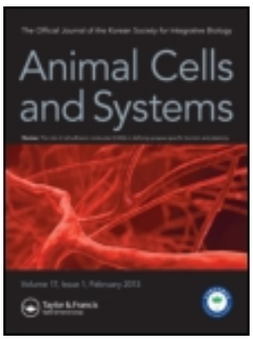

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Effects of LED spectral sensitivity on circadian rhythm-related genes in the yellowtail clownfish, *Amphiprion clarkii*

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Using clock genes in the yellowtail clownfish *Amphiprion clarkii*, we investigated the effects of light spectra and melatonin on circadian rhythms using light-emitting diodes (LEDs): red, green, and blue. To assess differences in circadian rhythms under different LED light spectra, we measured changes in the expressions of Melatonin receptor 1 (MT1) and *Period 2* (*Per2*) and *Cryptochrome 1* (*Cry1*) genes and changes in the plasma melatonin levels. Under red light, MT1 expression was significantly higher than that under other light spectra, but *Per2* and *Cry1* expressions were significantly lower than were those under other light spectra. Plasma glucose was significantly higher under red light than under other light spectra. These results indicate that, because red light is rapidly absorbed by water molecules, it cannot be detected by fish and acts as a stressor. In addition, melatonin regulates circadian rhythms via clock genes, and green and blue lights may be the most suitable for altering circadian rhythms in yellowtail clownfish, as these wavelengths generally penetrate seawater more efficiently than that by red light.

Keywords: LED; circadian rhythm; *Per2*; *Cry1*; gluconeogenesis

Introduction

Among the many factors that control circadian rhythms, light is the most important, because it affects many of the physiological and behavioral changes that occur within 24-h periods (Pierce et al. 2008). Because of the central importance of the day–night light cycle (the photoperiod) for the survival of organisms, light-sensitive circadian clocks have evolved in most animals, including fish. The photoperiod exerts an endogenous effect by causing rhythmic synthesis and release of the "time-keeping" hormone melatonin, which affects rhythmic physiological functions in fish (Bromage et al. 2001). Many activities in organisms vary according to periodic changes in environmental factors. Melatonin is mainly produced in the pineal organ and retina, and its concentration in the plasma is higher during the night than the day. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of the circadian rhythm but also affects seasonal biological rhythms (Reiter 1991).

The effects of melatonin are mediated by melatonin receptors (MTs), which belong to the G protein-coupled receptor superfamily (Reppert et al. 1996). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Reppert et al. 1996) and mediate various physiological functions of melatonin in these tissues. Reppert et al. (1996) used recent molecular techniques to examine amino acid structures and revealed the existence of three different subtypes of melatonin receptors, MT1–MT3. In particular, MT1 has been widely identified in vertebrates, and high levels of MT1 expression have been detected in the suprachiasmatic nucleus (SCN; Reppert et al. 1996), where the master circadian clock system is located in mammals (Masana et al. 2000). Therefore, photoperiod information conveyed via MTs can control the circadian rhythm in the SCN and regulate the circadian rhythm by feedback mechanisms involving the clock genes (Okamura et al. 2002; Park et al. 2007).

However, there are few studies of circadian rhythm regulation systems in the fish brain (Shi et al. 2004; Park et al. 2007). A recent report showed that the master circadian clock in teleost fishes may be the brain and pineal gland and that illumination during the night affects melatonin synthesis in a chromatic- and intensity-dependent manner in the pineal glands of all examined teleost fish species (Ekström & Meissl 1997).

Period (*Per*), which was first characterized in *Drosophila* (Reddy et al. 1984), is a key protein in the circadian system of animals. *Per* genes that code for a cytoplasmic heterodimer have been identified in vertebrates, and the role of these cytoplasmic heterodimers as circadian oscillators has been studied extensively. Four types of *Per* (*Per1–Per4*) have been reported in zebrafish (*Danio rerio*; Delaunay et al. 2000). In zebrafish, *Per2* is a circadian oscillator that is rapidly induced by light information from the SCN and appears to be involved in light-dependent clock resetting (Vallone et al. 2004).

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Cryptochrome 1 (*Cry1*) regulates gluconeogenesis in the liver and the biological clock (Hirota et al. 2012). Hirota et al. (2012) reported that the small molecule, KL001, regulates *Cry* activity. These authors found that *Cry* gene inhibits gluconeogenesis by blocking enzymes and activates the gluconeogenesis pathway, as indicated by an increase in the *Cry* expression when KL001 was injected into mouse hepatocytes.

