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Effect of LED light spectra on circadian rhythms in goldfish *Carassius auratus*: expression profiles following thermal stress

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We investigated the effects of light spectra on circadian rhythms under high thermal stress using red, green and purple light-emitting diodes (LEDs). To assess differences in circadian rhythms during high thermal stress under different LED light spectra, we measured changes in the expression of the melatonin receptor 1 (MT1), *period 2* (*Per2*), and *cryptochrome 1* (*Cry1*) genes and MT1 protein, and in the plasma melatonin and glucose levels. Under high water temperatures and red light conditions, MT1 expression and plasma melatonin and glucose were significantly higher than under low water temperatures and other light spectra, but *Per2* and *Cry1* expressions were significantly lower. Thus, high water temperatures induce oxidative stress and melatonin acts as an antioxidant molecule. Further, melatonin regulates circadian rhythms via clock genes, and green and purple lights may be suitable for maintaining circadian rhythms in goldfish *Carassius auratus*, as these wavelengths generally penetrate freshwater more efficiently than does red light.

Keywords: circadian rhythm; *Cry1*; glucose; melatonin receptor 1; *Per2*; thermal stress

1. Introduction

All organisms have daily circadian rhythms, known as circadian clocks, to regulate their physiological, biochemical and behavioural functions (King & Takahashi 2000). Among the many factors that control circadian rhythms, light is the most important (Jin et al. 2009). Light at various wavelengths can induce or inhibit organisms with photoreceptors, and influences not only physiological changes but also reproduction and behaviour (Pierce et al. 2008).

In aquatic organisms, water temperature also influences the regulation of circadian rhythms (Beckmann et al. 1990). In aquatic vertebrates such as fish, water temperature affects circadian rhythms, hormone activity and immunity (Chang et al. 2001).

Some organisms, including fish, have various clock genes, known as the circadian pacemaker, which regulate circadian rhythms (Nanako et al. 2012). For example, the typical clock genes regulating circadian rhythms are *period2* (*Per2*) and c*ryptochrome1* (*Cry1*); these clock genes are activated by light. *Per2* has high day-time expression but

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low night-time expression and appears to be involved in light-dependent clock resetting (Dunlap 1999; Kim et al. 2012). Moreover, among the circadian oscillators, *Cry1* is rapidly induced when it is stimulated and driven by light conditions; this oscillation dampens quickly after the transfer of cells to a dark environment (Cermakian et al. 2002; Besharse et al. 2004).

Melatonin is also known to regulate circadian rhythms. This hormone is produced mainly in the pineal organ and retina, and its plasma content is higher in the night-time than in the daytime. It plays an important role in the regulation of circadian rhythms, not only as a neuroendocrine messenger, but also in seasonal biological rhythms (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 & Swaab 2005; Kim et al. 2014).

The effects of melatonin are mediated by melatonin receptors (MTs), which belong to the G-protein-coupled receptor superfamily. MTs are distributed in the central nervous system and peripheral tissues of vertebrate species and mediate various physiological functions of melatonin in these tissues (Reppert et al. 1996; Park et al. 2013). Dubocovich et al. (2000) recently described three different subtypes of MTs: MT1, MT2 and MT3. In particular, MT1 has been identified in vertebrate brains, including in the hypophyseal pars tuberalis and hypothalamic suprachiasmatic nucleus, where it recognizes seasonal changes and regulates circadian rhythms (Reppert et al. 1996).

However, research on the genes regulating circadian rhythms in fish brains has not been systemically conducted, not only for MT1 but also for clock genes such as *Per2* and *Cry1*.

Recently, several studies have reported that fish circadian rhythms are regulated by irradiation with specific wavelengths of light-emitting diodes (LEDs) (Shin et al. 2011; Park et al. 2013). Also, Shin et al. (2012) reported on the benefits of narrow bandwidth light that promotes early growth and reproduction in yellowtail clownfish. LEDs, a new form of lighting technology that is still being developed, can be manufactured to emit specific wavelengths (Migaud et al. 2007).

In this study, we investigated the possible effects of LED lights of specific wavelengths on the control of circadian rhythms, including changes in mRNA expression of clock genes such as MT1, *Per2* and *Cry1*, by using goldfish *Carassius auratus* exposed to thermal stress (high water temperatures) under three wavelengths (red, green and mixed purple). In addition, we determined the effects of LED lights on circadian rhythm control in the fish by measuring changes in plasma melatonin and glucose concentrations.

2. Materials and methods

2.1. Experimental fish and conditions

For each experiment, common goldfish (length, 6.1 ± 0.5 cm; mass, 12.5 ± 0.4 *g*) were purchased from a commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in three 300-L circulation filter tanks in the laboratory. Each tank (an experimental group) contained 12 fish. The goldfish were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatize to the conditions for 24 h. The water temperature was then increased from 22 to 30 °C in daily increments of 2 °C.

