nm using a plate reader. LPO is expressed as nanomolars of MDA and 4-HNE per gram of protein.

2.8. Lysozyme activity

Lysozyme activity was quantified in hemolymph according to Santarem et al. (1994). The pooled hemolymph was centrifuged at 780 g for 10 min and the supernatant was collected. Hemolymph was frozen and stored at −80 °C before analysis. Hemolymph (10 μL) was then added to 190 μL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 5 min at 20 °C in a 96-well plate. The average decrease in absorbance per minute was determined for each enzyme solution and a standard curve of enzyme concentration vs. ΔA/min was drawn. One unit of lysozyme was defined as the amount of enzyme producing activity equivalent to 1 μg of lysozyme under the conditions described above. Results are expressed as micrograms of lysozyme per milligram of protein.

2.9. Plasma parameter analysis

Plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min), and then plasma glucose, AspAT (EC 2.6.1.1), and AlaAT (EC 2.6.1.2) levels were measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

2.10. Melatonin determination by ELISA

The melatonin concentration in the plasma was determined using the enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). Plasma samples were purified immediately after defrosting in the centrifuge. They were then purified with the extraction columns. After that, 50 μL of each sample was added to different wells of an ELISA plate that was precoated with capture antibody. The samples were incubated with the melatonin–biotin and antiserum solutions for 15 h at 4 °C. The wells were then washed with the assay buffer (phosphate buffer with Tween and stabilizer) and the plate was incubated with the enzyme-labeled solution (antibiotin–alkaline phosphatase in TRIS buffer with stabilizers) for 2 h at room temperature with constant shaking. After the plate was washed a second time, it was incubated with the *p*-nitrophenyl phosphate solution for 30 min before 50 μL of the stop solution (1 N NaOH with 0.25 M EDTA) was added. Absorbance was read at 405 nm.

2.11. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Tukey's post hoc test was used to assess statistically significant differences among the different days after starvation and different light spectra. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Expression and activity of antioxidant enzymes (SOD, CAT, and GPX) in the liver

We examined the antioxidant enzyme mRNA expression and activity during starvation among the light spectra using QPCR (Figs. 1 and 2). In all experimental groups, mRNA expression and activity levels were significantly high at 6 days and then decreased after starvation. Otherwise, mRNA expression and activity levels were significantly lower in the green-LED and blue-LED groups than in the other light spectra (Figs. 1 and 2). Western blot analysis revealed a protein with CAT-specific immunoreactivity and a mass that corresponded to the predicted mass of cinnamon clownfish CAT (64 kDa). The expression pattern of the protein resembled the pattern of the CAT mRNA expressed in cinnamon clownfish livers (Fig. 1A).

3.2. LPO and plasma H_2O_2 levels

We observed the LPO and plasma H_2O_2 levels during starvation among light spectra using a plate reader (Fig. 3). At first, LPO levels in all experimental groups were significantly increased at 3 days into the starvation period. However, in the green-LED and blue-LED groups, the LPO levels were significantly lower than those of other light spectra (Fig. 3A).

Furthermore, plasma H_2O_2 levels were significantly increased until 12 days in the starved-SNP and red-LED groups, whereas the levels in the green-LED and blue-LED groups were significantly increased at 6 days and then decreased to levels lower than the other light spectra (Fig. 3B).

3.3. Plasma glucose, AlaAT, and AspAT levels

We observed the plasma glucose, AlaAT, and AspAT levels during starvation among light spectra by biochemistry autoanalyzer (Fig. 4). At first, plasma glucose levels in all experimental groups were significantly increased at 6 days (starved-SNP, 4.8 ± 0.2 mmol/L; starved-red LED, 5.5 ± 0.3 mmol/L) and then decreased after starvation. However, in the green-LED and blue-LED groups, glucose levels were significantly lower than in the other light spectra (Fig. 4A).

