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Retinal light input regulates clock genes and immune function in yellowtail clownfish (*Amphiprion clarkii*)

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The study aimed to test differences in immune ability and clock gene expression between intact fish and ophthalmectomized yellowtail clownfish exposed to light-emitting diodes (LEDs). Clock genes (*Period 2, Per2; Cryptochrome 1, Cry1*) and melatonin receptor 1 mRNA and protein expression in all wavelengths groups were significantly lower in ophthalmectomized fish than in intact fish, but ophthalmectomized fish exposed to green and blue LEDs showed expression levels similar to intact fish. Furthermore, indicators of immune activity (immunoglobulin M; IgM mRNA and protein expression and lysozyme activity) were significantly higher in ophthalmectomized fish exposed to green and blue LEDs than other groups of ophthalmectomized fish. Also, plasma H_2O_2 levels in ophthalmectomized fish exposed to green and blue LEDs showed their levels similar to intact fish. These results suggest that short wavelengths affect the daily rhythms of fish and enhance the immune ability, even in the absence of an eye.

Keywords: daily rhythm; clock genes; immune ability; immunoglobulin M; light-emitting diodes; ophthalmectomy

Introduction

A large body of research has addressed the effects of photoperiod, temperature, pH, and dissolved oxygen on the physiological status of fish (Bowden 2008). Among the many factors that control circadian rhythms, light is the most important factor because it affects many of the physiological and behavioral changes that occur within a 24-h period (Pierce et al. 2008). Dramatic diel changes in skylight color are associated with concomitant changes in luminance, as well as cycles of alternating white light and darkness (Krull et al. 1985; Endler 1993). The spectral composition of incident light changes in underwater environments and rapid attenuation occurs with increasing depth (Lythgoe 1979). The short wavelength (blue) end of the visible spectrum predominates in deeper waters, whereas red light only penetrates shallow waters (McFarland 1991; Lythgoe et al. 1994; Myrberg & Fuiman 2002). Recent studies of light have been performed using light-emitting diodes (LEDs) instead of natural or metal halide light (Bapary et al. 2011; Shin et al. 2011). LEDs are a new form of lighting technology that can be manufactured to produce specific wavelengths of light (Migaud, Cowan, et al. 2007). Specific LED wavelengths influence physiological functions in fish. For example, they induce gonad development (Bapary et al. 2011) and enhance growth (Shin et al. 2012); however, they also create oxidative stress (Shin et al. 2011). When barfin flounder (*Verasper moseri*) were farmed

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using short wavelengths, such as those in the blue spectrum, growth rates increased (Yamanome et al. 2009). In addition, exposure to the blue spectrum prevents stress in Nile tilapia (*Oreochromis niloticus*) (Volpato & Barreto 2001).

However, the role that such chromatic changes might play in the setting of circadian activity and immune ability patterns has been largely ignored (Rani & Kumar 2000; Malik et al. 2004) in terms of eye presence.

Period 2 (*Per2*) and *Cryptochrome 1* (*Cry1*) gene expression drives circadian variations in neural and peripheral tissues, where circadian genes regulate the circadian rhythm of various physiological processes such as reproductive rhythm (Besharse et al. 2004). In vertebrates, *Cry1* is a negative element in the transcriptional feedback loop of the circadian clock, which drives the biological rhythms of organisms. Vertebrate *Cry* protein joins *Per* to form a complex that binds and blocks the protein complex formed by Circadian Locomotor Output Cycles Kaput and Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (ARNT)-Like (CLOCK:BMAL1), inhibiting *Cry* transcription (Okamura et al. 2002; Iuvone et al. 2005). Expression of *Per2* and *Cry1* is induced by environmental factors, including light (Takemura et al. 2008). These observations suggest the involvement of clock genes in the light-switch pattern of the circadian clock (Pando et al. 2001). In vertebrates, light-responsive clock genes have been reported in goldfish (*Carassius auratus*; Iigo et al. 2003), zebrafish (*Danio rerio*; Vallone et al. 2004), and reef fish (*Siganus guttatus*; Takemura et al. 2008), yet detailed studies into clock genes in fish are limited.

Furthermore, melatonin receptors (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 G-protein-coupled receptor 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. 2002).

There has been limited research into the relationship between light and immune function, and between light spectra and daily rhythms in ophthalmectomized fish exposed to LED lighting. Humoral adaptive immunity in fish is mediated by immunoglobulin (Ig); the immunoglobulin M (IgM) class is primary in most teleost fish (Voss & Sigel 1972). Lysozyme, another immune indicator, damages bacterial cell walls. It is abundant in secretions such as tears, saliva, and mucus, and has also been found in the semen of mammals (Mendeluk et al. 1997) and birds (Sotirov et al. 2002).

In fish under stress caused by environmental factors such as light, the primary stress responses induce secondary responses that affect energy requirements, such as increased reactive oxygen species, an indicator of oxidative stress, are also produced by light (Kim & Phyllis 1998).