We established a control group, exposed to a water temperature of 22 °C, and experimental groups, exposed to water temperatures of 26 or 30 °C. In the present study, the experimental design used for specific spectrums was modified from the methods of Shin et al. (2011). Briefly, the control and experimental groups were exposed to red (peak at 630 nm), green (530 nm) or purple (mixed 450 and 630 nm) LEDs (Daesin LED Co., Kyunggi, Korea), or light from a white fluorescent bulb (Figure 1). There was a 12-h light (L):12-h dark (D) photoperiod (lights on at 07:00 h and lights off at 19:00 h). The LEDs were placed 40 cm above the surface of the water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2. The fish were reared under these conditions with a daily feeding of commercial feed until the day prior to the sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA). The fish were anesthetized with 200 mg/L tricaine-methane-sulphonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) to minimize stress prior to blood collection. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, 5 min) and stored at −80 °C until analysis. The fish were euthanized by spinal transaction (the first sampling at 11:00 h) at 12 h sampling intervals to collect the hypothalamus and blood under dim fluorescent bulb light using an attenuated white fluorescent bulb.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using the TRIzol kit according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Three micrograms of total RNA was reverse transcribed in a total volume of 20 μL, using an oligo-$d(T)_{15}$ anchor and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for use in polymerase chain reaction (PCR) and quantitative PCR (QPCR).

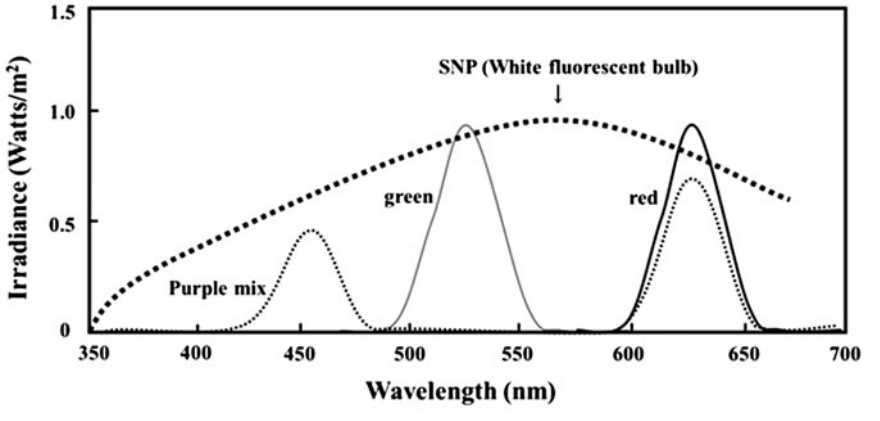

Figure 1. Spectral profiles of red (630 nm), green (530 nm), and purple [mixed blue (450 nm) and red (630 nm)] LEDs used in this study. The large dotted line is the spectral profile of white fluorescent light (SNP).

2.3. Quantitative PCR

To carry out this study, we have considered the recommendations of the minimum information for publication of QPCR experiments guidelines (Bustin et al. 2009). Total RNA was extracted from the hypothalamus using a TRIzol kit (Gibco/BRL) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer) according to the manufacturer's instructions. QPCR was performed using cDNA. QPCR was conducted to determine the relative expression levels of *Cry1* (GenBank accession No. EF690700), *Per2* (EF690697), MT1 (AB481372) and *β*-actin (AB039726) mRNA using total RNA extracted from the hypothalamus. The primers used for QPCR are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure the specificity of primers. The PCR amplification was conducted using a Bio-Rad iCycler iQ Multicolour Real-time PCR Detection System (Bio-Rad) 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 50 cycles each of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with *β*-actin, and all data were expressed relative to the corresponding *β*-actin-calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and the internal controls (*β*-actin) were calculated using the $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = 2\text{^} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$] (Livak & Schmittgen 2001).

2.4. Western blot analysis

Total protein from the brain and pituitary of goldfish was extracted using a protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL PMSF, and 0.15 mg/mL leupeptin, pH 7.4), sonicated and quantified using the Bradford method (Bio-Rad). A total of 25 µg of total protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad). For reference, a protein ladder (Bio-Rad) was also used. Samples were electrophoresed at 180 V. The gels were immediately transferred to a 0.2 µm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% milk in 0.04% Tris-buffered saline with Tween (TTBS) for 45 min and subsequently washed in TTBS. The membranes were incubated with MT1 antibodies [NOVUS BIOLOGICALS® Melatonin Receptor 1A Antibody (NBP1-28912); dilution, 1:4000; approximately 45 kDa], followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:4000; Bio-Rad) for 60 min. As an internal control, the

Table 1. Primers used for QPCR amplification.

