Comparative Biochemistry and Physiology, Part A 160 (2011) 221–228

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effects of LED light spectra on oxidative stress and the protective role of melatonin in relation to the daily rhythm of the yellowtail clownfish, *Amphiprion clarkii*

Hyun Suk Shin [a], Jehee Lee [b], Cheol Young Choi [a,*]

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

ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 2 June 2011
Accepted 3 June 2011
Available online 12 June 2011

Keywords:
Light emitting diodes
Light spectrums
Melatonin
Oxidative stress
Yellowtail clownfish

ABSTRACT

The present study aimed to test the effects of melatonin on oxidative stress in the yellowtail clownfish, *Amphiprion clarkii*, as produced by light emitting diodes (LEDs): red, green, and blue. We investigated the effects of the different LEDs on oxidative stress by measuring the mRNA expression of *arylalkylamine N-acetyltransferase* (*AANAT2*), the expression and activities of antioxidant enzymes (superoxide dismutase, SOD (EC 1.15.1.1); and catalase, CAT (EC 1.11.1.6)), and plasma H_2O_2 and plasma melatonin levels. In red light, the expression of *AANAT2*, SOD, and CAT mRNA was significantly higher than those under the other light spectra. SOD and CAT activities and plasma H_2O_2 and melatonin levels were also significantly higher for the red spectra than those for the other light spectra. These results indicate that red light induces oxidative stress. To investigate the effects of melatonin on oxidative stress, we injected melatonin into live fish (*in vivo*) or treated cultured pineal organ (*in vitro*) with melatonin. We found that *AANAT2*, SOD, and CAT mRNA expression levels, SOD and CAT activities, and plasma H_2O_2, lipid peroxidation (LPO) and melatonin levels were significantly lower than those for the controls. Therefore, our results indicate that red light induces oxidative stress and melatonin plays the role of a strong antioxidant in yellowtail clownfish.

1. Introduction

Among the many factors that control circadian rhythms, light is the most important, modifying many of the physiological and behavioral changes that occur within a 24-h period (Pierce et al., 2008). Because of the central importance of the day–night light cycle (the photoperiod) in the survival of organisms, light-sensitive circadian clocks have evolved in most animals, including fish. The photoperiod exerts an endogenous effect by causing a rhythmic synthesis and release of the "time-keeping" hormone melatonin, which affects rhythmic physiological functions in fish (Bromage et al., 2001). Also, the oscillation of the circadian rhythm is driven by an intracellular molecular clock and is self-sustaining, i.e. occurring even in the absence of environmental cues (Pierce et al., 2008). Melatonin is produced mainly in the pineal organ and retina, and its plasma content is higher at nighttime than in the daytime. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of circadian rhythm but also in seasonal biological rhythms (Reiter, 1991; Falcón et al., 2007).

In addition to its role in circadian regulation, melatonin 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 plays a role in antioxidant action by converting upon oxidation to a number of antioxidant compounds, including cyclic 3-hydroxymelatonin, N_1-acetyl-N_2-formyl-5-methoxykynuramine, and N_1-acetyl-5-methoxykynuramine (Reiter et al., 1997). Because of this array of compounds, melatonin is considered to be 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).

The *arylalkylamine N-acetyltransferase* (AANAT) enzyme is the precursor of melatonin; which is the rate-limiting enzyme of melatonin synthesis (Iuvone et al., 2005; Klein, 2007). AANAT catalyzes the conversion of serotonin to N-acetylserotonin, which is then catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT) to form melatonin (Klein et al., 1997). In addition, the nocturnal rise in melatonin production is due to an increase in the activity of AANAT (Klein, 2007).

Light emitting diodes (LEDs), a new form of lighting technology that is still being developed, can be manufactured to output specific wavelengths (Migaud et al., 2007). Furthermore, LEDs have lower power requirements, electrical running costs, and a longer life span than the standard metal halide bulbs (Migaud et al., 2007). Narrow bandwidth light using such new technologies, and especially a high-energy short wavelength, 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

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

1095-6433/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2011.06.002

emitting narrow bandwidths (Villamizar et al., 2009). It is known that the spectral composition of incident light changes differentially in underwater environments and that there is a rapid attenuation with increasing depth (Lythgoe, 1979); the short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates in shallow waters (McFarland, 1991; Lythgoe et al., 1994; Myrberg and Fuiman, 2002). When farming the barfin flounder, *Verasper moseri*, using short wavelengths such as those in the blue spectrum, the flounders showed a high growth rate (Yamanome et al., 2009). In addition, it was shown that the blue spectrum prevented stress in the Nile tilapia, *Oreochromis niloticus* (Volpato and Barreto, 2001), and that long wavelengths such as those in the red spectrum induced gonad development in the tropical damselfish *Chrysiptera cyanea* (Bapary et al., 2011).

However, certain LED spectra can have the negative effect of inducing stress in fish, including oxidative stress (Head and Malison, 2000; Van der Salm et al., 2004). Overproduction of ROS by environmental stress can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and induce DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity and can thereby accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003). Complex antioxidant defense systems maintain homeostasis in changing environments and protect aerobic organisms against ROS and subsequent oxidative stress-induced damage (Bagnyukova et al., 2007). Antioxidants can include enzymes such as superoxide dismutase (SOD), catalase (CAT), or glutathione peroxidase (GPX) and compounds such as melatonin, metallothionein, vitamin C, or vitamin E (α-tocopherol) (McFarland et al., 1999). Among these antioxidant compounds, melatonin plays a role in clearing free radicals and is known as a strong antioxidant within the antioxidant defense system (Wu and Swaab, 2005). Moreover, antioxidant enzymes found in the liver and kidneys of marine organisms and antioxidant compounds scavenge free radicals (Basha Siraj and Rani Usha, 2003). In fish, SOD and CAT are typical antioxidant enzymes that directly scavenge ROS.