Recently, studies of light effects have been performed by using light-emitting diodes (LEDs) instead of natural light or metal halide lights (Shin et al. 2011). LEDs are a new form of lighting technology that is still being developed and can be manufactured to output specific wavelengths (Migaud et al. 2007). Furthermore, LEDs have lower power requirements, lower electrical running costs, and longer life spans than standard metal halide bulbs (Migaud et al. 2007).

However, LED studies have only recently examined the effects of photoperiod and light intensity in fish (Pierce et al. 2008). Although studies have documented the responses of fishes to various spectra (Villamizar et al. 2009; Shin et al., 2011, 2012), investigations into changes in the control of fish circadian rhythms due to exposure to various light spectra remain very limited.

In the current study, we investigated the effects of specific spectra on the circadian rhythm of the yellowtail clownfish, *Amphiprion clarkii* (a high-value ornamental fish), by measuring changes in the expression of the MT1 gene, expression of the clock genes *Per2* and *Cry1*, and plasma glucose in relation to daily rhythms after exposure to red, green, or blue LED spectra.

Materials and methods

Experimental fish and conditions

Yellowtail clownfish ($n = 280$; length, 5.5 ± 0.3 cm; weight, 2.4 ± 0.5 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and were allowed to acclimate for two weeks in twelve 300-l circulation filter tanks in the laboratory. The fish were exposed to a simulated natural photoperiod (SNP) (Migaud et al. 2007). 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 (L):12-h dark (D) period (light 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) for 28 h. 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 spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA) (Figure 1).

Melatonin determination by ELISA

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

Plasma glucose analysis

Plasma samples were separated by centrifugation (4°C, $10{,}000 \times g$, 5 min), and then the plasma glucose level was measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

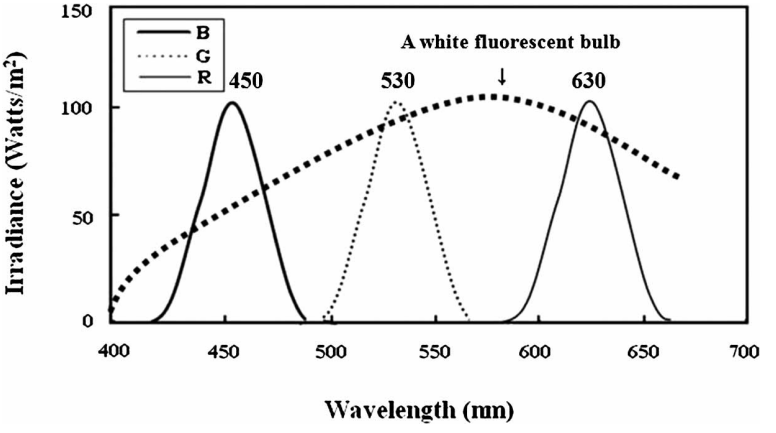

Figure 1. Spectral profiles of blue (B), green (G), or red (R) LEDs used in this experiment. Reprinted from Shin et al. (2011) with permission from Comparative Biochemistry and Physiology, Part-A.

Results

Expression of MT1, Per2, and Cry1 genes in the brain

We examined the effect of different LED light spectra on the expression of MT1, *Per2*, and *Cry1* genes in the brain by using quantitative real-time polymerase chain reaction (qPCR) (Figure 2). The expression of MT1 was significantly higher during the scotophase than during the photophase for all light spectra. In contrast, expressions of *Per2* and *Cry1* were significantly higher during the photophase than during the scotophase for all light spectra. MT1 expression in the group exposed to red LEDs was significantly higher than the expression in the groups exposed to other LEDs, and *Per2* and *Cry1* expressions in the groups exposed to green and blue LEDs were significantly higher than the expressions in the groups exposed to red LEDs.

