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Effects of retinal light input on circadian rhythm genes in the yellowtail clownfish (Amphiprion clarkii) as determined using LED light spectra

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The objective of this study was to test the effects of ophthalmectomy on the circadian rhythms in response to various wavelengths in yellowtail clownfish. The responses were evaluated by measuring rhodopsin (RH) and melatonin receptor 1 (MT1) mRNA and protein expression, and plasma melatonin levels. The RH, MT1 mRNA, and protein expression in ophthalmectomized fish were significantly lower than in intact fish. However, RH and MT1 mRNA and protein expression levels in green and blue light-emitting diode groups were similar between intact and ophthalmectomized fish. Furthermore, we found that the number of the RH-immunoreactive cells observed in the retina was almost in agreement with mRNA and protein expression patterns. These findings suggest that circadian rhythm involves the perception of light by the eye and the transduction of this signal through the circadian rhythm axis. Furthermore, short wavelengths lights may be most suitable for control of the circadian rhythm in fish.

Keywords: circadian rhythm; light-emitting diodes; melatonin receptor; ophthalmectomy; rhodopsin

Introduction

For several decades, cycles of white light alternating with darkness have been the standard stimuli used in laboratory studies of circadian behavioral patterns in organisms ranging from bacteria to primates (Tang et al. 1999; Morgan 2004). 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 a 24-h period (Pierce et al. 2008). In nature, the dramatic diel changes in skylight color are associated with concomitant changes in luminance (Endler 1993), but the role that such chromatic changes might play in the setting of circadian activity patterns has been largely ignored (Rani & Kumar 2000; Malik et al. 2004).

In recent years, the effects of lights on fish have been examined using light-emitting diodes (LEDs) instead of natural light or metal halide lights (Bapary et al. 2011; Shin et al. 2011). LEDs, which comprise a new form of lighting technology that is still under development, can be manufactured to output specific wavelengths (Migaud, Cowan et al. 2007). The spectral composition of incident light changes in various underwater

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environments, and rapid attenuation occurs with increasing depth (Lythgoe 1979). The short, or blue, end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates into shallow waters (Lythgoe et al. 1994; Myrberg & Fuiman 2002). Specific wavelengths from LEDs can affect the physiological functions of fish. For example, the wavelengths can have positive effects by inducing gonad development (Bapary et al. 2011) and enhancing growth (Shin et al. 2012), and they have also been reported to induce negative effects through oxidative stress (Shin et al. 2011). In addition, exposure to the blue spectrum has been shown to prevent stress in the Nile tilapia, *Oreochromis niloticus* (Volpato & Barreto 2001).

Because of the central importance of the day–night light cycle (the photoperiod) to 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).

Organisms respond to periodic changes in environmental factors (Bromage et al. 2001). Melatonin is mainly produced in the pineal organ and the retina, and its plasma content is higher at night than during the day. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of the circadian rhythms, but it also affects seasonal biological rhythms (Reiter 1991; Falcón et al. 2007). The photosensitive pineal organ of non-mammalian vertebrates transduces environmental light into a neural and neuroendocrine signal in the form of melatonin rhythms (Ekstrzm & Meissl 1997). The duration of nocturnal melatonin secretion is proportional to the length of the night. In that way, melatonin conveys diurnal and annual information to multiple biochemical and physiological processes in all vertebrates (Arendt 1995). Additionally, these actions are mediated via melatonin receptors (MTs), which belong to the G-protein-coupled receptor (GPCR) superfamily (Iigo et al. 1994; Reppert et al. 1996).

MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Dubocovich 1995; Reppert et al. 1996) and are thought to mediate various physiological functions of melatonin in these tissues. MT1 has a high affinity to [^{125}I] Mel and belongs to the GPCR family, and MT2, with a low affinity to [^{125}I] Mel, has also been identified (Reppert et al. 1996). In particular, MT1 has been widely identified in vertebrates (Roca et al. 1996), and high MT1 expression has been detected in the suprachiasmatic nucleus (SCN) (Dubocovich et al. 2003), where the master circadian clock system is located in mammals (Gauer et al. 1993). Therefore, MTs offer evidence that photoperiod information is conveyed to the SCN to control circadian rhythms (Masana et al. 2000).

Until recently, the photoperiodic and circadian responses (i.e. light perception and the melatonin entrainment pathway) in teleosts were believed to differ significantly from those of mammals. Photoperiodic response in mammals relies on a centralized organization where light information, perceived by retinal photoreceptors, is relayed via the retinohypothalamic tract to the master clock in the SCN. The SCN, in turn, directly controls melatonin production in the pineal organ (Ekstrom & Meissl 2003). Light also controls the expression of the gene that codes for rhodopsin (RH), a dim-light photoreceptor belonging to the GPCR family. RH is located in rod cells and transduces extracellular light signals into the cell interior (Khorana et al. 2002).