Head and Malison (2000) and Van der Salm et al. (2004) reported that light spectra can induce stress in fish, but there have only recently been LED studies dealing with the effects of photoperiod and light intensity in fish (Boeuf and Le Bail, 1999; Bayarri et al., 2002; Pierce et al., 2008). Although there are studies that show the response of fish to various spectra (Neumeyer, 1992; Villamizar et al., 2009; Bapary et al., 2011), investigations on the response of fish to various light spectra is still very limited (Head and Malison, 2000; Van der Salm et al., 2004).

Therefore, we investigated the expression changes of *AANAT2* mRNA in relation to the daily rhythm in experimental fish after exposure to red, green, and blue LED spectra, using the yellowtail clownfish, which is a high-value ornamental fish. In addition, we determined the oxidative stress levels and the antioxidant response mechanism for specific spectrums by measuring SOD and CAT expression and activities and plasma H_2O_2, lipid peroxidation (LPO) and melatonin levels in the yellowtail clownfish.

2. Materials and methods

2.1. Experimental fish and conditions

Yellowtail clownfish (n = 300; length, 5.2 ± 0.5 cm; mass, 2.1 ± 0.5 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and were allowed to acclimate for 2 weeks in twelve 300-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. The water temperature and photoperiod were 27 ± 1 °C and a 12-h light:12-h dark period (lights on 07:00 h and light off 19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h). For the experimental groups, the fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LEDs (Daesin LED Co. Kyunggi, Korea) (Fig. 1). The LEDs were set 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 fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) prior to blood collection. 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 4-h intervals, 07:00 h (ZT0), 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT24), to collect the pineal organ and liver under dim light.

2.2. Quantitative PCR (QPCR)

Total RNA was extracted from the livers using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. QPCR was performed using cDNA. QPCR was conducted to determine the relative expression levels of *AANAT2* (GenBank accession no. JN032590), SOD (0000), CAT (JN032592), and β-actin (JN039369) mRNA using total RNA extracted from the pineal organ and liver. The following QPCR primers were designed with reference to the known sequences of the yellowtail clownfish: *AANAT2* forward (5′-CAT TCG TCT CTG TGT CTG G-3′) and reverse (5′-AAA GCC TCT CCT TGT CCC-3′) primers; SOD forward (5′-CAC GAG AAG GCT GAT GAC-3′) and reverse (5′-GAT ACC AAT GAC TCC ACA GG-3′) primers; CAT forward (5′- GGG CAA ATT GGT CCT CAA-3′) and reverse (5′- CGA TGT GTG TCT GGG TAG-3′) primers; and β-actin forward (5′-CCA ACA GGG AGA AGA TGA C-3′) and reverse (5′-TAC GAC CAG AGG CAT ACA-3′) primers. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. The 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 an internal control, experiments were duplicated with β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all data were expressed relative to the corresponding β-actin and GAPDH calculated threshold cycle (CT) levels.

2.3. In vitro culture of the pineal organ and melatonin treatment

After the fish was anesthetized, the pineal organ was dissected out and placed in an ice-cold medium (pH 7.5) containing 150 mM NaCl, 3.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.7 mM NaH_2PO_4, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 10 mM HEPES, and 0.88 g/L Eagle's MEM (Sigma) containing antibiotics (0.06 g/L penicillin and 0.1 g/L

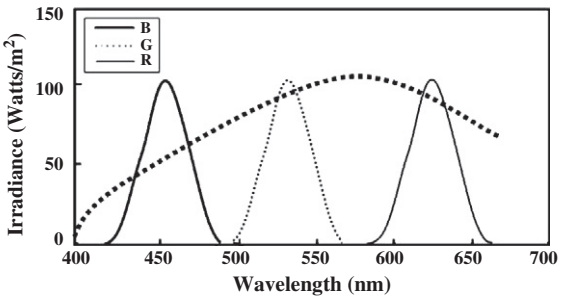

Fig. 1. Spectral profiles of the blue (B), green (G), and red (R) LEDs used in this study. Square dotted line shows the spectral profile of a white fluorescent light.

streptomycin; Penicillin–Streptomycin, Gibco, USA). The pineal organs were transferred to a well of a 24-well microplate containing 1 mL of the medium and incubated at 20 ± 1 °C in an incubator under LD conditions (12L:12D, light switched on at 07:00 h). The lights were set at 40 cm above the surface of the pineal-organ culture plate, and the irradiance at the surface was maintained at approximately 0.96 W/m^2 (fluorescent bulb). The LEDs were approximately 0.9 W/m^2 during the light phase. The pineal organ was sampled at 4 h intervals from ZT4 to ZT28. One milliliter of each sample was centrifuged (20 °C, 10,000 *g*, 15 s), and the supernatant was removed and remained tissues, in the bottom after centrifugation, stored at −80 °C until RNA extraction.

Melatonin (Sigma, USA), dissolved in 0.9% physiological saline at the appropriate doses, was added to the culture medium in the following ratio: 1/1000e (v/v) and the indicted concentrations of melatonin (10 μM) were obtained. Cultured pineal organs were treated for 4 to 28 h without renewing the medium. One milliliter of each sample was centrifuged (20 °C, 10,000 *g*, 15 s), and the supernatant was removed and removed and remained tissues, in the bottom after centrifugation, were stored at −80 °C until RNA extraction.