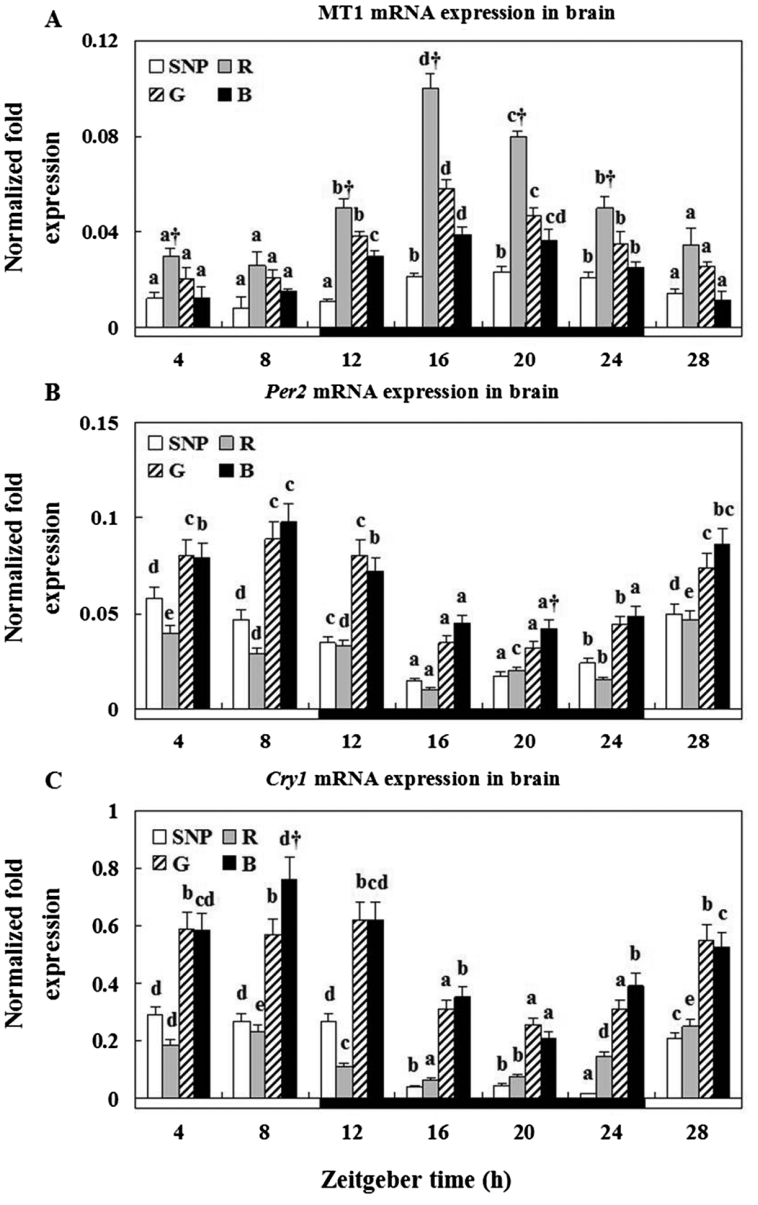

Figure 2. Changes in the expression levels of MT1 (A), *Per2* (B), or *Cry1* (C) genes in the brain of yellowtail clownfish under lighting conditions with red (R), green (G), and blue (B) light-emitting diodes and a simulated natural photoperiod, as measured by quantitative real-time polymerase chain reaction. The fish were reared under a light:dark (LD) cycle (12:12). Total brain RNA (2.5 μg) was reverse-transcribed and amplified. Results were expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different between the Zeitgeber times (ZTs) within the same light spectrum ($P < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT ($P < 0.05$). All values are means ± SD ($n = 5$).

Plasma melatonin levels

We observed the effects of the different light spectra on plasma melatonin levels by using a microplate reader (Figure 3). Plasma melatonin levels were significantly higher during the scotophase than during the photophase for all light spectra. Plasma melatonin levels in the group exposed to red LEDs were significantly higher than the levels in the groups exposed to other LEDs.

Plasma glucose levels

We observed the effects of the different light spectra on plasma glucose level by using a biochemistry autoanalyzer (Figure 4). Plasma glucose levels were significantly higher during the scotophase than during the photophase for all light spectra. However, glucose levels were significantly higher in the group exposed to red LEDs than in the other LED groups and the control group.

Discussion

In this study, we examined expression of the MT1, *Per2*, and *Cry1* genes in yellowtail clownfish that were exposed to different LED spectral groups (red, green, and blue). We also investigated the effects of specific LED spectra on the circadian rhythm of the fish by measuring plasma melatonin levels.