RH consists of a protein, opsin, and a retinal chromophore and is the primary photoreceptor responding to external light signals in the retina (Yokoyama 2000). The GPCR family is among the largest families of membrane receptors, and the family plays an important role in transmitting extracellular signals into the cell interior (Khorana et al.

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2002). Upon light activation, the isomerization of 11-*cis*-retinal into *all-trans*-retinal induces structural changes in the transmembrane domain (Khorana 2000). Thus, RH is able to transduce light signals into electrical responses via a series of signal amplification events, and those signals then reach the brain.

In the brain, the transmitted signal, especially when received by the SCN, which serves as the site of the central oscillator in the hypothalamus, controls circadian rhythms such as the diurnal rhythm (Reppert & Weaver 2001). The retina is thus entirely responsible for providing the brain with photic information (Foster 1998).

Therefore, we investigated the changes in the mRNA and protein expression levels of RH and MT1 and the immunohistochemistry (IHC) of RH expression in the retina during daily rhythms by exposing ophthalmectomized yellowtail clownfish (*Amphiprion clarkii*) to specific spectra. We also measured the changes in plasma melatonin levels to examine how ophthalmectomy changes an organism's circadian rhythm.

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Materials and methods

Experimental fish, ophthalmectomy, and conditions

Yellowtail clownfish ($n = 400$; length, 5.2 ± 0.5 cm; weight, 2.1 ± 0.5 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and were allowed to acclimate for two weeks in eight 300-l circulation filter tanks in the laboratory. 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 was 27 ± 1 °C, and the photoperiod was a 12-h light:12-h dark period (lights on 07:00 h and lights off 19:00 h). The fish were fed commercial feed twice daily (09:00 h and 17:00 h). For the experimental groups, the fish were exposed to blue (peak at 450 nm), green (530 nm), or red (630 nm) LEDs (Daesin LED Co. Kyunggi, Korea). The LEDs were set 50 cm above the surface of the water, and the irradiance at the surface was maintained at approximately 0.9 W/m^2. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Boulder, Colorado, USA). The fish ($n = 180$; length, 5.1 ± 0.5 cm; weight, 2.0 ± 0.4 g) were ophthalmectomized under anesthesia with 200 mg/l tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA). An incision was made around the one eye, which was then elevated with a forceps, and the optic nerve was cut to remove the one eye completely according to a method modified from Martinez-Chavez and Migaud (2009). Fish were ophthalmectomized just one eye for reducing infection and mortality. These ophthalmectomized fish were placed in 300-l circulation filter tanks under 12-h light:12-h dark period at 27 °C for one week. No mortalities were observed. 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 of the five fish 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 until analysis. The fish were euthanized by spinal transection at 4-h intervals for four times and then 8-intervals for three times, i.e. 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT0), 15:00 h (ZT8), 23:00 h (ZT16), and 07:00 h (ZT0) to collect the brain and retina under dim light.

Quantitative PCR

Total RNA was extracted from the brain and retina using a TRIzol kit (Gibco/BRL, USA). Reverse transcription was performed using M-MLV reverse transcriptase

(Bioneer, Korea) according to the manufacturer's instructions. Quantitative PCR (QPCR) was performed using cDNA. QPCR was conducted to determine the relative expression levels of MT1 (GenBank Accession No. **JN418212**), RH (**KC684925**), and *β*-actin (**JN039369**) mRNA using the total RNA extracted from the brain and retina. The following QPCR primers were designed with reference to the known sequences of the yellowtail clownfish: MT1 forward (5′-GTC ATC GGC TCC ATC TTC-3′) and reverse (5′-GTT TAT CGT ATT TGA GGC TGT G-3′) primers; RH forward (5′-TCA ACT TCC TCA CCC TCT A-3′) and reverse (5′-GAA TCC TCC AAG CAC CAT-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 internal controls, the experiments were duplicated with *β*-actin, and all data were expressed relative to the corresponding *β*-actin calculated threshold cycle (CT) levels. The efficiencies were found to be as follows: *β*-actin = 94.2%, MT1 = 94.5%, and RH = 94.3%. The calibrated Δ*C*t value (ΔΔ*C*t) for each sample and internal control (*β*-actin) was calculated $\left[\Delta\Delta Ct = 2^\wedge-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{internal control}})\right]$.