Genes (accession no.)	Primer	Sequences
Cry1 (EF690700)	Forward	5′-CGG AGA CCT GTG GAT CAG-3′
	Reverse	5′-GTG GAA GAA TTG CTB GAA-3′
Per2 (EF690697)	Forward	5′-CTG GAG CCG CAA AGT TTC-3′
	Reverse	5′-CTG GAT GTC TGA GTC TAA-3′
MT1 (AB481372)	Forward	5′-TTG GCA GTA GCG ATT TCT C-3′
	Reverse	5′-CTC ACG ACG GAA GTT CTG-3′
β-actin (AB039726)	Forward	5′-TTC CAG CCA TCC TTC CTA-3′
	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

β-tubulin primary antibody (dilution, 1:4000; ab6046, Abcam, UK) was followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:4000; Bio-Rad) for 60 min. Bands were detected using a sensitive electrochemiluminescence (ECL) system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc™ XRS$^+$ System (Bio-Rad).

2.5. *In vitro culture of brain cells*

The culture of goldfish brains was performed using enzymatic and mechanical procedures. Brain tissue was quickly removed and placed in 3 mL of ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, containing 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL fungizone; GIBCOBRL, Rockville, MD, USA). The isolated brain tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the brain tissues were chopped into small pieces with a pair of scissors. The brain cells and minced brain tissue were transferred to a flask and incubated for 10 min at room temperature while being slowly stirred. The mixture of dispersed brain cells and tissues was filtered, and the culture medium (neuro basal medium, without L-glutamine, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 1% foetal bovine serum, Gibco-BRL; with the medium's osmolarity adjusted to match goldfish plasma osmolarity of 353 mOs) was added. The cell suspension was centrifuged at $800 \times g$ for 10 min, and the cells were then resuspended in fresh culture medium.

The brain cells (1.2×106 cells $\times$ 800 μL^{-1} well) were applied to a 24-well tissue culture plate, which was started at 23:00 h and then sampled at 12- (L), 24- (D) h intervals. For the experimental groups, brain cells were exposed to either red (peak at 630 nm), green (530 nm) or purple (mixed 450 and 630 nm) LEDs (Daesin LED Co., Kyunggi, Korea) or a white fluorescent bulb. The LEDs were set 50 cm above the surface of the cell culture plate, and the irradiance at the surface of the plate was maintained at approximately 0.9 W/m^2, and a 12-h L:12-h D period (lights on 07:00 h and lights off 19:00 h). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD) and the temperature was maintained using a heat prevent system for the LEDs.

2.6. *Plasma parameter analysis*

2.6.1. *Plasma melatonin level*

Plasma samples were separated by centrifugation (4 °C, 10,000 $\times$ *g*, 5 min), and plasma melatonin levels were analyzed using the immunoassay technique with a Melatonin direct Saliva ELISA kit (IBL, Hamburg, Germany). An anti-antibody specific to the antibody of melatonin was pre-coated onto a microplate, following which 50 μL of plasma, 50 μL of HRP-conjugate and 50 μL of the antibody were added to each well. These were mixed well and then incubated for 2 h at 37 °C. Following the last wash, any remaining wash buffer was aspirated or decanted off, and 50 μL each of substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which they changed from colourless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each

well, resulting in the colour changing from blue to yellow. The optical density of the solution in each well was then determined within 10 min, using a microplate reader set to 450 nm.

2.6.2. Plasma glucose level

The plasma glucose level was measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

2.7. 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).

3. Results

3.1. Changes in Per2 and Cry1 mRNA expression levels

To compare the expression levels of *Per2* and *Cry1* mRNA during temperature changes under different LED light spectra, we performed QPCR using cDNA extracted from goldfish hypothalamuses in each experimental group. *Per2* and *Cry1* mRNA expression levels in the photophase group of simulated natural photoperiod (SNP) and all LED light spectra were significantly higher than in the scotophase, and the levels significantly decreased with thermal increase.

Additionally, *Per2* and *Cry1* mRNA expression levels in the green LED groups were significantly higher than in the red LED groups (Figure 2).

3.2. Changes in MT1 mRNA and protein expression levels

Based on the western blots using protein extracted from goldfish brains, the levels in the scotophase group of SNP and all LED light spectra were significantly higher than in the photophase group. Additionally, the levels significantly increased with thermal increase. In particular, MT1 protein levels in the high water temperature red LED group were higher than those in the other groups (Figure 3(a)).