Therefore, we investigated the correlation between changes in daily rhythm, light spectrum, and immune function by exposing intact and ophthalmectomized yellowtail clownfish (*Amphiprion clarkii*, a high-value ornamental fish) to specific spectra. We measured changes in mRNA and protein expression of clock genes (*Per2* and *Cry1*), MT1 and IgM, as well as changes in plasma lysozyme activity, and H_2O_2 levels over the course of daily rhythms, following exposure to red, green, or blue LED spectra.

Materials and methods

Experimental fish, ophthalmectomy, and conditions

Yellowtail clownfish (average 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. Also, each tank (each experimental group) was composed by number of 70 fish. The fish were exposed to a white fluorescent bulb (27 W), and it 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 (lights on 07:00 h and lights off 19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h). For the eight experimental groups including the intact and ophthalmectomized fish, the fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LEDs (Daesin LED Co. Kyunggi, Korea) including white fluorescent bulb (Control; Cont.) (Figure 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 reared under these conditions until the previous day to the sampling with a daily feeding of commercial feed. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA). The fish 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. The one eye was elevated with a forceps and the optic nerve was cut to remove the one eye completely. These ophthalmectomized fish were allowed to acclimate for one week in 300-l circulation filter tanks under LD 12:12 at 27 °C, and then they were separated to each experimental groups by exposed to LED spectrums. No mortalities were observed. The fish were anesthetized with 200 mg/l tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) for minimizing the stress, prior to blood collection. Blood was collected rapidly 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 until analysis. The fish were euthanized by spinal transection at 4-h sampling intervals as first cycle of 24-h, and 8-h sampling intervals as second cycle of 24-h, 07:00 h (ZT0; lights on), 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12; lights off), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT0; lights on), 15:00 h

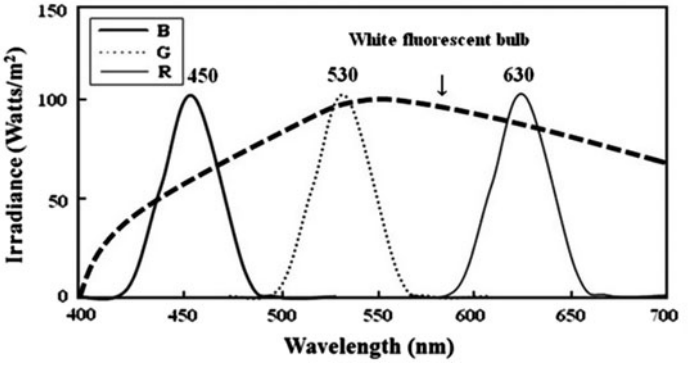

Figure 1. Spectral profiles of blue (B), green (G), and red (R) LEDs. Reprinted from Shin et al. (2011), with permission from Comparative Biochemistry and Physiology, Part-A.

(ZT8), 23:00 h (ZT16), 07:00 h (ZT0; lights on), to collect the brain and liver under dim red light during the nocturnal samplings and the experimental light during the diurnal samplings.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from the brain and liver using a TRIzol kit (Gibco/BRL, USA) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. RT-qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative expression levels of *Per2* (GenBank accession No. **JQ809468**), *Cry1* (**JQ809469**), IgM (**KC526955**), and *β*-actin (**JN039369**) mRNA using total RNA extracted from the brain and liver. The following RT-qPCR primers were designed with reference to the known sequences of the yellowtail clownfish: *Per2* forward (5′-CAG GAG GAA GAG AAA GTG AC-3′) and reverse (5′-TTT GTT GTT TGG GTT GGG-3′) primers; *Cry1* forward (5′-CAC TAA CAA CCC CTG CTT-3′) and reverse (5′-CAT GAT GGC GTC GAT CCA-3′) primers; MT1 forward (5′-GTC ATC GGC TCC ATC TTC-3′) and reverse (5′-GTT TAT CGT ATT TGA GGC TGT G-3′) primers; IgM forward (5′-AAG ACT CTG CTG TGT ATT ACT G-3′) and reverse (5′-GTG ACG GTG ACT GTT GTT-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 RT-qPCR was performed as follows: 95 °C for 5 min, followed by 35 cycles each of 95 °C for 20 s and 55 °C for 20 s. As internal controls, experiments were duplicated with *β*-actin and all data were expressed relative to the corresponding *β*-actin calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value ($\Delta\Delta Ct$) for each sample and internal controls (*β*-actin) was calculated using $2^{-\Delta\Delta Ct}$ Method, [$\Delta\Delta Ct = 2^{-(\Delta Ct\text{sample} - \Delta Ct\text{internal control})}$] (Livak & Schmittgen 2001).

Western blot analysis

Total protein isolated from the brain (*Per2*, *Cry1,* and MT1) and liver (Ig) 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. For reference, a protein ladder (Fermentas, Vilnius, Lithuania) was used. Samples were electrophoresed at 80 V through the stacking gel and 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 Ig (1:2000; LifeSpan BioSciences; LSBio, Seattle, WA, USA) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:2000; Bio-Rad), *Per2* (dilution, 1:4000; United States Biological; Massachusetts, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:2000; Bio-Rad), *Cry1* (dilution, 1:4000;

Abcam; Cambridge, MA, USA), followed by horseradish peroxidase-conjugated anti-mouse 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 (ECL Advance; GE Life Sciences, Sweden). They were then exposed to autoradiography sensitive film for 2 min.