First, we observed that MT1 gene expression was significantly higher in the group exposed to red LEDs (Figure 2(A)) than in groups exposed to either green or

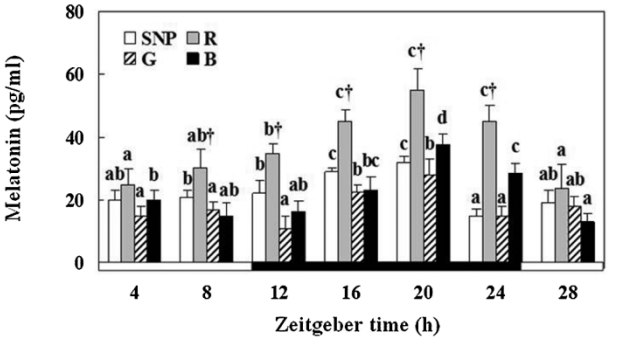

Figure 3. Plasma melatonin levels obtained in yellowtail clownfish under lighting conditions with red (R), green (G), or blue (B) light-emitting diodes and a simulated natural photoperiod, as measured using a microplate reader. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different between the Zeitgeber times (ZTs) within the same light spectrum ($P < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT ($P < 0.05$). All values are means ± SD ($n = 5$).

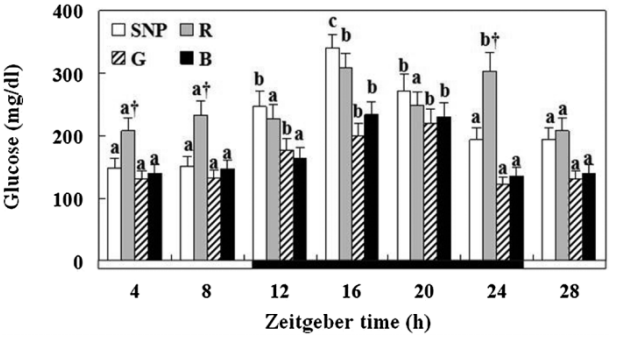

Figure 4. Plasma glucose levels obtained in yellowtail clownfish under lighting conditions with red (R), green (G), or blue (B) light-emitting diodes and a simulated natural photoperiod, as measured using a biochemistry autoanalyzer. The fish were reared under a light:dark (LD) cycle (12:12). The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different between the Zeitgeber times (ZTs) within the same light spectrum ($P < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT ($P < 0.05$). All values are means ± SD ($n = 5$).

blue LEDs. Migaud et al. (2006) reported that most light energy is wasted in the form of unsuitable wavelengths (longer wavelengths, red light) that are rapidly absorbed by water molecules and therefore cannot be detected by fishes. Meanwhile, Villamizar et al. (2009) reported that blue wavelengths stimulate the visual system of fishes to a degree that is sufficient to facilitate feeding. In addition, Falcon and Meissl (1981) and Villamizar et al. (2009) reported that the fish pineal gland has double spectral sensitivity to light in the blue–green region. In the present study, the fish could not detect red wavelengths because red light is rapidly absorbed by water molecules and does not reach the depths where the fish reside. We hypothesized that MT1 expression would be significantly higher in the group of fish exposed to red LEDs than in the groups exposed to other LEDs. We found that, at night, the levels of MT1 mRNA were significantly higher in these fish than in those in the SNP group and the groups exposed to blue–green LEDs. We hypothesized that fish cannot detect red light and thus perceive it as night; therefore, MT1 mRNA expression levels would be significantly higher in the group exposed to red LED than to other light spectra.

To date, the use of molecular approaches to examine the circadian system has been limited to only few model fish species. However, the importance of clock genes in the circadian rhythms of reproduction and feeding in several aquaculture fishes has been recently proposed. Furthermore, studies of the circadian system in fish using various spectra are very limited. We, therefore, measured expression of clock

genes in fish exposed to various LED spectra. Expressions of the clock genes, *Per2* and *Cry1*, were significantly higher in groups of fish exposed to blue–green LEDs (Figures 2(B) and 2(C)) than in those exposed to red LEDs. While MT1 expression increased significantly during periods of darkness (Figure 2(A)), clock gene expression increased significantly during periods of light. *Per2* and *Cry1* are induced by light exposure (Okamura et al. 2002). Hur et al. (2012) reported that the clock genes in the brain of the threespot wrasse, *Halichoeres trimaculatus*, have different daily and circadian patterns. The opposite results for clock gene expression and MT1 expression suggest that a feedback loop exists between melatonin and clock genes. Cahill and Besharse (1995) reported that the circadian rhythm-controlling hormones, melatonin and dopamine, play opposing roles in the regulation of circadian rhythms. Dopamine, an amacrine and interplexiform cell neurotransmitter, functions as a chemical signal for light, producing light-adaptive physiology (Dowling & Ehinger 1978). Melatonin, on the other hand, has dark-adaptive effects. Melatonin inhibits the release of dopamine by acting on the melatonin receptors, and dopamine inhibits the synthesis and release of melatonin from photoreceptor cells by acting on dopamine-like receptors. Dopamine can also induce *Per2* and entrain the circadian clock (Cahill & Besharse 1991). Thus, the melatonin-secreting photoreceptors and dopamine-secreting amacrine/interplexiform cells form a cellular feedback loop that functions to regulate circadian physiology (Iuvone et al. 2005). Our results support the hypothesis that the clock genes *Per2* and *Cry1* are inhibited by melatonin.