We performed QPCR using cDNA extracted from goldfish hypothalamuses in each experimental group to compare the expression levels of MT1 mRNA. The levels of MT1 mRNA expression in the scotophase were significantly higher than in the photophase and the levels significantly increased with thermal increase. In particular, the levels in the red LED group were significantly higher than those of the other LED spectra groups, but the levels in the green LED group were significantly lower than the other groups (Figure 3(b)).

3.3. Changes in Per2 and Cry1 mRNA expression in cultured brain cells (in vitro)

We investigated changes in the *Per2* and *Cry1* mRNA levels in brain cells cultured for 2 days under SNP or red, green or purple LED light conditions at intervals of 12 h in the daytime (11:00 h; ZT4, ZT28) and night-time (23:00 h; ZT16, ZT40). *Per2* and *Cry1* mRNA levels significantly decreased with culture time and the levels in the photophase were significantly higher than those in the scotophase. In particular, the levels in

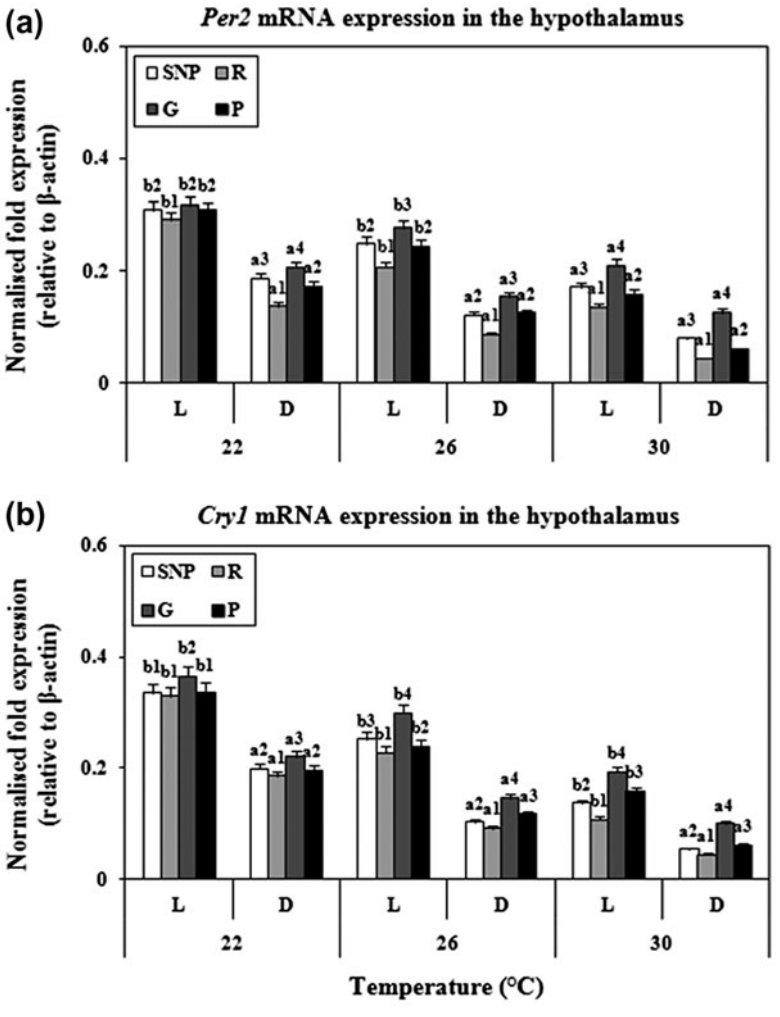

Figure 2. Changes in the expression levels of *Per2* mRNA (a) and *Cry1* mRNA (b) in the hypothalamus of goldfish at 22 °C (control), 26 and 30 °C using red (R), green (G) and purple (P) LEDs and a SNP, as measured by quantitative real-time PCR. The total RNA from the hypothalamus (2.0 μg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the *β*-actin levels in the same sample. Values with different characters are significantly different at different times in fish exposed to the same temperature ($p < 0.05$). The numbers indicate significant differences between different LEDs within the same temperature ($p < 0.05$). All values are means ± SE ($n = 5$).

the green LED group were significantly higher than those of the other LED spectra groups, but the levels in the red LED group were significantly lower than in the other groups (Figure 5).