Western blot analysis

The total protein isolated from the retina (RH) and brain (MT1) of yellowtail clownfish 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 phenylmethylsulfonyl fluoride, and 0.15 mg/ml leupeptin). It was then sonicated and quantified using the Bradford method (Bio-Rad). Total protein (30 µg) was loaded in separate lanes on 4% acrylamide stacking gel and 12% acrylamide resolving gel. A protein ladder (Fermentas, Vilnius, Lithuania) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and at 150 V through the resolving gel until the bromophenol blue dye front had run off of the gel. The gels were then immediately transferred to a 0.2-µm polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. The membranes were then blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min and then washed in TBS. The membranes were incubated with RH (1:4000; Abcam, Cambridge, MA, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:2000; Bio-Rad), and MT1 (dilution, 1:4000; Novus; Littleton, CO, USA) and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:2000; Bio-Rad) for 60 min. The internal control was *β*-tubulin (dilution, 1:5000; ab6046, Abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:5000; Bio-Rad) for 60 min. Bands were detected using standard enhanced chemiluminescence (ECL) and the more sensitive ECL systems (GE Life Sciences, Sweden). The membranes were then exposed to autoradiography sensitive film for 2 min.

Immunohistochemistry

Retina cells were detected immunocytochemically according to a method modified from Iwata et al. (2010). Four-µm-thick rehydrated tissue sections were incubated overnight at 4 °C with the rabbit anti-RH (dilution 1:4000, Abcam) and 30 min at 37 °C with the

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secondary antibody (HRP-conjugated a-rabbit immunoglobulin, 1:100 dilution). The antibodies were diluted in 2% bovine serum albumin in TBS pH 7.6 EnVision (K4001; Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine (DAB^+) (K3468; Dako) were used as the detection system. Slides were counterstained with Meyer's hematoxylin, dehydrated, and mounted with Canada balsam for observation under a light microscope (DM 100; Leica, Wetzlar, Germany). Images were captured with a digital camera (DFC 290; Leica).

Melatonin determination by ELISA

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

Statistical analysis

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

Results

Expression of RH mRNA and protein in the retina

We examined the effects of light spectra and the differences between intact and ophthalmectomized fish on the expression of RH mRNA and protein in the retina using QPCR (Figure 1). RH mRNAs were expressed at significantly higher levels during the photophase than during the scotophase for all groups, and the levels in green and blue LED groups were significantly higher than those observed in the other spectra groups. Additionally, whole RH mRNA and protein expression levels in the ophthalmectomized fish were significantly lower than those in the intact fish.

Immunocytochemistry (IHC) of RH

We used IHC to detect the location and expression of RH in the retina compared to RH mRNA and protein expression. The number and area of RH-IR neurons and the intensity of immunostaining in the outer nuclear layer of intact fish retina were compared with those of the ophthalmectomized fish (Figure 2). The green and blue LED-illuminated intact fish and the retinas of ophthalmectomized fish appeared to have more RH-IR neurons than the group exposed to a white fluorescence bulb (control) and the group with red LED-illuminated retina. We found a distinct difference in the distribution of retina RH-IR cells associated with various light spectra and the presence of an eye that was visualized via immunolocalization of the retina.

Expression of MT1 mRNA and protein in the brain

We examined the effects of the different light spectra and the differences between intact and ophthalmectomized fish on the expression of MT1 mRNA and protein in the

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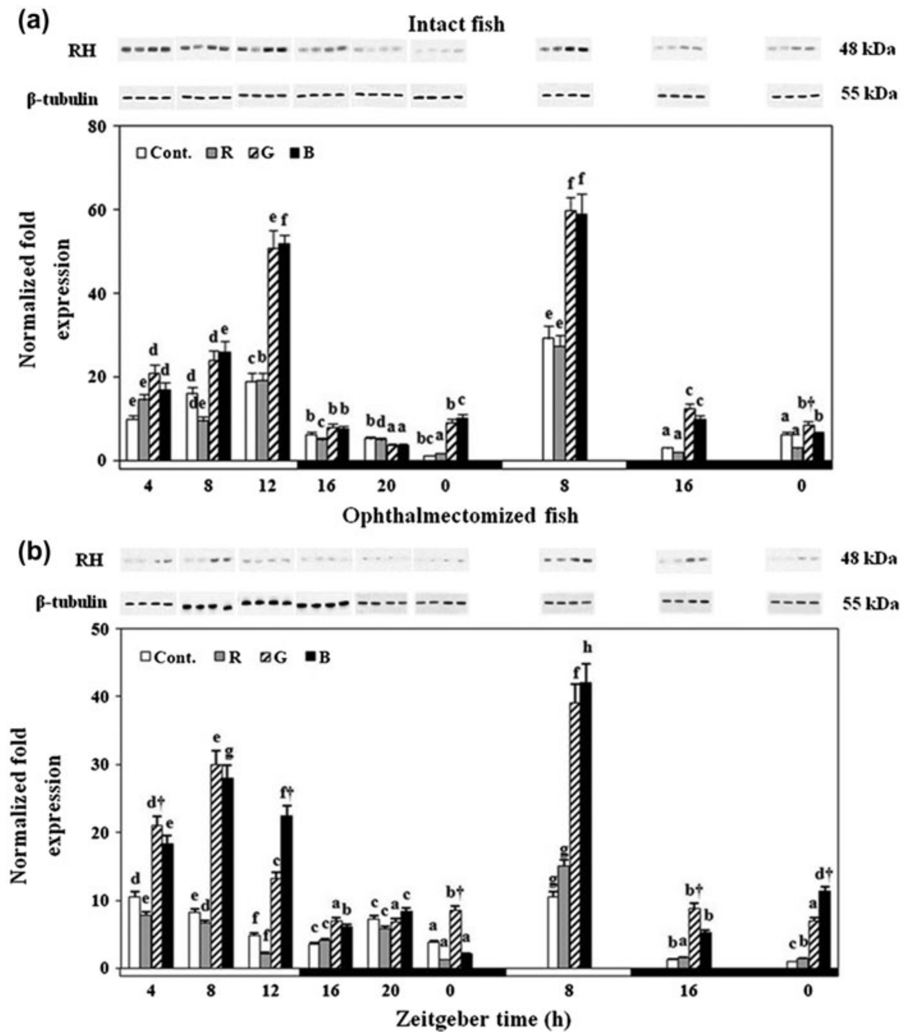