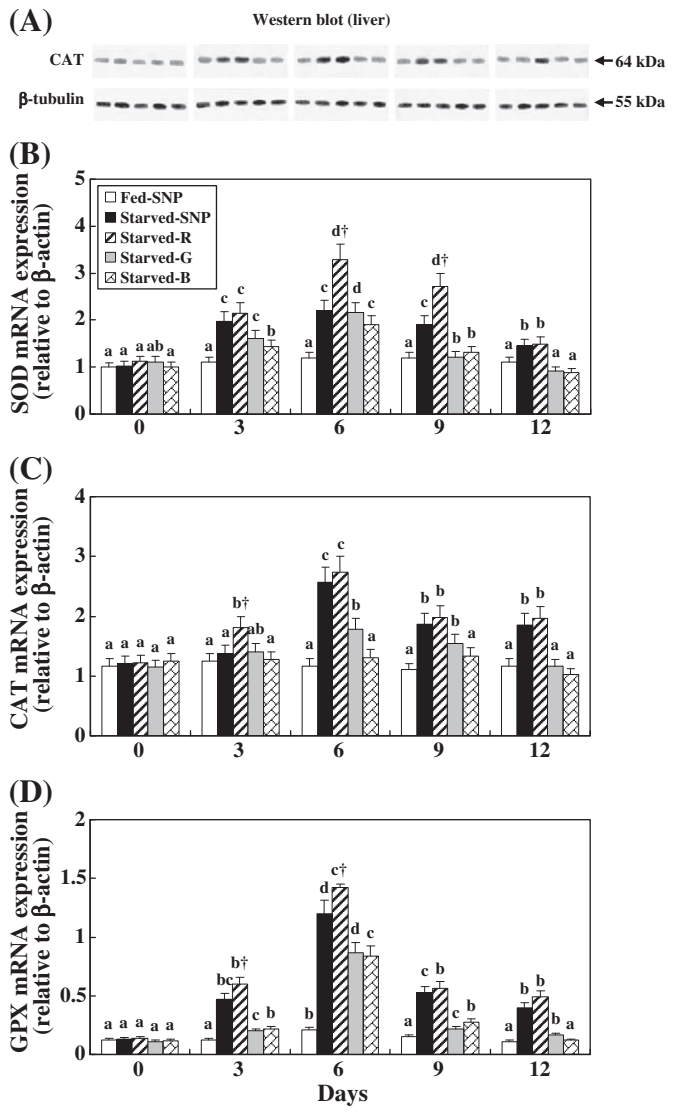

Fig. 1. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) mRNA expression levels in the livers of cinnamon clownfish during starvation under lighting conditions using red (R), green (G), and blue (B) light-emitting diodes (LEDs) and a simulated natural photoperiod (SNP). (A) Western blot using CAT (64 kDa) to examine protein expression in the livers of cinnamon clownfish during starvation. The 55-kDa β-tubulin was used as the internal control. SOD (B), CAT (C), and GPX (D) mRNA levels relative to the β-actin mRNA levels in the livers of cinnamon clownfish during starvation under lighting conditions using red (R), green (G), and blue (B) LEDs and an SNP as measured by quantitative real-time polymerase chain reaction. The fish were reared under a light:dark cycle (12:12). 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 different characteristics are significantly different between starvation days within the same light spectrum ($P<0.05$). The cross (†) indicates a significant difference among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

Furthermore, plasma AlaAT and AspAT levels were significantly increased at 3 days (AlaAT [starved-SNP, 1932 ± 96.6 U/L; starved-red LED, 2217 ± 110.85 U/L], AspAT [starved-SNP, 426 ± 21.3 U/L; starved-red LED, 556 ± 27.8 U/L]) and then decreased in all experimental groups, but levels in the green-LED and blue-LED groups were significantly lower than in the other light spectra (Fig. 4B, C).

3.4. Lysozyme activity

We examined the immune indicator, lysozyme activity levels, during starvation among light spectra using a plate reader (Fig. 5). In all experimental groups, lysozyme activity levels were significantly decreased at 3 days and then increased after starvation. Otherwise, in the green-LED and blue-LED groups, lysozyme activity levels were significantly higher than in the other light spectra.