Lysozyme activity

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

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 yellowtail clownfish plasma were added to each well of flat-bottom 96-well microtitre plates. Concentrations are expressed as nmol peroxide/ml.

Statistical analysis

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

Results

Expression of Per2 and Cry1 mRNA and proteins in the brain

We examined the effects of different light spectra, and differences between intact fish and ophthalmectomized fish on the expression of *Per2* and *Cry1* mRNA and proteins in the brain (Figures 2 and 3). In all fish, *Per2* and *Cry1* mRNAs significantly increased during the photophase and decreased during the scotophase. Levels in the green and blue LED groups were significantly higher than in groups exposed to white fluorescent and red LED light. In addition, most *Per2* and *Cry1* mRNA expression levels were significantly lower by about 2.4 and 6.7-fold in the ophthalmectomized fish than in intact fish ($p < 0.05$).

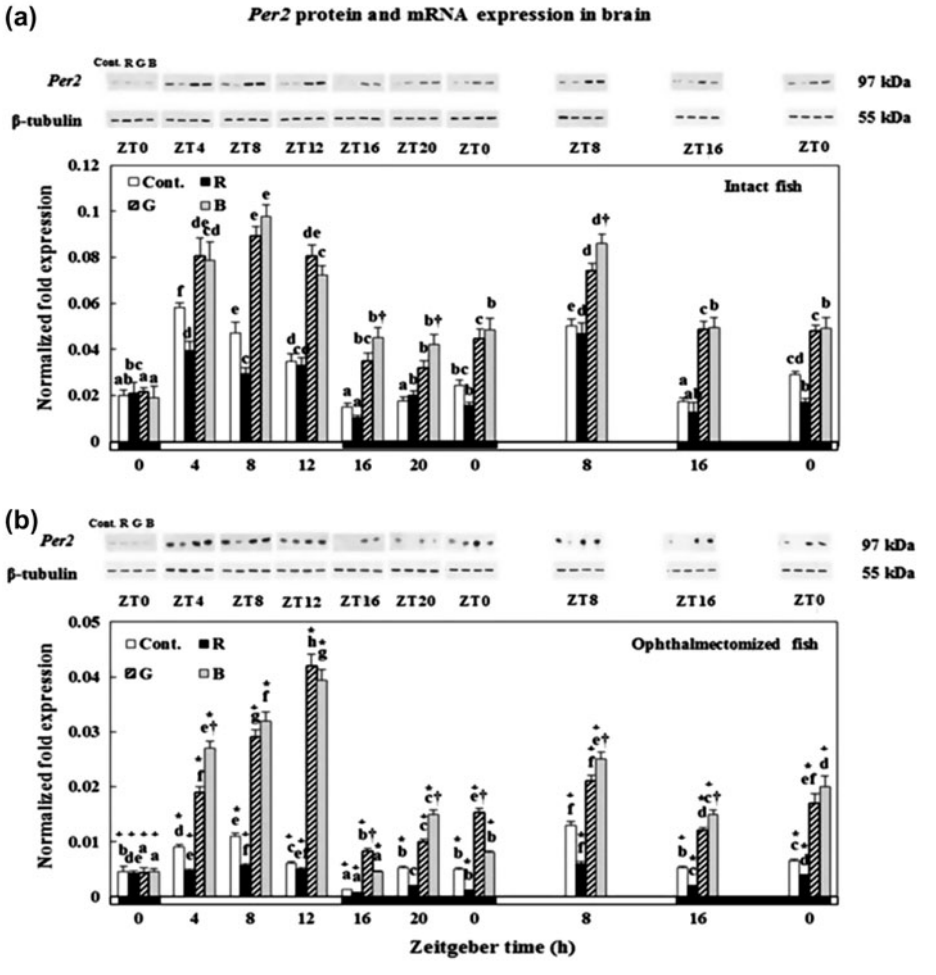

Figure 2. Expression of *Per2* in the brain of intact (a) and ophthalmectomized (b) yellowtail clownfish (Cont., R, G and B) under red (R), green (G), blue (B) LEDs, and white fluorescent bulb (Cont.) illumination, as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) cycle (12:12) h. Total brain RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as fold expression normalized to *β*-actin. The white bar represents the photophase and the black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within the same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

Furthermore, *Per2* protein expression during the photoperiod significantly increased then decreased during the scotophase; the levels in green and blue LED groups were significantly higher than in all other spectrum groups in intact and ophthalmectomized fish (Figure 2(a) and (b)). In addition, *Cry1* protein expression levels during the photoperiod were significantly higher as similar level than during the scotophase, and levels in the green and blue LED groups were as high as *Per2* expression in intact and ophthalmectomized fish (Figure 3(a) and (b)). Furthermore, *Per2* and *Cry1* expression levels in intact fish were higher than in the ophthalmectomized fish group.