Figure 1. Changes in the expression levels of RH mRNA and protein in the retina of intact (a) and ophthalmectomized yellowtail clownfish fish (b) under lighting conditions using red (R), green (G), or blue (B) LEDs or a white fluorescent bulb (Cont.). The fish were reared under a 12-h light:12-h dark cycle. The total retina RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as normalized expression levels with respect to the *β*-actin in the same sample. The white bar represents the photophase, and the black bar represents the scotophase. Values with different characters are significantly different between the Zeitgeber times (ZT) within the same light spectrum ($p < 0.05$). The cross (†) indicates a significant difference between different light spectra within the same ZT ($p < 0.05$). All values represent the means ± SD ($n = 5$).

brain (Figure 3). MT1 mRNA and protein were expressed at significantly higher levels during the scotophase than during the photophase for all groups. However, MT1 mRNA and protein expressions levels in the intact fish were clearly highest in the red LED groups ($p < 0.05$). MT1 mRNA and protein expression levels in the ophthalmectomized fish were significantly higher in the green and blue LED groups, and they were similar to the levels observed in the green and blue LED groups of intact fish.

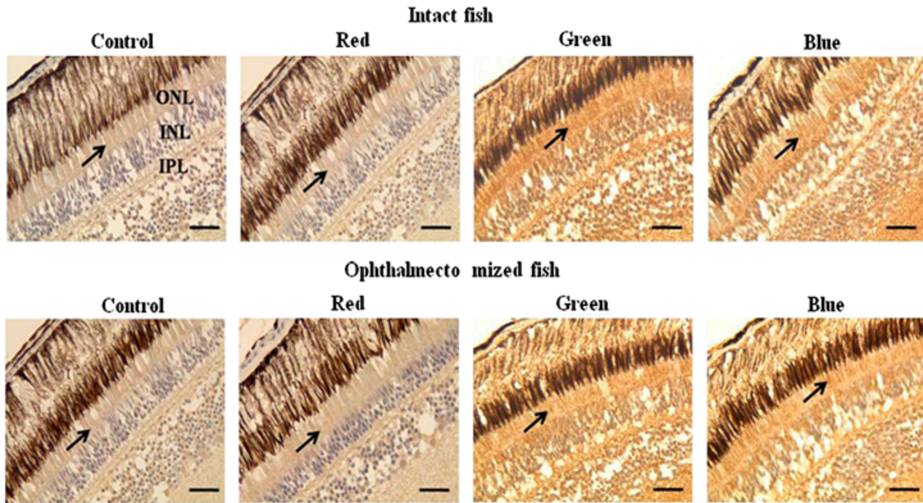

Figure 2. (Colour online) Immunohistochemical localization of retina RH-immunoreactivity (RH-IR) in cross-sections of intact and ophthalmectomized fish under red (R), green (G), blue (B) LEDs, or a white fluorescent bulb (Cont.) at ZT8 on the second day. Arrows indicate RH-IR cells, which are stained brown. ONL = outer nuclear layer; INL = inner nuclear layer; IPL = inner plexi form layer; GCL = ganglion cell layer. Scale bars = 250 μm.

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Plasma melatonin levels

We observed the effects of light spectra and the differences between intact and ophthalmectomized fish on plasma melatonin levels using a microplate reader (Figure 4). Plasma melatonin levels were significantly higher during the scotophase than during the photophase for all groups. However, plasma melatonin levels in the intact fish were highest in the red LED groups. Plasma melatonin levels in the ophthalmectomized fish were highest in the green and blue LED groups, which were similar to the levels observed in the same groups of intact fish.

Discussion

To investigate changes in circadian rhythms, we examined the mRNA and protein levels of RH and MT1 and examined plasma melatonin levels during daily cycles by exposing ophthalmectomized yellowtail clownfish to red, green, and blue LEDs.