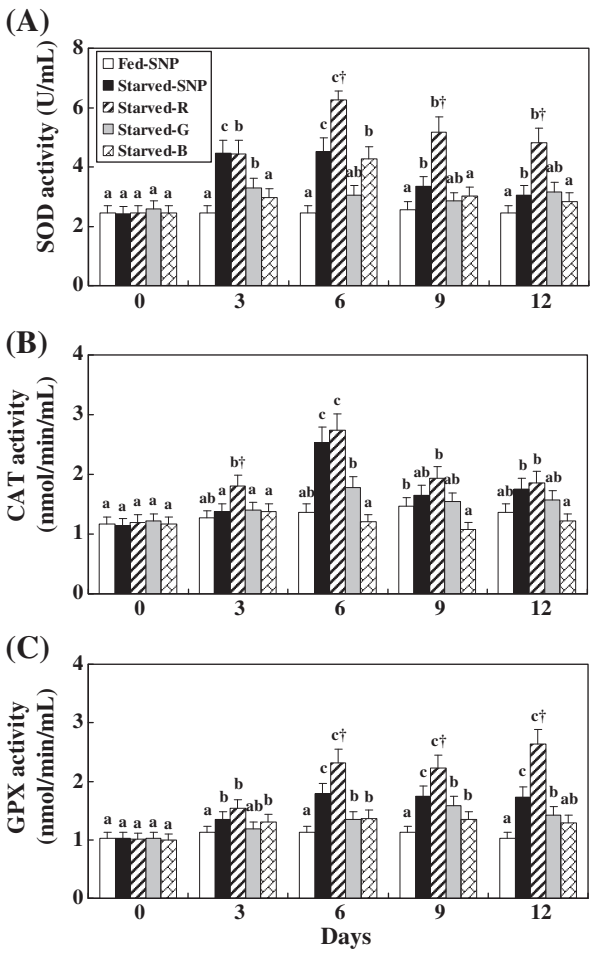

Fig. 2. Changes in the activity levels of superoxide dismutase (SOD) (A), catalase (CAT) (B), and glutathione peroxidase (GPX) (C) in the liver during starvation of cinnamon clownfish under lighting conditions using red (R), green (G), blue (B) light-emitting diodes (LEDs) and a simulated natural photoperiod (SNP) as measured using a microplate reader. Letters indicate significant differences between starvation days within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

3.5. Plasma melatonin levels

We analyzed the plasma melatonin levels during starvation among light spectra using ELISA (Fig. 6). In the starved-SNP and red-LED groups, plasma melatonin levels were increased at 6 days (starved-SNP, 48.8 ± 1.9 pg/mL; starved-red LED, 60 ± 3.9 pg/mL) compared with initial levels (11.2 ± 2.5 pg/mL), and the levels remained stable until the end of the experimental period (12 days). Also, in the green-LED and blue-LED groups, melatonin levels were significantly increased at 6 days but were lower (32.8 ± 1.9 pg/mL and 25.8 ± 1.9 pg/mL, respectively) than those in the other light spectra.

4. Discussion

In this study, we examined the mRNA expression and activities of the antioxidant enzymes SOD, CAT, and GPX as well as LPO; the correlation between plasma H_2O_2 and immune function; and the levels of melatonin, a well-known antioxidant material, to examine the

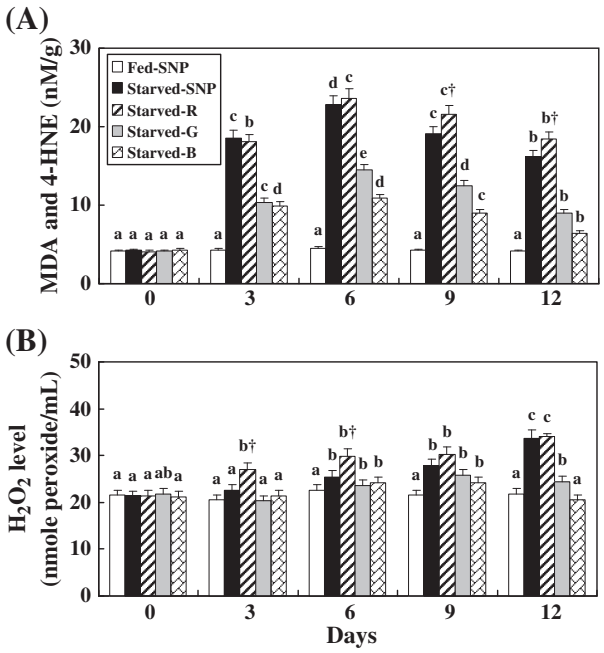

Fig. 3. Lipid peroxidation (A) and plasma H_2O_2 concentrations (B) during starvation of cinnamon clownfish under lighting conditions using red (R), green (G), and blue (B) light-emitting diodes and a simulated natural photoperiod, as measured using a microplate reader. The fish were reared under a light:dark cycle (12:12). Letters indicate significant differences between the starvation days within the same light spectrum ($P<0.05$). The crosses (†) indicate significant differences among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

effects of different LED spectra (red, green, and blue within a visible light) on oxidative stress during 12-day starvation to investigate the possibility of controlling oxidative stress using specific LED light sources and molecular physiological effects.