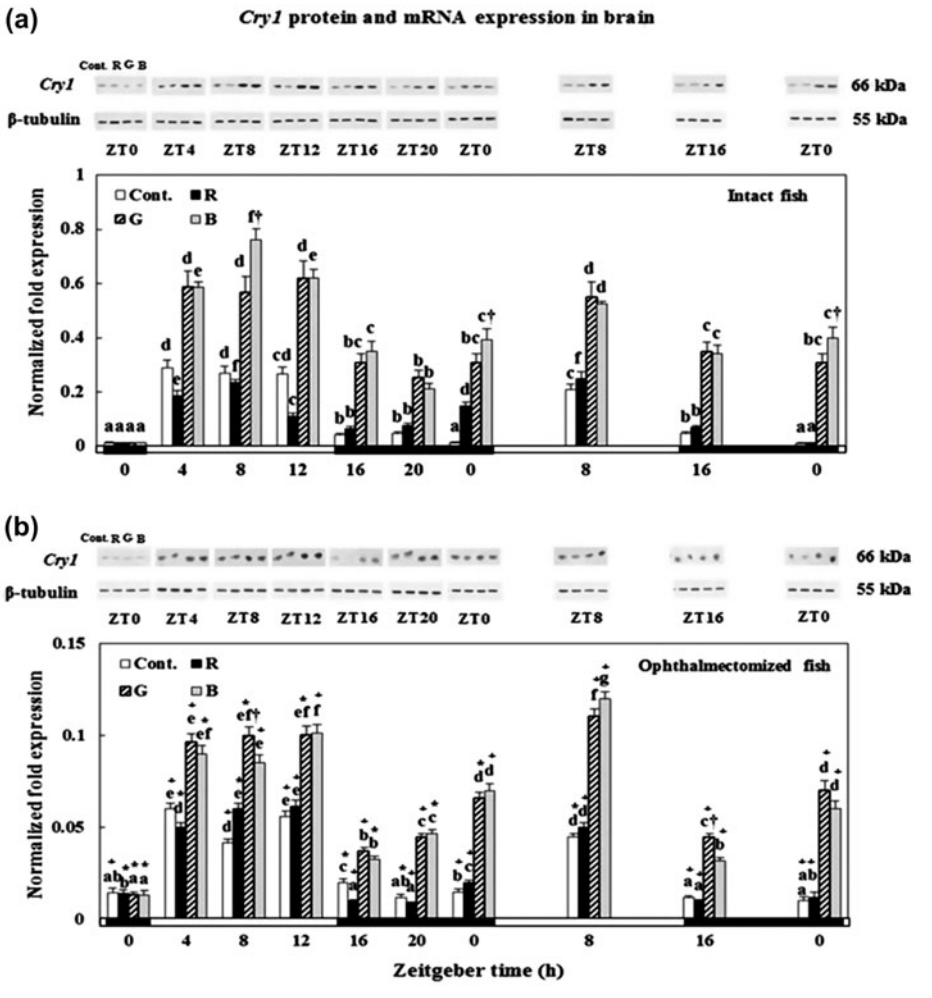

Figure 3. Expression of *Cry1* in the brain of intact (a) and ophthalmectomized (b) yellowtail clownfish under red (R), green (G), blue (B) LED, and white fluorescent bulb (Cont.) illumination, as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) cycle (12:12) h. Total brain RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as fold expression normalized to *β*-actin. The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

Expression of MT1 mRNA and MT1 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 brain (Figure 4). MT1 mRNA and protein were 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