We observed that the mRNA and protein expression levels of RH in the retina, where light penetrates first, were significantly increased during the photophase compared to the scotophase in all groups. Additionally, RH mRNA and protein expression levels in ophthalmectomized fish were significantly lower than those observed in intact fish, and the levels in green and blue LED groups were significantly higher than those observed in the other spectra groups (Figure 1). Furthermore, we examined the distribution of RH-IR in ZT8 of photophase using IHC between ophthalmectomized and intact fish, and the result showed less RH-IR in ophthalmectomized fish than in intact fish, as well as changes in RH mRNA expression.

However, we have observed that the RH-IR levels in green and blue LED groups were higher than in the other spectra groups (Figure 2). These results indicate that light is the one of the environmental factors that synchronizes the circadian clock. Im et al.

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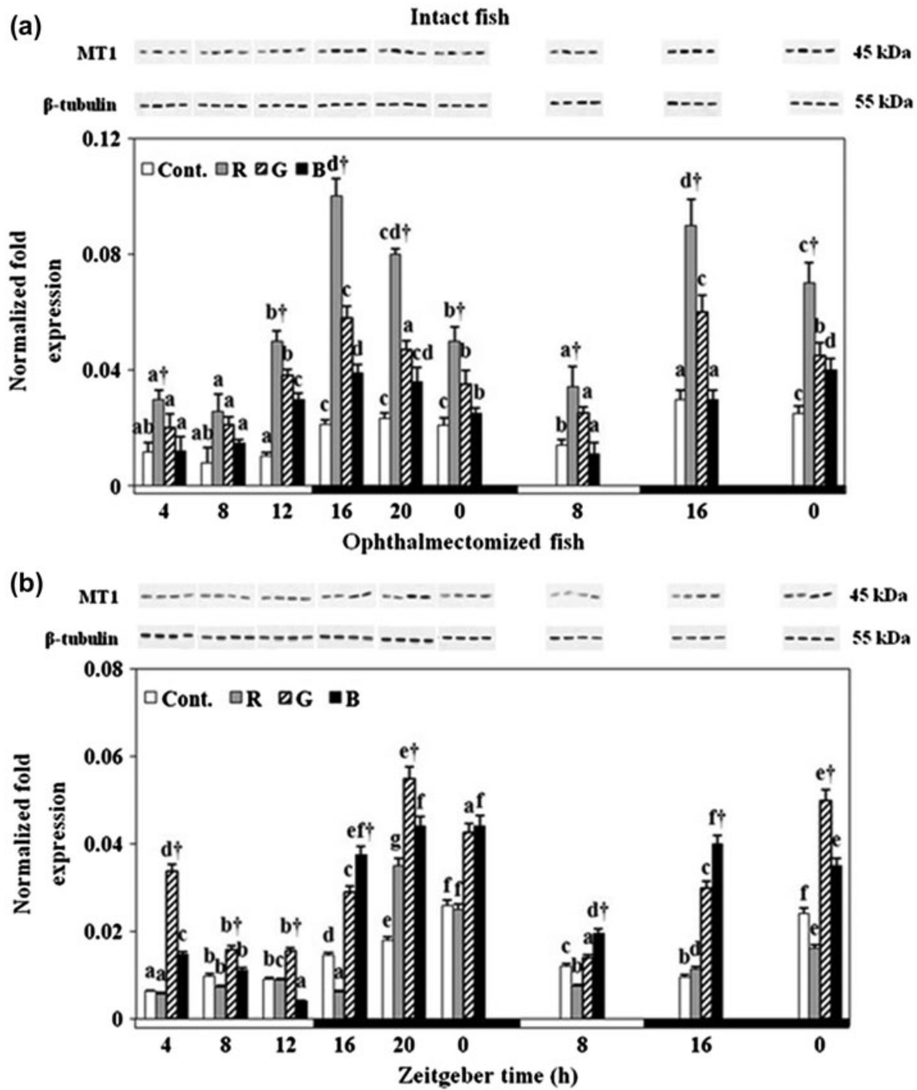

Figure 3. Changes in the expression levels of MT1 mRNA and protein in the brains of intact (a) and ophthalmectomized yellowtail clownfish (b) kept under lighting conditions using red (R), green (G), blue (B) LEDs, or a white fluorescent bulb (Cont.), as measured using quantitative real-time PCR. The fish were reared under a 12-h light:12-h dark cycle. Total brain RNA (2.5 μg) was reverse-transcribed and amplified. The 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 with different characters are significantly different between the ZT within the same light spectrum ($p < 0.05$). The cross (†) indicates a significant difference between different light spectra within the same ZT ($p < 0.05$). All values represent the means ± SD ($n = 5$). Reprinted with permission from Shin et al. (2013). [DOI:10.1080/09291016.2013.870757]

(2007) reported that light is primarily responsible for synchronizing peripheral clocks to the environment. In the majority of cases, it is still unclear how these cells "see" the changes in light, so the previous authors investigated the expression of RH mRNA