3.4. Changes in MT1 mRNA expression levels by cultured brain cells (in vitro)

We investigated the changes in the MT1 mRNA levels in brain cells cultured for 2 days under SNP or red, green or purple LED light conditions at intervals of 12 h in the

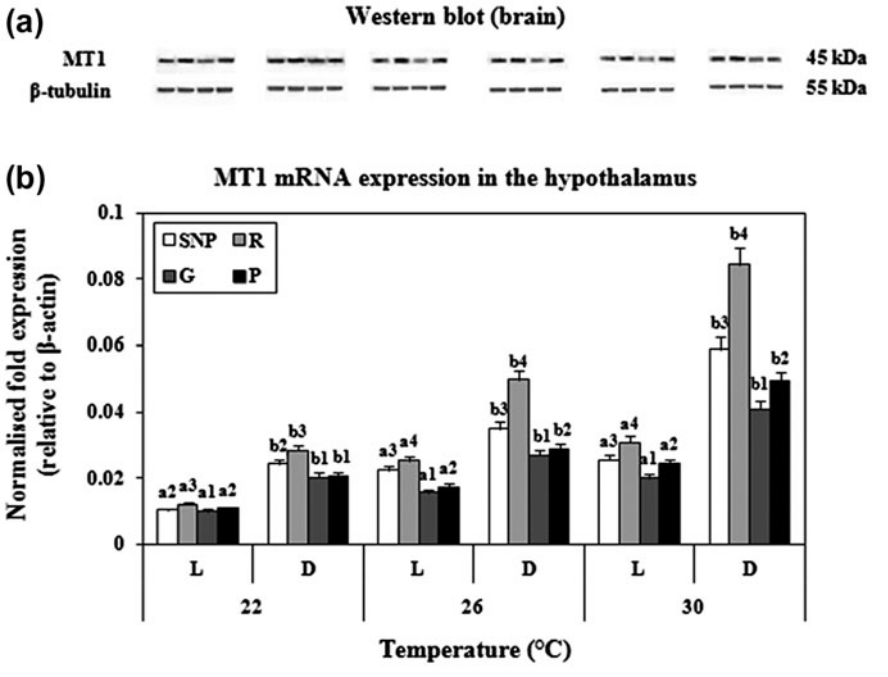

Figure 3. Changes in the expression levels of MT protein (anti-goldfish MT; a polyclonal rabbit antibody; 45 kDa) in the brain (a) and MT1 mRNA in the hypothalamus of goldfish at 22 °C (control), 26 and 30 °C using red (R), green (G) and purple (P) LEDs and a SNP, as measured by quantitative real-time PCR (b). Values with different characters are significantly different at different times in fish exposed to the same temperature ($p < 0.05$). The numbers indicate significant differences between different LEDs within the same temperature ($p < 0.05$). All values are means ± SE ($n = 5$).

daytime (11:00 am; ZT4, ZT28) and night-time (11:00 pm; ZT16, ZT40). MT1 mRNA expression levels significantly increased with culture time and the levels in the scotophase were significantly higher than those in the photophase. In particular, the levels in the red LED group were significantly higher than those of the other LED spectra groups, but the levels in the green LED group were significantly lower than in the other groups (Figure 6).

3.5. Plasma melatonin and glucose levels

Plasma melatonin and glucose levels significantly increased with increasing temperatures under all LED light spectra. In particular, the melatonin and glucose levels in the scotophase were significantly higher than that in the photophase (Figure 4). The plasma melatonin level in the 22 °C group in the photophase (11:00 h) and SNP was the lowest (12.5 ± 0.6 pg/mL) and this level increased with thermal increase. In particular, the level in the 30 °C group of red LEDs was the highest (83.4 ± 4.1 pg/mL) (Figure 4(a)).

The plasma glucose level in the 22 °C group in the photophase (11:00 h) under SNP was the lowest (2.5 ± 0.1 mmol/L), similar to the plasma melatonin levels, and the level increased with increasing temperatures. In particular, the levels in the 30 °C group with red LEDs was the highest (10.2 ± 0.5 mmol/L). The levels in all temperature groups with red LEDs were significantly higher than that with the other LED groups (Figure 4(b)).