Moreover, melatonin influences the molecular clock to phase its circadian activity. Although it is not known whether the SCN of fish is a circadian oscillator, the master clocks are possibly located in the hypothalamus in addition to the eyes and pineal organ (Falcón et al. 2007).

The expressions of *Per2* and *Cry1* were induced by environmental factors and light. These observations are consistent with the involvement of clock genes and the light switch pattern of the circadian clock (Pando et al. 2001; Kim et al. 2012). In vertebrates, light-responsive clock genes have been reported in goldfish (*Carassius auratus*; Iigo et al. 2003) and zebrafish (Vallone et al. 2004), but detailed studies of clock genes in fish are limited.

Zhang et al. (2010) suggested that the clock component, *Cry1*, is a circadian regulator of hepatic gluconeogenesis as well as circadian rhythm. Through interaction with the Gs α subunit of G proteins, *Cry1* appears to modulate G protein-coupled receptor signaling. This may lead to temporal regulation of glucagon signaling, which is involved in the activation of hepatic gluconeogenesis. In the present study, we observed that plasma glucose levels were significantly higher throughout the entire photoperiod in fish exposed to a red LED spectrum than in those exposed to other light spectra, and that *Cry1* expression levels showed the opposite trend (Figure 4). Hirota et al. (2012) reported that *Cry1* inhibits gluconeogenesis, and we found that *Cry1* mRNA expression was significantly lower under red LEDs than under blue or green LEDs. On the other hand, plasma glucose was significantly higher under red LEDs than under blue or green LEDs. These results are in agreement with a study by Karakatsouli et al. (2007) in which the plasma glucose of rainbow trout, *Oncorhynchus mykiss*, was significantly higher in fish raised for 11 weeks under red light than in those raised under blue light. These authors concluded that red light induced stress and increased energy demands. Shin et al. (2012) measured the plasma H_2O_2 and lipid peroxidation levels in yellowtail clownfish and examined oxidative stress induced by red-spectrum lighting. Among the fish studied by Shin et al. (2012), plasma glucose levels were highest in the red LED group. In addition, new lighting technology, such as LED units, may be beneficial to the aquaculture industry, because the operating cost is relatively low and the bandwidth of the light can be specified. It is common knowledge that the blue–green end of the visible spectrum penetrates seawater more efficiently than longer wavelengths (Wagner 1990), and a number of reports suggest that fish are more sensitive to blue–green light than to red light (Max & Menaker 1992; Ekström & Meissl 1997; Bayarri et al. 2002).

In this study, we observed that plasma melatonin levels were significantly higher during the scotophase than during the photophase for all LED spectra. Moreover, melatonin levels were significantly higher throughout the entire photoperiod when fish were exposed to a red LED spectrum than to other spectra (Figure 3). We hypothesized that fish cannot detect red light; therefore, they perceive illumination by red light as darker than illumination by other spectra, and melatonin levels remain high. This result is in agreement with those of various teleost studies, such as those on goldfish (Iigo et al. 2003). In these studies, teleost melatonin levels were significantly higher during the scotophase than during the photophase.

In conclusion, we demonstrated that because red light could not be detected by the visual system of the yellowtail clownfish, due to the lack of photons, the fish perceived areas lit with red light as being darker than areas illuminated with blue–green wavelengths. In addition, red light induces stress and affects gluconeogenesis because of *Cry1* expression. MT1 expression was significantly higher in fish exposed to red LEDs

than in those exposed to blue–green LEDs, further supporting the hypothesis that yellowtail clownfish cannot detect red light because of the lack of photons. The expression of the clock genes *Per2* and *Cry1* was significantly higher in fish exposed to blue–green LEDs than in those exposed to red LEDs, suggesting that red light inhibits the circadian rhythms and is a stressor.

Acknowledgments

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