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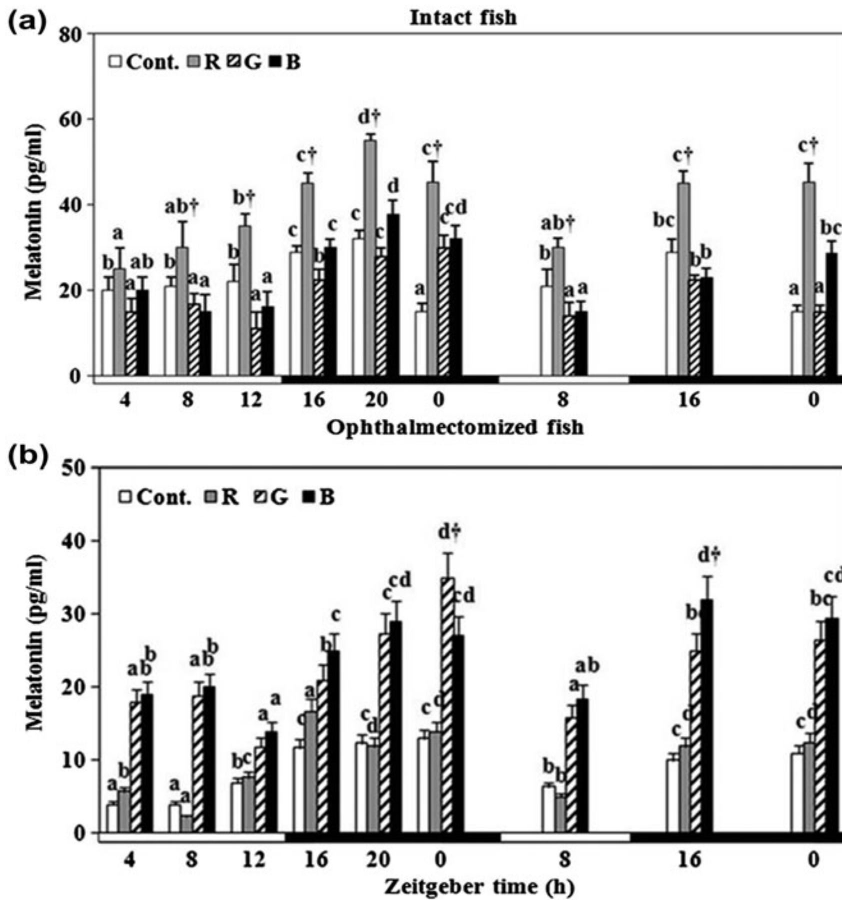

Figure 4. ELISA melatonin plasma levels in the intact (a) and ophthalmectomized yellowtail clownfish (b) in under lighting conditions using red (R), green (G), blue (B) LEDs, or a white fluorescent bulb (Cont.), as measured using a microplate reader. The fish were reared under a 12-h light:12-h dark cycle. The white bar represents the photophase, and the black bar represents the scotophase. Values with different characters are significantly different between the ZT within the same light spectrum ($p < 0.05$). The cross (†) indicates a significant difference between different light spectra within the same ZT ($p < 0.05$). All values represent the means ± SD ($n = 5$).

throughout the daily cycle and found that RH was regulated by the cycles in a circadian rhythm based on light.

We examined the RH mRNA and protein expression levels under various spectra of lights and in response to changes in light and dark. The expression levels were highest in the green and blue LED groups for both intact and ophthalmectomized fish. We know that the short, or blue, end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates shallow waters, so we hypothesized that the fish retina would detect more green and blue lights than red (Lythgoe et al. 1994; Myrberg & Fuiman 2002). This finding is in accordance with the few reports of light sensitivity in erythrophores cultured from fish, which respond to wavelengths of 470–530 nm with pigment granule dispersion (Oshima 2001; Sato et al. 2004).

We also observed the expression levels of MT1 mRNA and protein to examine the effect of ophthalmectomy on circadian rhythm. The expression levels in ophthalmectomized fish were significantly lower than those in intact fish (Figure 3). Migaud, Davie et al. (2007) reported that plasma melatonin was completely suppressed in ophthalmectomized fish. These results thus confirmed that the pineal gland requires the eyes to produce and secrete melatonin in a normal manner. According to Migaud, Davie et al. (2007), the expression of MT1 mRNA in ophthalmectomized fish was significantly lower than in intact fish, thus indicating that the retina plays an important role in regulating circadian rhythm.

Meanwhile, there were no significant differences in the response to the short wavelengths, i.e. green and blue LED lights, between intact and ophthalmectomized fish. This result underscores the importance of other spectra affecting circadian rhythms in fish in addition to the role of the eye. Loew and McFarland (1990) reported that a significant amount of light energy is wasted in the form of unsuitable wavelengths (longer wavelength, red light), which are rapidly absorbed by water molecules and therefore cannot be detected by fish. We hypothesized that light would have a diminished capacity to affect circadian rhythms in fish without eyes. Our results confirm Falcón and Meissl's report (1981) that the fish pineal organ has double spectral sensitivity in the blue-green regions.