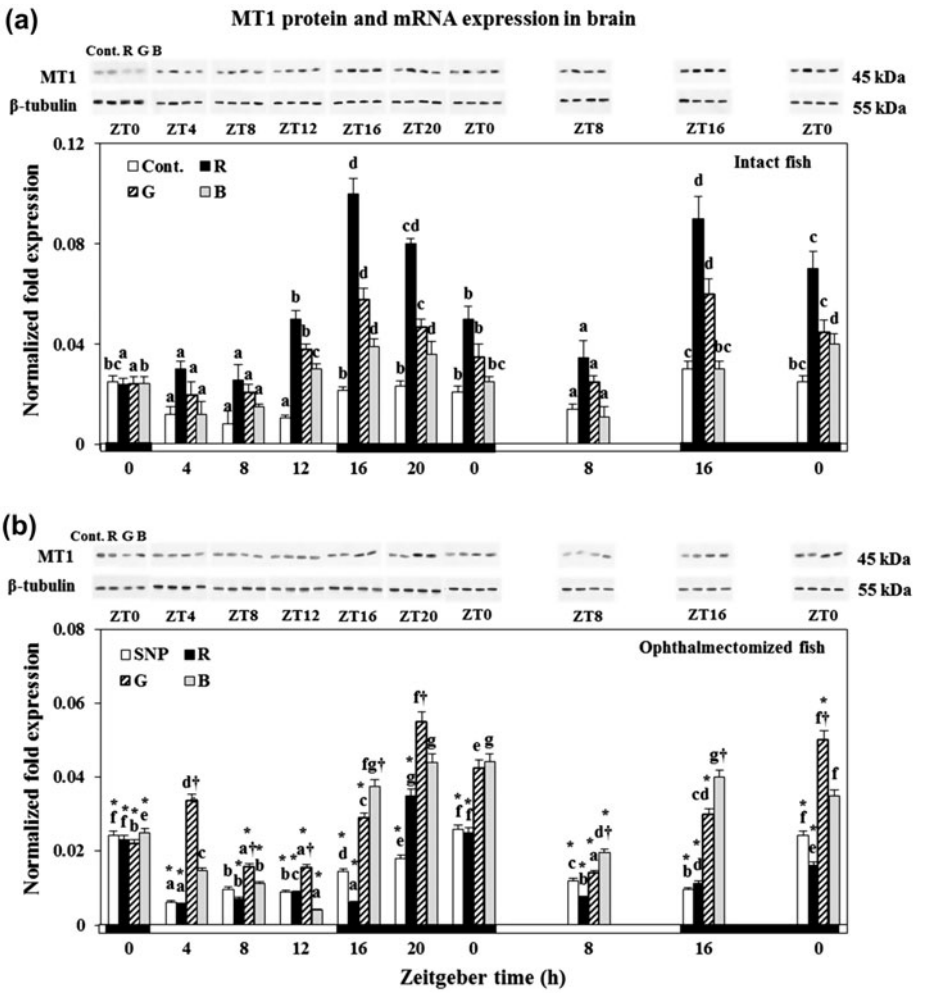

Figure 4. Expression of MT1 in the brain of intact (a) and ophthalmectomized (b) yellowtail clownfish under red (R), green (G), blue (B) LED, and white fluorescent bulb (Cont.) illumination, as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) cycle (12:12) h. Total brain RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as fold expression normalized to β-actin. The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

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.

Expression of IgM mRNA and Ig protein in the liver

We examined different light spectra and the expression of IgM mRNA and Ig protein in intact and ophthalmectomized fish (Figure 5). IgM mRNA expression levels were

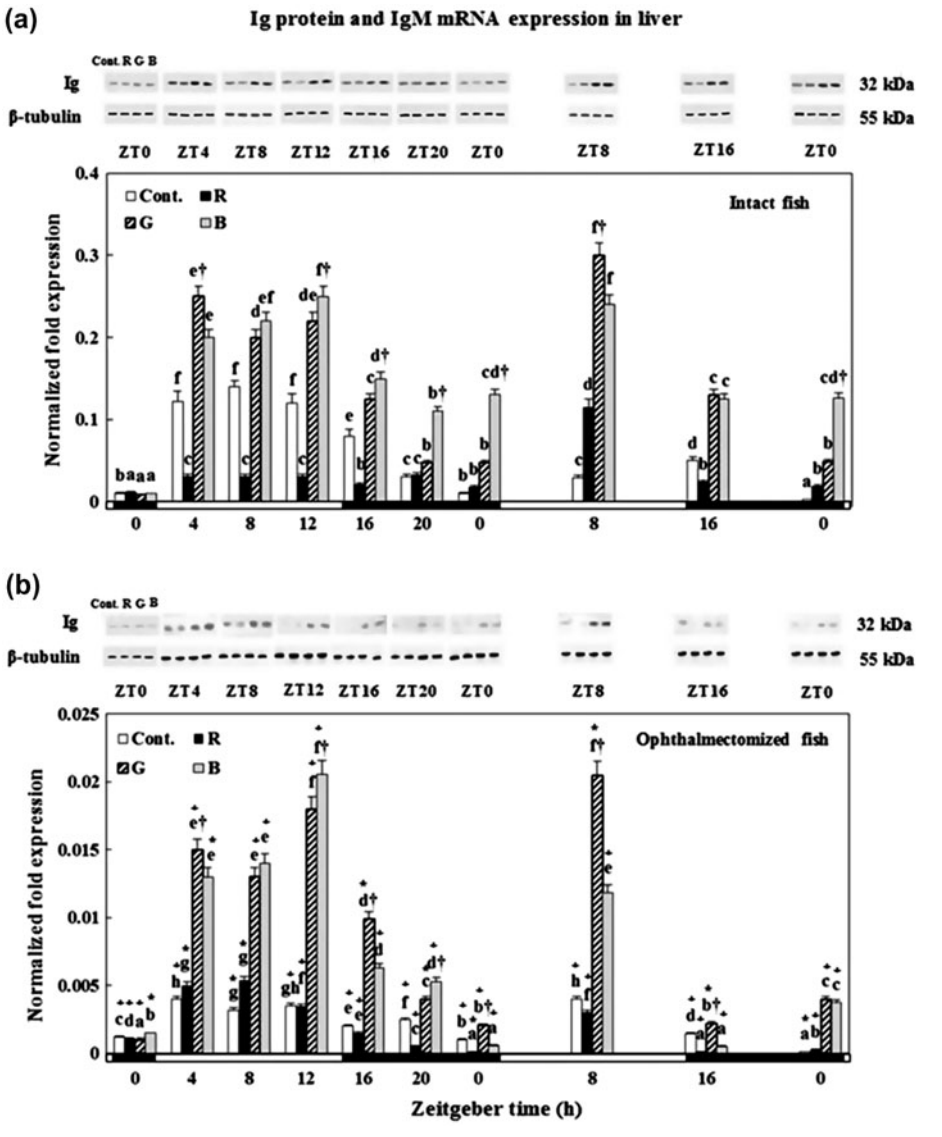

Figure 5. IgM expression in intact (a) and ophthalmectomized (b) yellowtail clownfish, as measured by quantitative real-time PCR and a microplate reader. The fish were reared under a light:dark (LD) cycle (12:12) h. Total liver RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as fold expression normalized to *β*-actin. The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

significantly higher levels during the photophase than during the scotophase for all groups. In particular, the levels in the green and blue LED groups were significantly higher than in the groups exposed to white fluorescent bulb and red LED light, amongst

intact fish and ophthalmectomized fish. Furthermore, IgM mRNA expression was significantly lower (~16-fold) in ophthalmectomized fish than in intact fish ($p < 0.05$).