2.4. Melatonin injection

Melatonin (Sigma) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the yellowtail clownfish (5.2 ± 0.5 g) were intraperitoneally injected at a dose of 200 μg melatonin/g body mass (BW) at a volume of 10 μL/g BW. We injected the fish at 07:00, and 5 fish were then sacrificed by decapitation at 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT24), and 11:00 h (ZT28).

After injection, the pineal organ and liver were sampled from 5 fish at 4-h intervals from ZT4 to ZT28. All of the fish survived the experimental period.

2.5. SOD and CAT activity analysis

Liver tissues were homogenized in ice-cold 0.1 M phosphate-buffered saline (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 analyses. SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities were determined using commercial kits supplied by Cayman Chemical (USA).

Each assay was performed in duplicate, and the enzyme units were recorded as U/mL, and the CAT 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). Twenty microliters of olive flounder serum were added per well to flat-bottom 96-well microtitre plates. 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), which are the degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides (Esterbauer et al., 1991), with a Lipid Hydroperoxide Assay Kit (Cayman Chemical) according to the manufacturer's instructions. LPO was expressed in terms of nmoles of MDA and 4-HNE per gram protein.

2.8. Melatonin determination by ELISA

The melatonin concentration in the plasma was determined using the enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). The absorbance was read at 405 nm.

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey's *post hoc* test was used to assess statistically significant differences among the different time points of the daily and circadian variation and the different light spectra. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Expression of *AANAT2* mRNA in the pineal organs

We examined the effect of the different LED light spectra on the expression of *AANAT2* mRNA in the pineal organ by using QPCR (Fig. 2). *AANAT2* mRNA was expressed at significantly higher levels during the scotophase than during the photophase for all light spectra. Moreover, for the pineal organ (*in vivo*) and cultured pineal organ (*in vitro*), *AANAT2* mRNA was expressed at significantly higher levels for the red LED group relative to the other LED ones (Fig. 2A and C).

Following melatonin injection into living fish or melatonin treatment of cultured pineal organs, the *AANAT2* expression patterns of the different LED groups during the daily rhythm were similar to the untreated LED ones. However, the expression levels of the melatonin-treated LED groups were significantly lower than the untreated LED ones (Fig. 2B and D).

3.2. Expression of SOD and CAT mRNA in the liver

We used QPCR to examine the effects of the different LED regimes on the expression of SOD and CAT mRNA in the liver (Fig. 3). Both genes were expressed at significantly higher levels during the photophase than during the scotophase for all light spectra. Meanwhile, SOD and CAT mRNA levels were significantly higher in the red LED group than in the other LED groups (Fig. 3A and C). Moreover, after the injection of melatonin, the expression patterns of both genes during the daily rhythm were similar between treated and untreated LED groups, but the expression levels were significantly higher for the untreated LED groups (Fig. 3B and D).

3.3. SOD and CAT activities in the liver

We examined the effect of the different LED spectra on SOD and CAT activities in the liver by using a microplate reader (Fig. 4). SOD and CAT activities were at significantly higher levels during the photophase than during the scotophase for all light spectra. In addition, the activities of both enzymes was significantly higher in the red LED groups than in the other LED ones and the control (Fig. 4A and C). In addition, the activity-level patterns of both enzymes during the daily rhythm were similar between the melatonin-injected LED groups and the uninjected ones, but the activity levels were significantly lower in the injected LED groups (Fig. 4B and D).

3.4. Plasma H_2O_2 levels

We observed the effects of the different LED spectra on the plasma H_2O_2 levels by using a microplate reader (Fig. 5). The plasma H_2O_2 levels were at significantly higher levels during the photophase than during the scotophase for all light spectra, whereas the H_2O_2 levels