The changes in plasma melatonin levels in response to changes in spectra were similar to the changes in MT1 mRNA expression in relation to ophthalmectomy and the regulation of circadian rhythms (Figure 4). This result agrees with various teleost studies, such as those on 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.

Such phase shift differences between pineal and retinal melatonin production could be due to the different functional roles of melatonin from the pineal gland and eyes. The former provides a reliable endocrine indicator of the day–night cycle (Falcón et al. 2007), while the latter could be involved in the paracrine protection and adaptation of the retina (Falcón et al. 2003). Therefore, we suggest that the retina, in addition to the pineal gland, secretes melatonin and is therefore crucial to regulating circadian rhythm. Migaud, Davie et al. (2007) reported that a different circadian system could be at work in European sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) because ophthalmectomy resulted in a significant decrease in nighttime production of melatonin. Such results are in accordance with previous reports in European sea bass (Migaud, Davie et al. 2007) as well as birds (Brandstätter 2003) and amphibians (bullfrog, *Rana catesbeiana*) (Wright et al. 2006). In all of these species, the findings suggest that both the eyes and the pineal gland are required to sustain the full amplitude of melatonin rhythms, meaning that the light perceived by the eyes could regulate melatonin synthesis via the pineal gland, most likely through neural projections into the brain (Jimenez et al. 1995).

We considered the possibility that the stress of the ophthalmectomy could affect the accuracy of our results. Therefore, we examined cortisol as stress indicator in intact and ophthalmectomized fish and found no significant difference between the groups (data not shown). It appears that ophthalmectomy causes only slight stress in experimental fish, and we doubt that the melatonin production and secretion were affected by post-surgery stress.

The MT1 mRNA expression levels in green and blue LED groups of ophthalmectomized fish were similar with the same groups of intact fish, which suggests that short wavelengths influence the maintenance of circadian rhythms in fish.

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In conclusion, we demonstrated that when ophthalmectomized yellowtail clownfish were illuminated by short wavelengths, e.g. green and blue, they were able to maintain normal circadian rhythms. We also traced the regulatory tract of circadian rhythms that conveys the detected light from retina to brain, as measured by the expression of the retina photoreceptor, RH.

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References

Arendt J. 1995. Melatonin and the mammalian pineal gland. London: Chapman and Hall; p. 210–285.

Bapary MAJ, Amin MN, 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.

Brandstätter R. 2003. Encoding time of day and time of year by the avian circadian system. J Neuroendocrinol. 15:398–404.

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.

Dubocovich ML. 1995. Melatonin receptors: are there multiple subtypes? Trends Pharmacol Sci. 16:50–56.

Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. 2003. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front Biosci. 8:1093–1108.

Ekstrom P, Meissl H. 2003. Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors. Philos TR Soc B: Biol Sci. 358:1679–1700.

Ekstrzm P, Meissl H. 1997. The pineal organ of teleost fishes. Rev Fish Biol Fisher. 7:199–284.

Endler JA. 1993. The color of light in forests and its implications. Ecol Monogr. 63:1–27.

Falcón J, Besseau L, Sauzet S, Fuentès M. 2007. Mélatonine et régulations neuroendocrines *chez* le poisson. J Soc Biol. 201:21–29.

Falcón J, Gothilf Y, Coon SL, Boeuf G, Klein DC. 2003. Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. J Neuroendocrinol. 15:378–382.

Falcón J, Meissl H. 1981. The photosensory function of the pineal organ of the pike (*Esox lucius* L.) correlation between structure and function. J Comp Physiol A. 144:127–137.

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

Foster RG. 1998. Shedding light on the biological clock. Neuron. 20:829–832.

Gauer F, Masson-Pevet M, Skene DJ, Vivien-Roels B, Pevet P. 1993. Daily rhythms of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei: evidence for a regulation of melatonin receptors by melatonin itself. Neuroendocrinology. 57:120–126.

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

Iigo M, Kezuka H, Suzuki T, Tabata M, Aida K. 1994. Melatonin signal transduction in the goldfish, *Carassius auratus*. Neurosci Biobehav Rev. 18:563–569.

Im LHJ, Isoldi MC, Scarparo AC, Visconti MA, de Lauro Castrucci AML. 2007. Rhythmic expression, light entrainment and α-MSH modulation of rhodopsin mRNA in a teleost pigment cell line. Comp Biochem Physiol A: Mol Integr Physiol. 147:691–696.

Iwata E, Nagai Y, Sasaki H. 2010. Immunohistochemistry of brain arginine vasotocin and isotocin in false clown anemonefish *Amphiprion ocellaris*. Open Fish Sci J. 3:147–153.