Ig protein expression levels during the photoperiod were significantly higher as similar level than scotophase, and levels in the green and blue LED groups were higher than

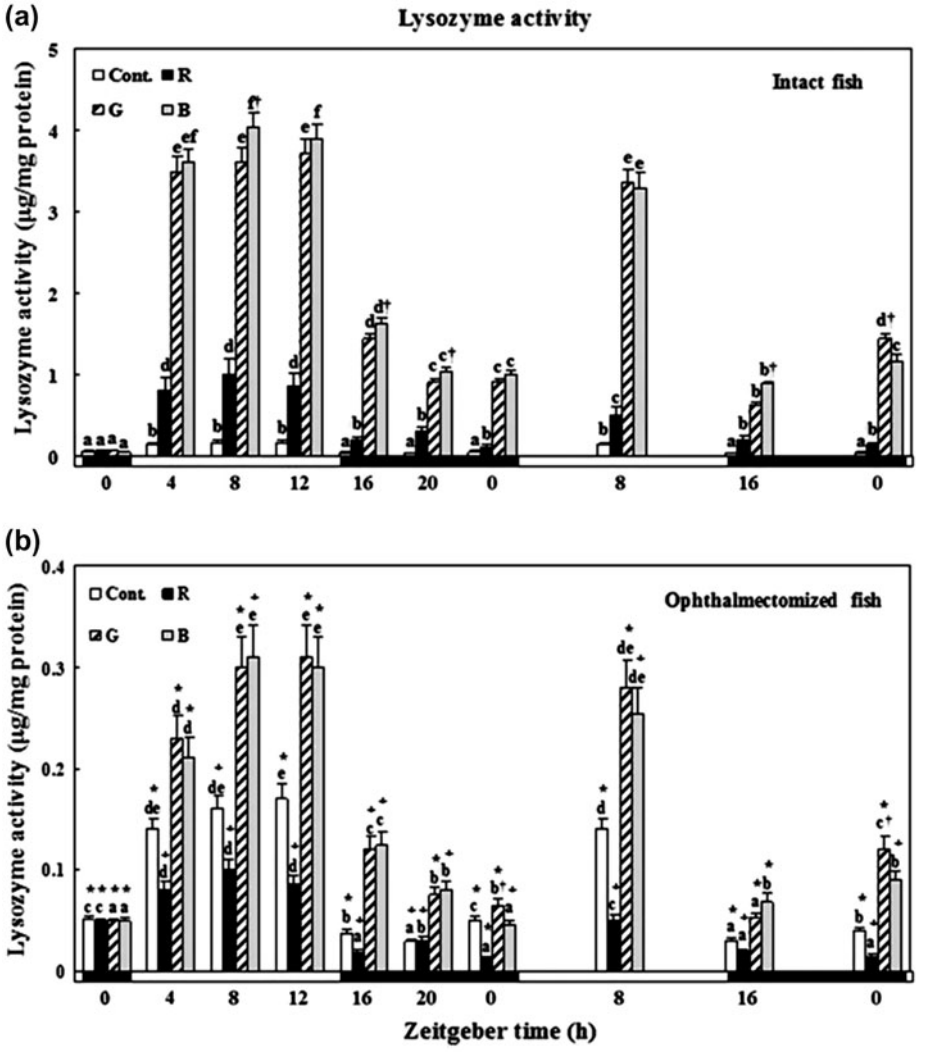

Figure 6. Lysozyme activity in intact (a) and ophthalmectomized (b) yellowtail clownfish under red (R), green (G), blue (B) LEDs, and white fluorescent bulb (Cont.) illumination, as measured by quantitative real-time PCR and a microplate reader. The fish were reared under a light:dark (LD) cycle (12:12) h. Total liver RNA (2.5 μg) was reverse-transcribed and amplified. The results are expressed as fold expression normalized to *β*-actin. The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

in other spectrum groups of intact and ophthalmectomized fish. Ig expression levels in intact fish were higher than in ophthalmectomized fish (Figure 5(a) and (b)).