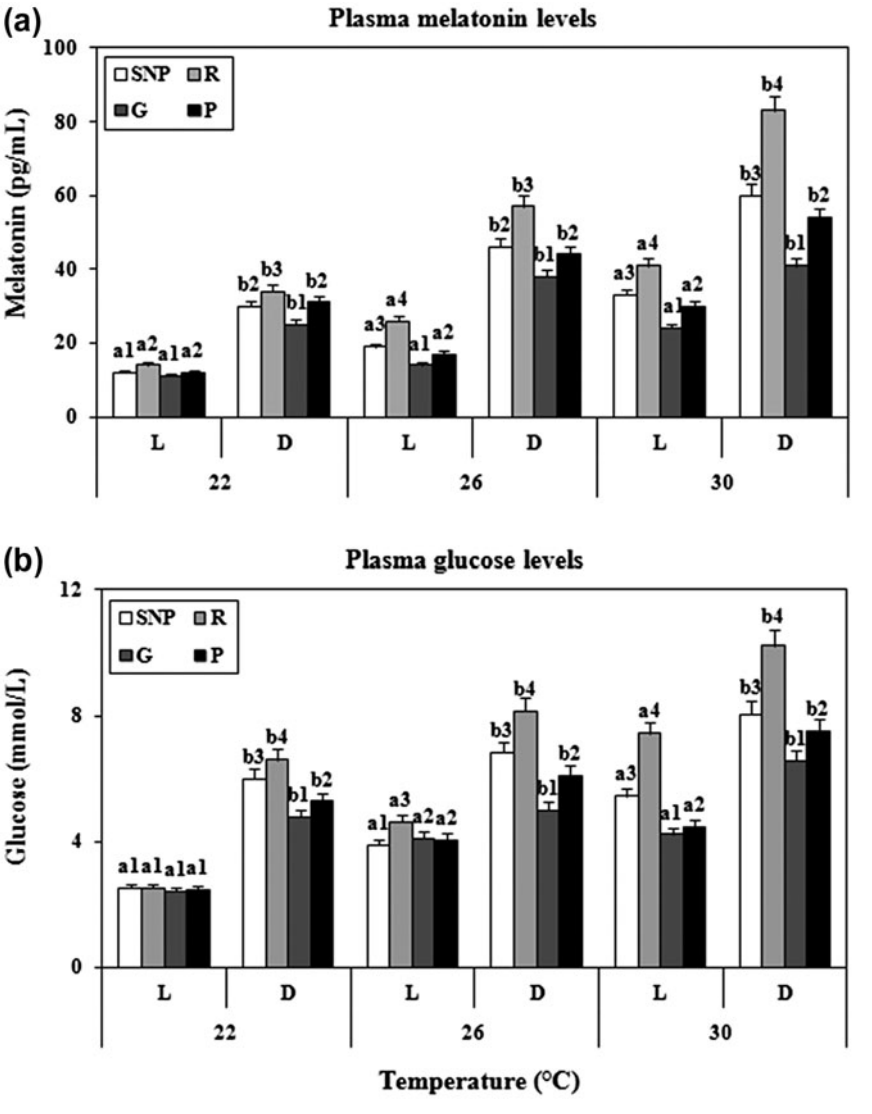

Figure 4. Changes in the levels of plasma melatonin (a) and glucose (b) in goldfish at 22 °C (control), 26 and 30 °C using red (R), green (G) and purple (P) LEDs and a SNP, as measured by a plate reader. Values with different characters are significantly different at different times in fish exposed to the same LEDs ($p < 0.05$). The numbers indicate significant differences between different LEDs within the same time ($p < 0.05$). All values are means ± SE ($n = 5$).

Plasma melatonin and glucose levels in all temperature groups with green or purple LEDs were significantly lower than that in the other groups.

4. Discussion

To identify how the circadian rhythm changes of goldfish exposed to thermal stress are regulated by LEDs, we investigated the expression of clock gene (MT1, *Per2* and *Cry1*)

mRNA and MT1 protein levels in goldfish under different LED spectra (red, green and purple) after exposure to thermal stress (22, 26 and 30 °C).

First, the goldfish were irradiated with SNP or red, green or purple LEDs after exposure to thermal stress. The expression of *Per2* and *Cry1* mRNA was decreased at the high water temperature, while the expression of MT1 mRNA was increased (Figures 2 and 3). These results indicate that oxidative stress increased in the goldfish at the high water temperatures, so melatonin levels were increased to act as antioxidants. The generation of CLOCK-BMAL heterodimers is inhibited by increased melatonin (Gerstner & Yin 2010), and it has been hypothesized that *Per2* and *Cry1* act as a feedback with CLOCK-BMAL heterodimers, and are also decreased by melatonin.

Per2 and *Cry1* mRNA expression levels in the photophase group of SNP and all LED light spectra were significantly higher than in the scotophase within the same experimental temperature. However, *Per2* and *Cry1* mRNA expression levels in the red LED experimental group were the lowest, while the expression levels in the green and purple LED experimental groups were the highest (Figure 2).

MT1 mRNA expression levels in the photophase group of SNP and all LED light spectra were significantly higher than those in the scotophase (Figure 3(b)).

These results are consistent with a previous study, which reported that the expression levels of MT1 mRNA in yellowtail clownfish were the highest in the red LED group and the expression levels of *Per2* and *Cry1* mRNA were the highest in the green LED group. Migaud et al. (2006) also reported that the wavelength energy of red LED lights, which have a longer wavelength, is wasted by being rapidly absorbed in the water column; therefore, the penetration of light is convulsively declined and the fish cannot detect the lights.

In this study, the expression levels of the mRNA of *Per2* and *Cry1*, which respond to light, were significantly lower and MT1, which increases in the scotophase, and mRNA were significantly higher in the red LED group than in the other LED groups because the goldfish did not perceive the light because the red LED energy was absorbed by the water.