We first found that the expression and activity levels of the antioxidant enzymes SOD, CAT, and GPX and that the CAT protein level changes were significantly increased and then decreased in all of the spectra during the 12-day starvation at 3-day intervals. However, the expression and activity levels in the red-LED group were significantly higher than those in the white fluorescent bulb group (Figs. 1 and 2). These results indicate that oxidative stress was induced by starvation, a finding that is in agreement with the report of Pascual et al. (2003) that antioxidant enzyme activity and LPO were found in parallel with food restriction in the gilthead sea bream *Sparus aurata* during 46-day starvation, and thus starvation induced the oxidative stress. Therefore, these results suggest that the short wavelength green and blue spectra inhibit starvation-induced oxidative stress. These results are in agreement with those of Shin et al. (2011) that the expression and activity of the antioxidant enzymes SOD and CAT in the green and blue groups were significantly lower than those in the other spectrum groups.

We hypothesized that the short wavelength green and blue LEDs would more effectively inhibit oxidative stress than the other spectra. With regard to these results, LPO and plasma H_2O_2 concentrations were increased according to increased starvation days, but the ones in the green-LED and blue-LED groups were relatively lower than those in the other spectrum groups (Fig. 3). These results indicate that lipid peroxidation material increased and then damaged tissues by starvation-induced ROS accumulation, whereas the short wavelength green and blue LEDs inhibited the starvation-induced oxidative stress. Shin et al. (2011) measured the LPO and H_2O_2 concentrations in yellowtail clownfish after red, green, and blue LED illumination, and found that the levels were significantly lower in the green-LED and blue-LED groups than those in the other spectrum groups.

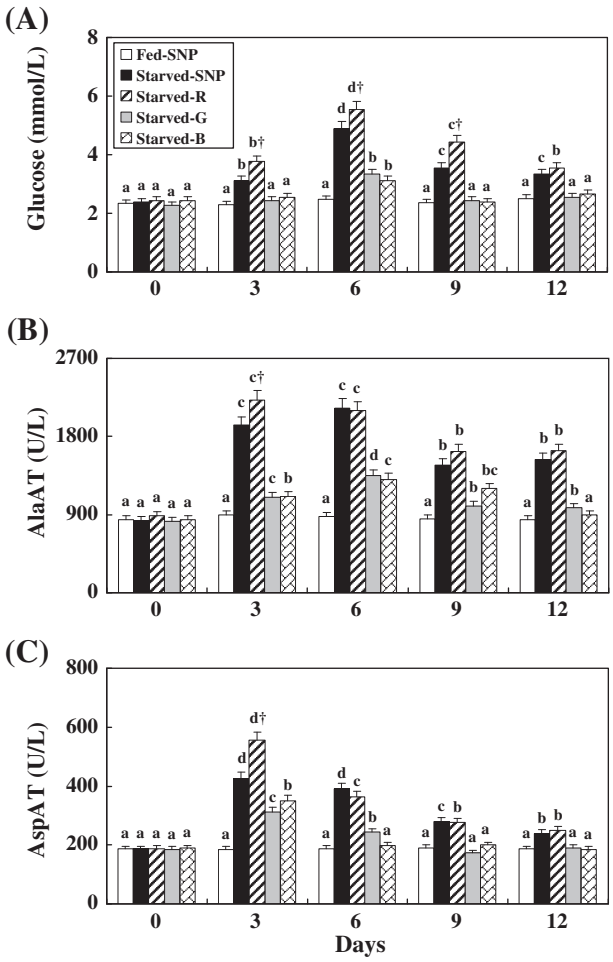

Fig. 4. Plasma glucose (A), alanine aminotransferase (AlaAT) (B) and aspartate aminotransferase (AspAT) (C) levels during starvation of cinnamon clownfish under lighting conditions using red (R), green (G), and blue (B) light-emitting diodes and a simulated natural photoperiod as measured using a biochemistry autoanalyzer. The fish were reared under a light:dark cycle (12:12). Letters indicate significantly differences between starvation days within the same light spectrum ($P<0.05$). The crosses (†) indicate significant differences among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

Starvation induces stress in fish, and the primary stress responses induce secondary responses that affect energy requirements, such as increases in plasma glucose levels (Carmichael et al., 1984). AlaAT and AspAT, well-known liver damage indicators, are also increased by stress (Pan et al., 2003). In this study, plasma glucose, AlaAT, and

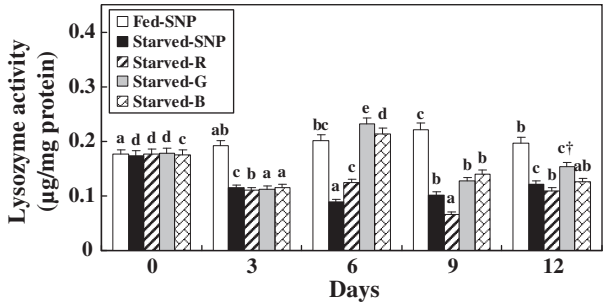

Fig. 5. Lysozyme activity levels during starvation of cinnamon clownfish under lighting conditions using red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod as measured using a microplate reader. The fish were reared under a light:dark cycle (12:12). Letters indicate significant differences among starvation days within the same light spectra ($P<0.05$). The crosses (†) indicate significant differences among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