Lysozyme activity levels

We examined the effects of the different light spectra on lysozyme activity in intact and ophthalmectomized fish (Figure 6). Lysozyme activity was significantly higher during the photophase than during the scotophase in all groups. Activity in the green and blue

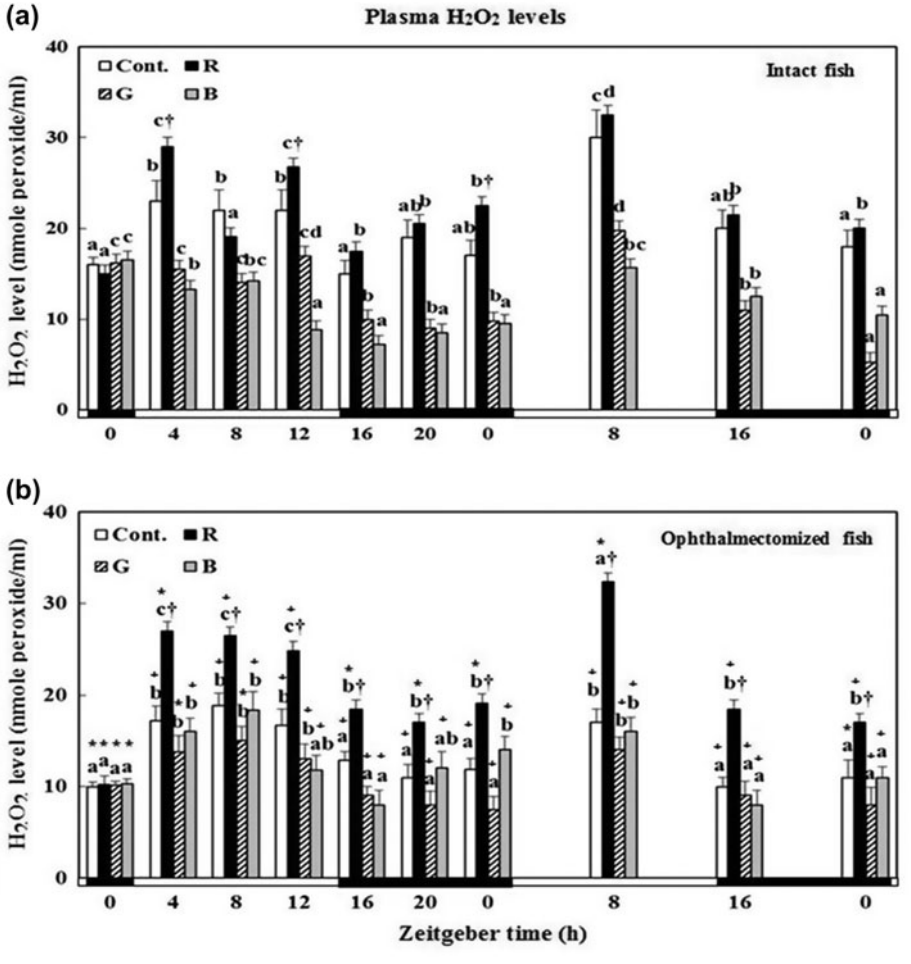

Figure 7. Plasma H_2O_2 concentrations in intact (a) and ophthalmectomized (b) yellowtail clownfish under red (R), green (G), and blue (B) LEDs and white fluorescent bulb (Cont.) illumination, as measured using a microplate reader. The fish were reared under a light:dark (LD) cycle (12:12) h. The white bar represents the photophase, and the black bar represents the scotophase. Values with different characters are significantly different at different Zeitgeber times (ZT) in fish exposed to the same light spectrum ($p < 0.05$). The cross (†) indicates significant differences between different light spectra within the same ZT, and the star (*) indicates significant differences between intact and ophthalmectomized fish within same ZT and light spectra ($p < 0.05$). All values are means ± SD ($n = 5$).

LED groups was significantly higher than in groups exposed to white fluorescent and red LED light. Furthermore, most lysozyme activity levels were significantly lower (~12.5-fold) in the ophthalmectomized fish than in the intact fish ($p < 0.05$).

Plasma H_2O_2 levels

We observed the effects of different light spectra on plasma H_2O_2 levels in intact and ophthalmectomized fish (Figure 7). Plasma H_2O_2 was significantly higher during the photophase than during the scotophase for all light spectra; however, the intact and ophthalmectomized fish were highest in the Cont. and red LED groups. In addition, the intact and ophthalmectomized fish groups had similar H_2O_2 levels ($p < 0.05$).

Discussion

In this study, we examined the correlation between changes in daily rhythm and immune function by measuring the expression of clock genes (*Per2* and *Cry1*), MT1, IgM, and lysozyme activity as well as differences in plasma H_2O_2 levels in response to various light spectra (red, green, and blue LEDs) in intact and ophthalmectomized yellowtail clownfish.

Expression of *Per2* and *Cry1* was significantly higher in intact fish than in ophthalmectomized fish, and expression was significantly higher in green and blue LED groups than in groups exposed to white fluorescent and red LED light (Figures 2 and 3). The photoperiodic and circadian organization (involving light perception and the melatonin entrainment pathway) in teleosts has, until recently, been believed to differ significantly from the centralized organization of mammals, in which light information, perceived by retinal photoreceptors, is relayed via the retinohypothalamic tract (Reiter 1993; Herzog & Tosini 2001; Ekstrom & Meissl 2003); therefore, we hypothesized that fish perceive external light and daily rhythm is maintained by the existence of both eyes.

Clock gene expression in all light spectra was significantly lower in ophthalmectomized groups than in intact groups. This result indicated that the expression of clock genes, which control daily rhythm, was reduced by loss of light perception due to the absence of one eye.

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 4). 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 Falcon and

Meissl's report (1981) that the fish pineal organ has double spectral sensitivity in the blue–green regions.