The plasma melatonin concentration in goldfish was significantly higher in the high-water temperature group (Figure 4(a)). These results are consistent with those of a previous study where goldfish exposed to high water temperatures increased their plasma melatonin concentration (Kim et al. 2014). Kim et al. (2014) reported that a high-water temperature environment acted as a factor in oxidative stress, and melatonin, which is both a circadian regulation hormone and antioxidant, increased; but a melatonin decrease induced oxidative stress in high water temperatures. Also, the plasma melatonin concentration was significantly lower than under the green LED lights (Figure 4(a)). These results were described in the previous study of Shin et al. (2011), which showed that the effects of green LED lights protected yellowtail clownfish from oxidative stress.

Based on our observations of the plasma melatonin levels in goldfish exposed to high water temperatures, we suggest that thermal stress confuses the circadian rhythm of goldfish (Figure 4(a)). However, although the goldfish were exposed to thermal stress, we observed significantly lower plasma melatonin levels in the green and purple LED light groups than in the other LED light group. Therefore, we suggest that the green and purple LED lights reduced stress, thus stabilizing and maintaining circadian rhythms.

In addition to plasma melatonin levels, plasma glucose levels in the red LED group were significantly higher than in the other LED groups (Figure 4(b)). These results demonstrate that when goldfish are stressed, the hypothalamus stimulates the secretion of a releasing factor that regulates the pituitary, and the pituitary stimulates the adrenocorti-

cotropic hormone (ACTH) in the plasma. This hormone stimulates the adrenal cortex to secrete cortisol (Krishnan et al. 1991). This is because cortisol is a promoter of gluconeogenesis, which increases plasma glucose levels (Krishnan et al. 1991).

Furthermore, although plasma glucose levels were low in the photophase, Hirota et al. (2012) reported that *Cry1*, which is expressed in the photophase, inhibits gluconeogenesis. Additionally, we hypothesized that plasma glucose levels with green LEDs