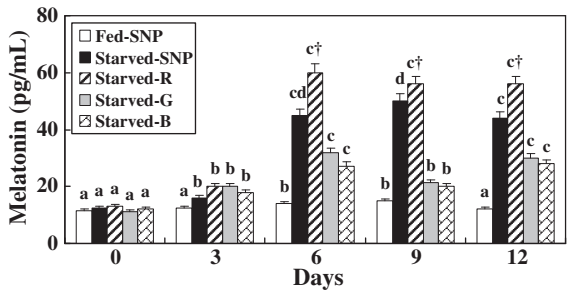

Fig. 6. Enzyme-linked immunosorbent assay of melatonin plasma levels during starvation of cinnamon clownfish under lighting conditions using red (R), green (G), and blue (B) light-emitting diodes and a simulated natural photoperiod as measured using a microplate reader. The fish were reared under a light:dark cycle (12:12). Letters indicate significant differences among starvation days within the same light spectra ($P<0.05$). The crosses (†) indicate significant differences among light spectra on the same starvation day ($P<0.05$). All values are means ± SD ($n=5$).

AspAT levels in the green-LED and blue-LED groups were significantly lower than those in the other spectrum groups during starvation (Fig. 4). Volpato and Barreto (2001) reported that cortisol levels were significantly lower in the blue illumination group, when *O. niloticus* were accommodated into high-density tank under green, blue, and white spectra for 48 h, and cortisol levels under the blue spectrum were significantly lower than those in 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.

Other negative factors induced by starvation affect immune function in addition to inducing stress in all organisms including fish (Pascual et al., 2006). Therefore, we investigated that difference of immune function among LED spectra and then measured the immune function indicator lysozyme activity. As a result, we found that activity levels were low during the entire starvation period but that the levels in the green-LED and blue-LED groups were significantly decreased at 3 days and then increased at 6 days (Fig. 5). These results suggest that the short wavelength enhances immune ability. The study related with these results has not yet been reported, but Alfereza et al. (2012) investigated the effect of blue light exposure on *Penicillium digitatum*, one of the most important disease-causing fungi in tangerines, and found that tangerines exposed to blue light had relatively lesser infections than controls, and this result indicates that the blue wavelength positively affects plant immune function (Alfereza et al., 2012).

When oxidative stress is induced in fish, antioxidant compounds and enzymes are secreted (Basha Siraj and Rani Usha, 2003). Among these antioxidant compounds, melatonin, a known strong antioxidant within the antioxidant defense system, plays a role in clearing free radicals, is thought to directly detoxify free radicals, and effectively protects the cell membrane against oxidative damage (Reiter et al., 1997). In this study, we found lower melatonin levels in the green-LED and blue-LED groups than in the other groups during starvation (Fig. 6). This result is in agreement with the ROS result showing lower H_2O_2 concentrations in the green-LED and blue-LED groups (Fig. 3B) than in the other groups; thus, levels of melatonin, which can reduce ROS, were low. Meanwhile, melatonin levels in the fluorescence bulb and red-LED groups were significantly high, suggesting that in the presence of a high level of ROS, melatonin was secreted to clear ROS. Shin et al. (2011) reported that ROS were eliminated by melatonin in yellowtail clownfish under red, green, and blue LEDs and that melatonin levels in the green-LED and blue-LED groups were significantly lower than those in the other groups. Therefore, this result and that reported by Shin et al. (2011) suggested that starvation in the short wavelength green-LED and blue-LED groups induced relatively less oxidative stress.

On the other hand, we hypothesized that because red light could not be detected by the cinnamon clownfish visual system due to the lack of photons, the fish perceived areas lit with red light as being darker than areas illuminated with blue and green wavelengths, thus red light acts as a stressor induced oxidative stress.

In conclusion, we hypothesized that short wavelength green and blue LEDs inhibit starvation-induced oxidative stress through antioxidant enzymes, ROS and LPO, and play a role in protecting against harmful ROS in cinnamon clownfish. Green and blue wavelengths within a visible light also effectively enhance immune function. Additional studies will be needed to identify mechanism of protective capacity using photoreceptors and various stress factors in fish related with different light spectra at various molecular levels.

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