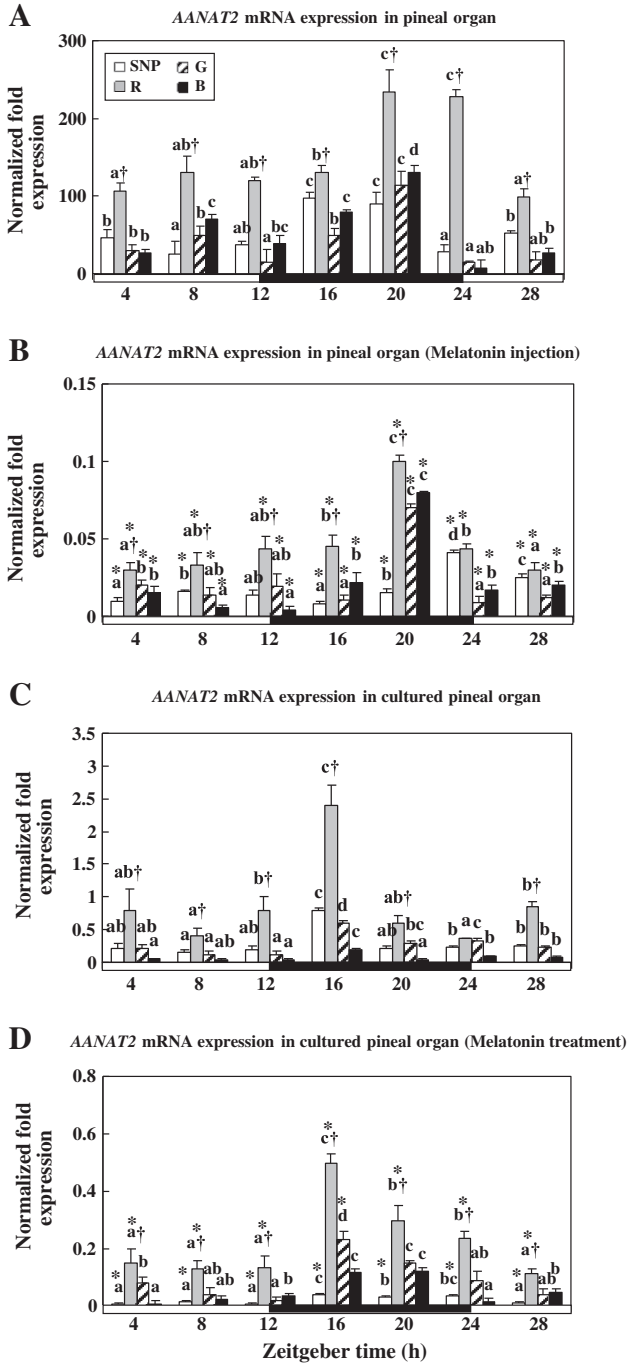

Fig. 2. Changes in the expression levels of *AANAT2* mRNA in the pineal organ (*in vivo*) (A), pineal organ injected with melatonin (*in vivo*) (B), cultured pineal organ (*in vitro*) (C), and cultured pineal organ treated with melatonin (*in vitro*) (D) under lighting conditions using red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) cycle (12:12). Total pineal organ 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. The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

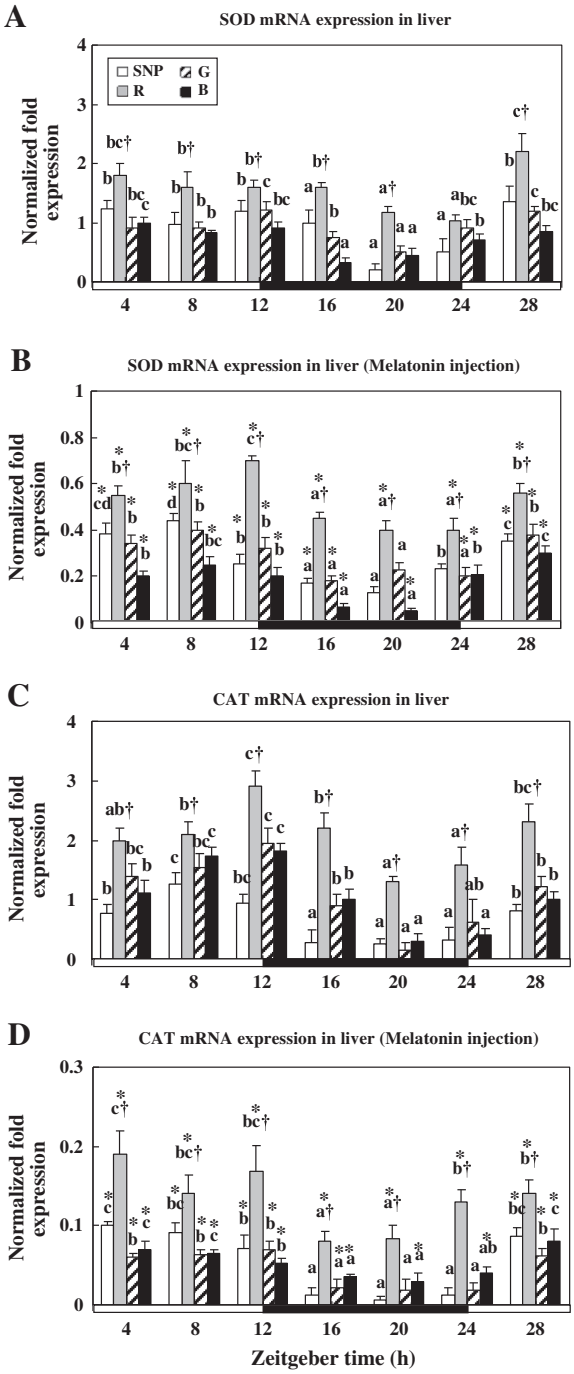

Fig. 3. Changes in the expression levels of SOD and CAT mRNA in the liver (A) and (C) and in the melatonin-injected liver (B) and (D) of yellowtail clownfish under red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) 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. The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

were significantly higher for the red LED group than for the other LED groups and the control (Fig. 5A). After the injection of melatonin, the pattern of H_2O_2 plasma levels during the daily rhythm were similar between the injected LED groups and the uninjected groups, but the plasma levels were significantly lower for the injected groups (Fig. 5B).