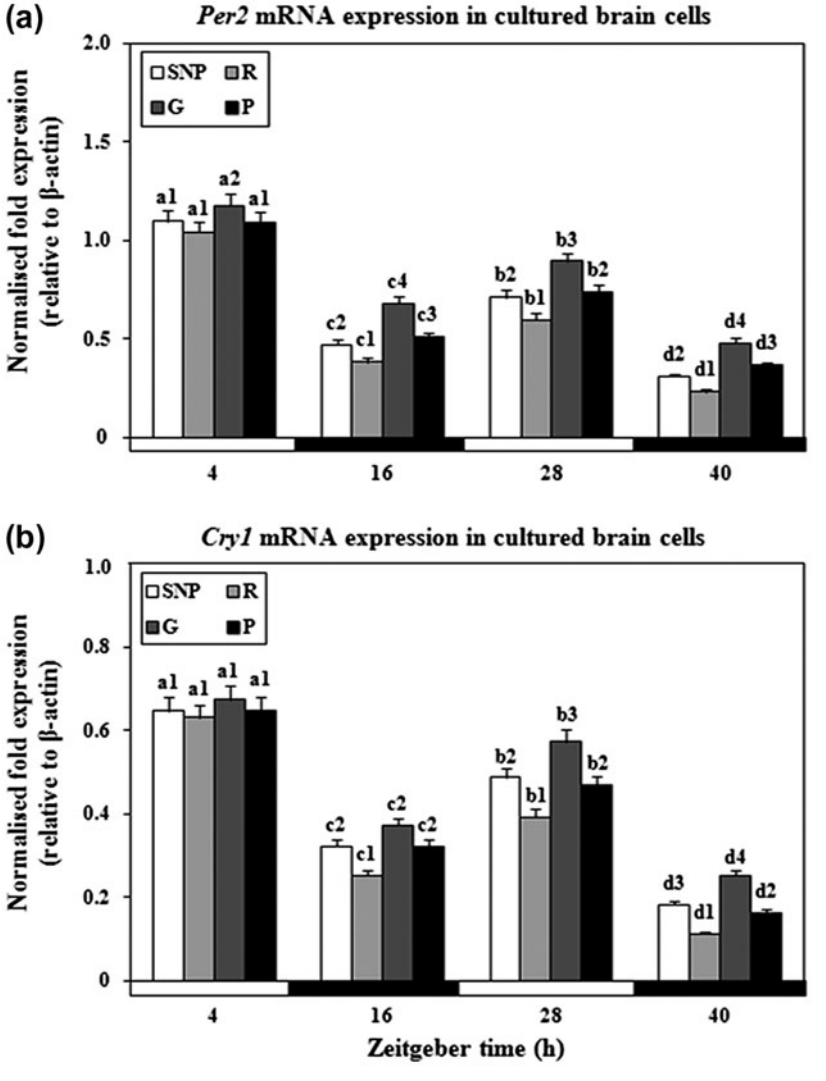

Figure 5. Changes in the expression levels of *Per2* (a) and *Cry1* (b) mRNA in the cultured brain cells of goldfish under red (R), green (G) and purple (P) LEDs and a SNP, as measured by quantitative real-time PCR. The sampling was accomplished twice, in light and darkness, during 48 h. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are significantly different at different times in fish exposed to the same LEDs ($p < 0.05$). The numbers indicate significant differences between different photoperiods within the same day ($p < 0.05$). All values are means ± SE ($n = 5$).

were lower than in the other LED groups; it seems that green LEDs stabilize physiological effects by reducing stress (Park et al. 2013).

To examine whether the LED spectra play the same roles in the *in vitro* environment, we examined the expression of *Per2*, *Cry1* and MT1 mRNA in brain cells cultured under SNP or red, green or purple LED light conditions. *Per2* and *Cry1* mRNA levels were significantly increased in the red LED group, and the levels at ZT4 and 28 were lower than at ZT16 and ZT40 (Figure 5). MT1 mRNA expression levels in the red LED group were significantly lower than in the other spectra groups, and the levels at ZT16 and 40 were higher than at ZT4 and ZT28 (Figure 6). These results were similar to the *in vivo* results. This is in agreement with Martinez-Chavez and Migaud (2009), who reported that the pineal organ of the Nile tilapia *Oreochromis niloticus niloticus* was photosensitive and, when placed *in vitro*, increased night-time melatonin levels. We hypothesize that the observed increase in ZT16 and 40 means that photosensitivity was stimulated in the night-time as culture time passed. Oliveira et al. (2009) reported that pineal organs maintained a rhythmic production of melatonin *in vitro* when exposed to an LD cycle.

We suggest that these results are in agreement with previous studies (Delaunay et al. 2003; Velarde et al. 2009) showing that *Per2*, *Cry1* and MT1, as clock genes that control circadian rhythms, play important roles in environments that vary in temperature, light source or photoperiod. We also suggest that green and purple wavelengths are especially effective for maintaining circadian rhythms.

In summary, when goldfish were exposed to high water temperatures in the red LED group, *Per2* and *Cry1* mRNA expression levels were the lowest, and MT1 mRNA expression, plasma melatonin and glucose levels were higher than in the other groups. These results suggested that oxidative stress in goldfish increased with exposure to high

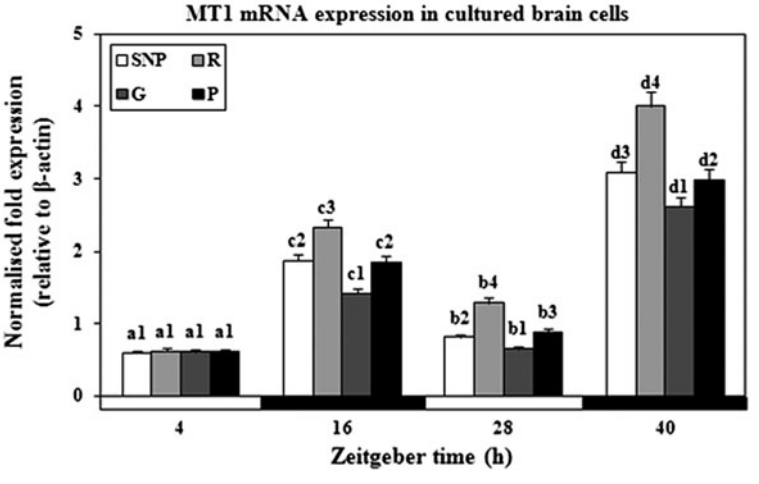

Figure 6. Changes in the expression of MT1 mRNA in the brain cells of goldfish cultured under red (R), green (G) and purple (P) LEDs and a SNP, as measured by quantitative real-time PCR. The sampling was performed twice, in light and darkness, during 48 h. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters were significantly different at different times in fish exposed to the same temperature ($p < 0.05$). The numbers indicate significant differences between different LEDs within the same temperature ($p < 0.05$). All values are means ± SE ($n = 5$).

water temperatures. When antioxidant activity occurs to reduce oxidative stress in goldfish, the clock genes are controlled to maintain circadian rhythms. Oxidative stress likely increases in the long wavelength environment, such as under the red LEDs, and this has an effect on controlling clock genes and melatonin. However, oxidative stress was controlled in short wavelength environments such as under the green LEDs, and this effectively maintains circadian rhythms such as controlling physiological changes in high-temperature stress environments.

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