Jimenez AJ, Fernandez-Llebrez P, Perez-Figares JM. 1995. Central projections from the goldfish pineal organ traced by HRP-immunocytochemistry. Histol Histopathol. 10:847–852.
Khorana HG. 2000. Molecular biology of light transduction by the mammalian photoreceptor, rhodopsin. J Biomol Struct Dyn. 17:1–16.
Khorana HG, Reeves PJ, Kim JM. 2002. Structure and mechanism in G protein-coupled receptors. Am Pharm Rev. 9:287–294.
Loew ER, McFarland WN. 1990. The underwater visual environment. The visual system of fish. London: Chapman and Hall; p. 1–43.
Lythgoe JN. 1979. The ecology of vision. Oxford: Clarendon Press.
Lythgoe JN, Muntz WRA, Partridge JC, Shand J, Williams DMcB. 1994. The ecology of visual pigments of snappers (*Lutjanidae*) on the Great Barrier Reef. J Comp Physiol A. 174:461–467.
Malik S, Rani S, Kumar V. 2004. Wavelength dependency of light-induced effects on photoperiodic clock in the migratory blackbird bunting (*Emberiza melanocephala*). Chronobiol Int. 21:367–384.
Martinez-Chavez CC, Migaud H. 2009. Retinal light input is required to sustain plasma melatonin rhythms in Nile tilapia *Oreochromis niloticus*. Brain Res. 1269:61–67.
Masana MI, Benloucif S, Dubocovich ML. 2000. Circadian rhythm of mt1 melatonin receptor expression in the suprachiasmatic nucleus of the C3H/HeN mouse1. J Pineal Res. 28:185–192.
Migaud H, Cowan M, Taylor J, Ferguson HW. 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.
Migaud H, Davie A, Martinez Chavez CC, Al-Khamees S. 2007. Evidence for differential photic regulation of pineal melatonin synthesis in teleosts. J Pineal Res. 43:327–335.
Morgan E. 2004. Ecological significance of biological clocks. Biol Rhythm Res. 35:3–12.
Myrberg AA Jr, Fuiman LA. 2002. The sensory world of coral reef fishes. Coral reef fishes. San Diego(CA): Academic Press; p. 123–148.
Oshima N. 2001. Direct reception of light by chromatophores of lower vertebrates. Pigm Cell Res. 14:312–319.
Pierce LX, Noche RR, Ponomareva O, Chang C, Liang JO. 2008. Novel functions for period 3 and exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ. Brain Res. 1223:11–24.
Rani S, Kumar V. 2000. Phasic response of the photoperiodic clock to wavelength and intensity of light in the redheaded bunting, *Emberiza bruniceps*. Physiol Behav. 69:277–283.
Reiter RJ. 1991. Melatonin: the chemical expression of darkness. Mol Cell Endocrinol. 79: C153–C158.
Reppert SM, Weaver DR. 2001. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol. 63:647–676.
Reppert SM, Weaver DR, Godson C. 1996. Melatonin receptors step into the light: cloning and classification of subtypes. Trends Pharmacol Sci. 17:100–102.
Roca AL, Godson C, Weaver DR, Reppert SM. 1996. Structure, characterization, and expression of the gene encoding the mouse Mel1a melatonin receptor. Endocrinology. 137:3469–3477.
Sato M, Ishikura R, Oshima N. 2004. Direct effects of visible and UVA light on pigment migration in erythrophores of Nile tilapia. Pigm Cell Res. 17:519–524.
Shin HS, Kim NN, Choi YJ, Choi CY. 2013. Retinal light input regulates clock genes and immune function in yellowtail clownfish (*Amphiprion clarkii*). Biol Rhythm Res., in press.
Shin HS, Lee JH, Choi CY. 2011. 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*. Comp Biochem Physiol A: Mol Integr Physiol. 160:221–228.
Shin HS, Lee JH, Choi CY. 2012. Effects of LED light spectra on the growth of the yellowtail clownfish, *Amphiprion clarkii*. Fish Sci. 78:549–556.
Tang I, Murakami DM, Fuller CA. 1999. Effects of square-wave and simulated natural light–dark cycles on hamster circadian rhythms. Am J Physiol. 276:1195–1202.
Volpato GL, Barreto RE. 2001. Environmental blue light prevents stress in the fish Nile tilapia. Braz J Med Biol Res. 34:1041–1045.

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Wright ML, Francisco LL, Scott JL, Richardson SE, Carr JA, King AB, Noyes AG, Visconti RF. 2006. Effects of bilateral and unilateral ophthalmectomy on plasma melatonin in *Rana* tadpoles and froglets under various experimental conditions. Gen Comp Endocrinol. 147:158–166.

Yokoyama S. 2000. Molecular evolution of vertebrate visual pigments. Prog Retin Eye Res. 19:385–419.

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