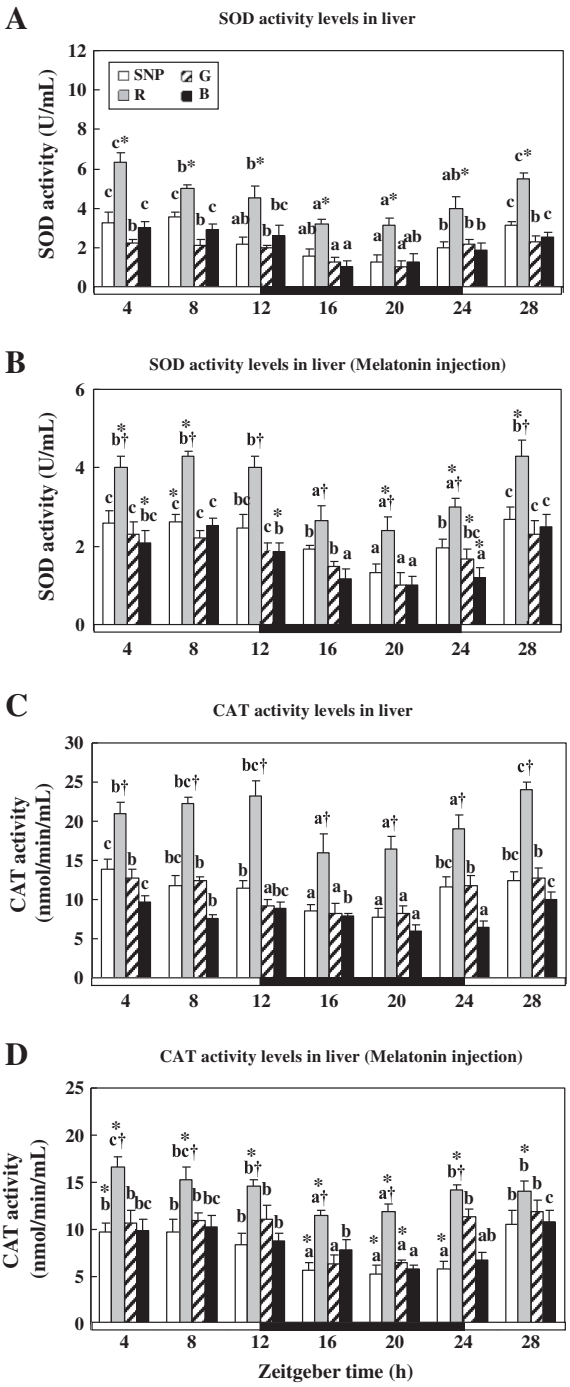

Fig. 4. Changes in the activity levels of SOD and CAT in the liver (A) and (C) and the melatonin-injected liver (B) and (D) of yellowtail clownfish under red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by a microplate reader. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

3.5. LPO levels

We observed the effects of the different LED spectra on the LPO levels (expressed in terms of the amounts of malondialdehyde [MDA] and 4-hydroxy nonenal) by using a microplate reader (Fig. 6). The LPO

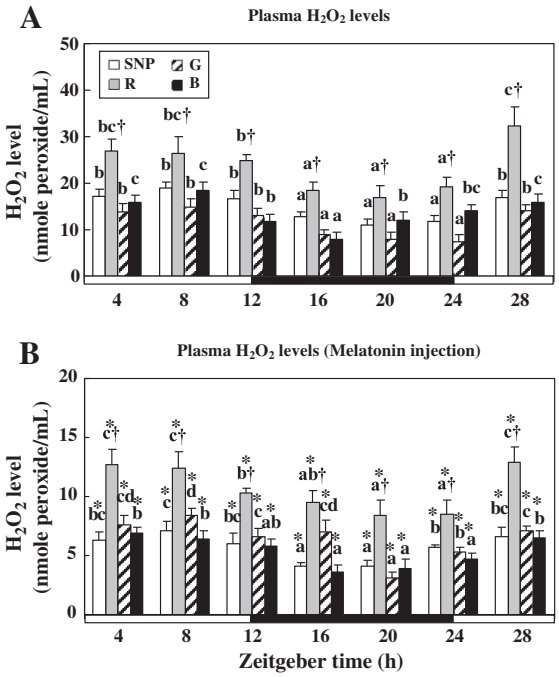

Fig. 5. Plasma H_2O_2 concentrations before (A) and after melatonin injection (B) in yellowtail clownfish under red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by microplate reader. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

levels were at significantly higher levels during the photophase than during the scotophase for all light spectra, whereas the LPO levels were significantly higher for the red LED group than for the other LED groups and the control (Fig. 6A). After the injection of melatonin, the pattern of LPO levels during the daily rhythm were similar between the injected LED groups and the uninjected groups, but the plasma levels were significantly lower for the injected groups (Fig. 6B).

3.6. Plasma melatonin levels

We observed the effects of the different light spectra on plasma melatonin levels by using a microplate reader (Fig. 6). Plasma melatonin levels were significantly higher during the scotophase than during the photophase for all light spectra. However, the melatonin levels were at significantly higher levels for the red LED group than for the other LED groups and the control (Fig. 6A). Following the injection of melatonin, the pattern of plasma melatonin levels during the daily rhythm was similar between the injected and uninjected groups, whereas the plasma levels were significantly higher in the injected groups (Fig. 6B).

4. Discussion

In this study, we examined the mRNA levels of the *AANAT2* enzymes, which synthesizes the precursor of melatonin, the mRNA expression and activities of the antioxidant enzymes SOD and CAT, and plasma H_2O_2 and melatonin levels to examine the effects of different LED spectra (red, green, and blue) on oxidative stress in order to investigate the antioxidant response mechanisms. In addition, we observed the *AANAT2* mRNA expression levels in the pineal organ and SOD/CAT mRNA expression and activity changes in the liver of the yellowtail

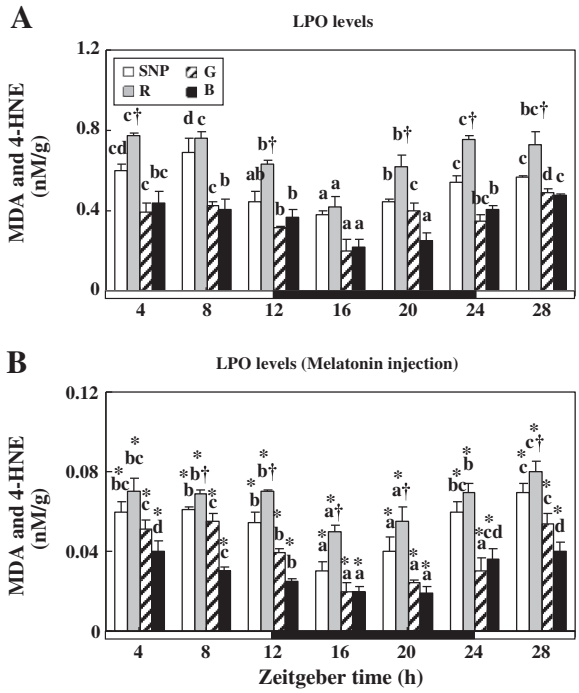

Fig. 6. Malondialdehyde (MDA) and 4-hydroxy nonenal (4-HNE) levels before (A) and after melatonin injection (B) in yellowtail clownfish under red (R), green (G), and blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by microplate reader. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicates significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

clownfish during the daily rhythm to examine the antioxidant capacity of melatonin.