Furthermore, Migaud, Davie, et al. (2007) reported that a different circadian system could be at work in sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) as ophthalmectomy significantly reduced night-time production of melatonin. These results are consistent with previous reports in tilapia (Martinez-Chavez & Migaud 2009; Nikaido et al. 2009), birds (Jimenez et al. 1995; Brandstatter 2003), and amphibians (Wright et al. 2006). In all these species, findings suggest both eyes and the pineal gland are required to sustain full-amplitude melatonin rhythms, meaning that light perceived by the eyes may regulate melatonin synthesis via the pineal gland, probably through neural projections into the brain (Jimenez et al. 1995; Yanez & Anadon 1998; Bayarri et al. 2003).

In ophthalmectomized groups, clock gene expression was significantly higher in groups exposed to green and blue LED. Pulses of differently colored lights (blue, green, and red) affect circadian rhythms in various ways, with fish tissues displaying higher sensitivity to short wavelengths (Bayarri et al. 2002; Bayarri et al. 2003). In addition, Loew and McFarland (1990) and Migaud et al. (2006) reported that most light energy is wasted in the form of unsuitable wavelengths (longer wavelengths and red light) that are rapidly absorbed by water molecules and cannot be detected by fish. Many fish farmers empirically choose to use "true-light" tubes, tungsten filament, fluorescent, or metal halide lights, which create bright point light sources with spectral emissions of unnatural wavelengths that may not be detected by fish and/or could compromise their welfare (Boeuf & Le Bail 1999; Migaud, Cowan, et al. 2007). Therefore, we hypothesized that white fluorescent and red light cannot be detected by fish, thus explaining why white fluorescence and red LED light yielded similar results in this study.

Meanwhile, Migaud, Cowan, et al. (2007) have reported that blue–green LED are effectively suitable wavelengths for enhancing fish growth side. In addition, Villamizar et al. (2009) have reported that blue wavelengths stimulate the visual system enough to facilitate feeding by fishes.

Underwater twilight and dawn spectrums are characterized by a narrow irradiance spectrum in the blue region, as opposed to the broader, flattened, spectral irradiance of sunlight, that is centered around 560 nm (McFarland & Munz 1975). Falcon and Meissl (1981) have reported that the double spectral sensitivity of the fish pineal gland to the blue–green region is justified.

This result is consistent with other studies in which indicators of immune activity, IgM mRNA, Ig protein, and lysozyme exhibit higher expression and activity levels in the blue and green LED groups of ophthalmectomized fish, and significantly higher than in all fish groups exposed to white fluorescent and red LED light fish (Figures 5 and 6). These results suggest that light is the most important factor because it affects many physiological and behavioral changes. Moreover, these results are consistent with Bowden et al. (2007), who reported that changes in circadian rhythm induced by light can impact the immune system and general health. The results of this study indicate that specific spectra affect fish immune systems, which may be enhanced by artificial use of these spectra. Shin et al. (2012) measured plasma H_2O_2 and lipid peroxidation in yellowtail clownfish and examined oxidative stress induced by red-spectrum lighting.

We compared intact and ophthalmectomized groups exposed to white fluorescent light and found that IgM mRNA and Ig protein expression and lysozyme activity were significantly lower in ophthalmectomized groups than in the control group (with two eyes). These results suggest that light and immunity are tightly associated. However,

expression levels of IgM mRNA and Ig protein and lysozyme activity in groups exposed to blue and green LED were significantly higher than in groups exposed to other spectra. Thus, IgM and lysozyme activity levels increased to counter the reduced immunity resulting from ophthalmectomy, and light, wavelength, and immune function were closely related in our experiments. We hypothesized that blue and green wavelengths enhance immune function and that these wavelengths could be used to artificially regulate fish immune function.

These results are consistent with those of Bowden (2008) who showed that IgM and lysozyme activity differ seasonally, with summer levels significantly higher than winter levels. Therefore, these results suggest the possibility that IgM levels and lysozyme activity are correlated with the amount of light, and fish detect blue and green wavelengths best because the short (or blue) end of the visible spectrum predominates in deeper waters, whereas red light is rapidly absorbed by water molecules and does not reach the depths where fish reside. Similar research has been carried out using plants. Xu et al. (2012), for example, reported that treatment with blue LED light increased fruit yield, quality, and disease resistance in tomato crops.

This result is consistent with the finding that plasma H_2O_2 levels in groups exposed to blue and green LED were significantly lower than in other groups, and intact fish had levels similar to those of ophthalmectomized fish (Figure 7). This result is consistent with those of Shin et al. (2011), who reported that exposure to short wavelength green and blue LED induced less oxidative stress in fish exposed to starvation.

We demonstrated that different light spectra influence the daily rhythmicity of clock gene expression, immune function, and stress indicators in yellowtail clownfish focused on retinal function between intact fish and ophthalmectomized fish. Short wavelengths such as blue and green specifically regulate the daily rhythm by maintaining *Per2* and *Cry1* expression more effectively than white fluorescent bulbs and red LED in ophthalmectomized fish. Furthermore, blue and green wavelengths enhance immune function more effectively than other wavelengths.

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