First, we found that the *AANAT2* mRNA expression were significantly higher for the red LED spectrum than other spectra for both the pineal organ (*in vivo*) and cultured pineal organ (*in vitro*) (Fig. 2). Furthermore, we found that SOD and CAT mRNA expression and SOD and CAT activities were significantly higher for the red LED spectrum than for the other spectra (Fig. 3). Hence, these results suggest that red LED spectra produce oxidative stress in the yellowtail clownfish. Villamizar et al. (2009) have reported that the larvae of the European sea bass, *Dicentrarchus labrax*, when exposed to red LED spectra, showed a significantly lower growth and food intake rate relative to other spectra. With regard to these results, Villamizar et al. (2009) discussed the effect of the lack of appropriate photons for stimulating the visual system. In this study, the high expression levels of *AANAT2* mRNA in the red LED group suggest that melatonin was being synthesized as an antioxidant to protect against oxidative stress. Since melatonin synthesis in the pineal organ, the pattern of melatonin synthesis usually parallels *AANAT2* activity (Iuvone et al., 2005). *AANAT2* is the precursor of melatonin; which is the rate-limiting enzyme of melatonin synthesis (Iuvone et al., 2005; Klein, 2007). AANAT catalyzes the conversion of serotonin to N-acetylserotonin, which is then catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT) to form melatonin (Klein et al., 1997). Furthermore, melatonin is a strong antioxidant that can protect cell membranes from oxidative damage more effectively than other antioxidants (Reiter et al., 1997). Therefore, we analyzed *AANAT2* mRNA expression and SOD and CAT expression and activity in yellowtail clownfish injected (*in vivo*) or treated (*in vitro*) with melatonin to examine whether melatonin plays a role as an antioxidant against oxidative stress induced by specific LED spectra. Blanco-Vives et al. (2010) study reported that larvae exposed to red light exhibited a delay in growth and thus the yolk sac was visible, while in the blue light the yolk sac was completely absorbed. Larvae responded best to blue light, which appeared to be the most efficient light spectrum on the development on Senegal sole larvae (Blanco-Vives et al., 2010). These observations agree with our results that *AANAT2*, SOD and CAT expression and activity were of low levels in short wavelength such as blue and green LED lights, which appeared to be the most efficient light spectrums on the oxidative stress inhibition. Thus, the potential benefits of short wavelength such as blue light to adult fish as well as larvae. Also, we observed that the expression levels for AANAT2, SOD, and CAT were significantly lower for the melatonin-treated or -injected groups than for the uninjected or untreated ones (Figs. 2 and 3). These results indicated that melatonin reduces oxidative stress and are in agreement with those of Reiter et al.'s (2007) study, which reported that melatonin reduces oxidative stress. Therefore, we hypothesized that red LED light induced oxidative stress in yellowtail clownfish, and melatonin injection or treatment removed the ROS, as a strong antioxidant, that had occurred in fish. So, *AANAT2* gene expression, as a precursor of melatonin, indicated indirect antioxidant parameters against red LED light spectrum in this study. Also, Rosa Nogués et al. (2006) reported that there is sufficient melatonin to scavenge free radicals, thus antioxidant enzymes (SOD and CAT) have no need to increase and may even decrease, as we found in our results. Melatonin is thought to directly detoxify free radicals, such as highly toxic hydroxyl radicals, via electron donation, with an electron-rich aromatic indole ring that functions as an electron donor, thereby reducing electrophilic radicals (Reiter et al., 1997; Martinez et al., 2005; Wu and Swaab, 2005).

Consistent with this model for melatonin function, the plasma H_2O_2 levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~2 fold in melatonin-injected fish than the uninjected ones (Fig. 4). These results are in agreement with the studies of Allegra et al. (2003) and Martinez et al. (2005), which reported that melatonin scavenges ROS induced by red spectrum. In addition, the plasma H_2O_2 levels in the rat, *Rattus norvegicus*, treated with the environmental toxicants, polychlorinated biphenyls (PCB), were significantly higher than those in rats that intraperitoneally received melatonin (Venkataraman et al., 2008). Venkataraman et al. (2008) concluded that melatonin plays a strong antioxidant role by scavenging ROS.

Induced ROS from red LED spectrum can oxidatively damage the cellular elements in its target organ such as muscle. In this study, LPO levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~100 fold in melatonin-injected fish than the uninjected ones (Fig. 7). Red LED spectrum induced oxidative stress and then caused oxidative damage in tissue of yellowtail clownfish. These results are in agreement with Baydas et al. (2002) and Venkataraman et al. (2008), which reported that melatonin prevents oxidative stress induced by PCB, and then LPO levels were lower than the unsupplemented melatonin group in adult rats. It seems to be melatonin prevents oxidative stress induced by red LED spectrum.

In this study, we measured plasma melatonin levels and found that they were at a significantly higher level during the scotophase than during the photophase for all LED spectra. Moreover, the melatonin levels were significantly higher for the red LED spectrum than for the other spectra over the course of the entire photoperiod (Fig. 5). These results indicated that the red spectrum induced oxidative stress in yellowtail clownfish and that more melatonin was produced under the red LED to scavenge for increased ROS. In addition, plasma melatonin levels rapidly increased after melatonin injection. We can conclude that melatonin levels were temporarily increased by the injection of the

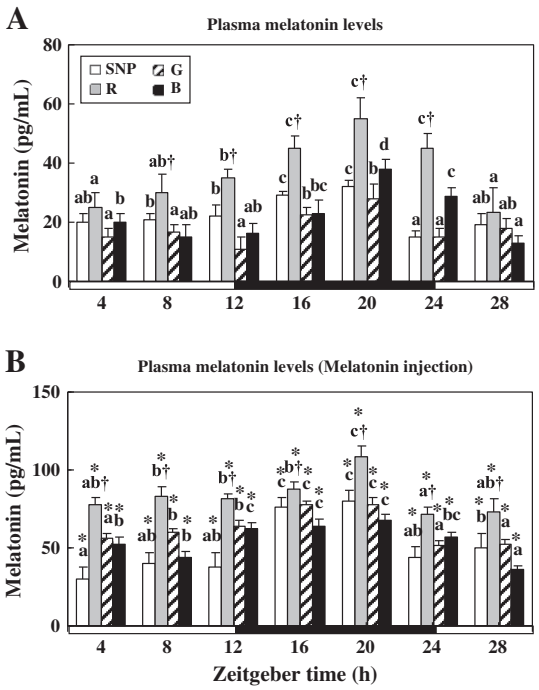

Fig. 7. Enzyme-linked immunosorbent assay melatonin plasma levels (A) and levels of melatonin injection (B) in yellowtail clownfish under lighting conditions using red (R), green (G), blue (B) LEDs and a simulated natural photoperiod (SNP), as measured by microplate reader. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($P<0.05$). The cross (†) indicate significant differences between different light spectra within the same Zeitgeber time (ZT) ($P<0.05$), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group ($P<0.05$). All values are means ± SD ($n=5$).

highest dose of melatonin. This result is in agreement with those of various teleost studies such as those on bloch (Renuka and Joshi, 2010), gilthead sea bream (Falcón et al., 1996), and goldfish (Iigo et al., 2003). In these studies, the teleost melatonin levels were significantly higher during the scotophase than during the photophase, similar to the results from this study. Increasing plasma melatonin levels have been shown to increase following induction of *AANAT* mRNA synthesis (Namboodiri et al., 1987; Ganguly et al., 2002).

In conclusion, the red spectrum is a factor in inducing oxidative stress in yellowtail clownfish. We also found that melatonin likely plays a strong antioxidant role to reduce oxidative stress in fish. Light is known as a factor that affects various physiological changes in fish. Hence, additional studies will be necessary to understand the oxidative stress mechanism in fish in response to different light spectra.

Acknowledgments

This study was supported by the Technology Development Program for Fisheries, Ministry for Food, Agriculture, Forestry, and Fisheries, Korea, and by the MKE (The Ministry of Knowledge Economy), Korea, under the ITRC (Information Technology Research Center) support program supervised by the NIPA (National IT Industry Promotion Agency (NIPA-2011-C1090-1021-0015)).

References

Allegra, M., Reiter, R.J., Tan, D.X., Gentile, C., Tesoriere, L., Livrea, M.A., 2003. The chemistry of melatonin's interaction with reactive species. J. Pineal Res. 34, 1–10.

Bagnyukova, T.V., Lushchak, O.V., Storey, K.B., Lushchak, V.I., 2007. Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23°C. J. Therm. Biol. 32, 227–234.

Bapary, M.A.J., Amin, M.N., Takeuchi, Y., Takemura, A., 2011. The stimulatory effects of long wavelengths of light on the ovarian development in the tropical damselfish, Chrysiptera cyanea. Aquaculture 314, 188–192.

Basha Siraj, P., Rani Usha, A., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxical. Environ. Saf. 56, 218–221.

Bayarri, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., 2002. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. J. Pineal Res. 32, 34–40.

Baydas, G., Yilmaz, O., Celik, S., Yasar, A., Ferit Gursu, M., 2002. Effects of certain micronutrients and melatonin on plasma lipid, lipid peroxidation, and homocysteine levels in rats. Arch. Med. Res. 33, 515–519.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O., Sánchez-Vázquez, F.J., 2010. Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 306, 137–145.

Boeuf, G., Le Bail, P.Y., 1999. Does light have an influence on fish growth? Aquaculture 177, 129–152.

Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63–98.

Esterbauer, H., Schaur, R.J., Zoliner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 81–128.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin and neuroendocrine regulations in fish. J. Soc. Biol. 201, 21–29.

Falcón, J., Molina-Borja, M., Collin, J.-P., Oaknin, S., 1996. Age-related changes in 2-[125I]-iodomelatonin binding sites in the brain of sea bass breams (*Sparus aurata* L). Fish Physiol. Biochem. 15, 401–411.

Ganguly, S., Coon, S.L., Klein, D.C., 2002. Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res. 309, 127–137.

Head, A.B., Malison, J.A., 2000. Effects of lighting spectrum and disturbance level on the growth and stress responses of yellow perch *Perca flavescens*. J. World Aquacult. Soc. 31, 73–80.

Iigo, M., Furukawa, K., Tabata, M., Aida, K., 2003. Circadian variations of melatonin binding sites in the goldfish brain. Neurosci. Lett. 347, 49–52.

Iuvone, P.M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D.C., Chaurasia, S.S., 2005. Circadian clocks, clock networks, Arylalkylamine N-Acetyltransferase, and melatonin in the retina. Prog. Retin. Eye Res. 24, 433–456.

Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Bégay, V., Falcón, J., Cahill, G.M., Cassone, V.M., Baler, R., 1997. The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog. Horm. Res. 52, 307–357.

Klein, D.C., 2007. Arylalkylamine N-acetyltransferase: "the Tim enzyme". J. Biol. Chem. 282, 4233–4237.

Kim, M.O., Phyllis, E.B., 1998. Oxidative stress in critical care: is antioxidant supplementation beneficial? J. Am. Diet. Assoc. 98, 1001–1008.

Lythgoe, J.N., 1979. The Ecology of Vision. Clarendon Press, Oxford.

Lythgoe, J.N., Muntz, W.R.A., Partridge, J.C., Shand, J., Williams, D. McB, 1994. The ecology of visual pigments of snappers (Lutjanidae) on the Great Barrier Reef. J. Comp. Physiol. 174A, 461–467.

Martinez, G.R., Almeida, E.A., Klitzke, C.F., Onuki, J., Prado, F.M., Medeiros, M.H., Di Mascio, P., 2005. Measurement of melatonin and its metabolites: importance for the evaluation of their biological roles. Endocrine 27, 111–118.

McFarland, W.N., 1991. The visual world of coral reef fishes. In: Sale, P.F. (Ed.), The Ecology of Fishes on Coral Reefs. Academic Press, San Diego, pp. 16–38.

McFarland, V.A., Inouye, L.S., Lutz, C.H., Jarvis, A.S., Clarke, J.U., McCant, D.D., 1999. Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown bullhead, *Ameiurus nebulosus*. Arch. Environ. Contam. Toxicol. 37, 236–241.

Migaud, H., Cowan, M., Taylor, J., Ferguson, H.W., 2007. 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.

Myrberg Jr., A.A., Fuiman, L.A., 2002. The sensory world of coral reef fishes. In: Sale, P.F. (Ed.), Coral Reef Fishes. Academic Press, San Diego, pp. 123–148.

Namboodiri, M.A., Dubbels, R., Klein, D.C., 1987. Arylalkylamine N-acetyltransferase from mammalian pineal gland. Methods Enzymol. 142, 583–590.

Neumeyer, C., 1992. Tetrachromatic color vision in goldfish: evidence from color mixture experiments. J. Comp. Physiol. A 171, 639–649.

Nouroozzadeh, J., Tajaddinisarmadi, J., Wolff, S.P., 1994. Measurement of plasma hydroperoxide concentrations by ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 200, 403–409.

Pandey, S., Parvez, S., Sayeed, I., Haques, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl & Schn.). Sci. Total. Environ. 309, 105–115.

Pierce, L.X., Noche, R.R., Ponomareva, O., Chang, C., Liang, J.O., 2008. Novel function for period 3 and Exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ. Brain Res. 1223, 11–24.

Reiter, R.J., 1991. Melatonin: the chemical expression of darkness. Mol. Cell. Endocrinol. 79, C153–C158.

Reiter, R.J., Carneiro, R.C., Oh, C.S., 1997. Melatonin in relation to cellular antioxidative defense mechanisms. Horm. Metab. Res. 29, 363–372.

Reiter, R.J., Tan, D.X., Pilar, T.M., Flores, L.J., Czarnocki, Z., 2007. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochim. Pol. 54, 1–9.

Renuka, K., Joshi, B.N., 2010. Melatonin-induced changes in ovarian function in the freshwater fish *Channa punctatus* (bloch) held in long days and continuous light. Gen. Comp. Endocrinol. 165, 42–46.

Rosa Nogués, M., Giralt, M., Romeu, M., Mulero, M., Sánchez-Martos, V., Rodríguez, E., Acuña-Castroviejo, D., Mallol, J., 2006. Melatonin reduces oxidative stress in erythrocytes and plasma of senescence-accelerated mice. J. Pineal Res. 41, 142–149.

Van der Salm, A.L., Martínez, M., Flik, G., Wendelaar Bonga, S.E., 2004. Effects of husbandry conditions on the skin colour and stress response of red porgy *Pagrus pagrus*. Aquaculture 241, 371–386.

Venkataraman, P., Krishnamoorthy, G., Vengatesh, G., Srinivasan, N., Aruldhas, M.M., Arunakaran, J., 2008. Protective role of melatonin on PCB (Aroclor 1254) induced oxidative stress and changes in acetylcholine esterase and membrane bound ATPases in cerebellum, cerebral cortex and hippocampus of adult rat brain. Int. J. Dev. Neurosci. 26, 585–591.

Volpato, G.L., Barreto, R.E., 2001. Environmental blue light prevents stress in the fish Nile tilapia. Braz. J. Med. Biol. Res. 34, 1041–1045.

Villamizar, N., García-Alcazar, A., Sánchez-Vázquez, F.J., 2009. Effect of light spectrum and photoperiod on the growth, development and survival of European sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 292, 80–86.

Wu, Y.H., Swaab, D.F., 2005. The human pineal gland and melatonin in aging and Alzheimer's disease. J. Pineal Res. 38, 145–152.

Yamanome, T., Mizusawa, K., Hasegawa, E., Takahashi, A., 2009. Green light stimulates somatic growth in the barfin flounder, *Verasper moseri*. J. Exp. Zool. 311A, 73–79.