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ARTICLE

Effects of various photoperiods and specific wavelengths on circadian rhythm in ornamental cleaner shrimp *Lysmata amboinensis*

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ABSTRACT

The cleaner shrimp, *Lysmata amboinensis*, is an ornamental crustacean that is important both commercially, in the aquarium industry, and ecologically, owing to its symbiotic relationship with host fishes. Cleaner shrimp display high daytime activity, unlike other crustaceans, and are highly sensitive to light, particularly, in the 480–540 nm range. However, there have been few studies on the physiological characteristics of this species. In this study, we investigated effect of photoperiod and specific wavelength on the circadian rhythm of cleaner shrimp. Circadian rhythm was evaluated by quantifying the mRNA expression of clock genes such as lysmata amboinensis cryptochrome1 (*laCry1*) and lysmata amboinensis period 2 (*laPer2*) and melatonin concentration. The mRNA levels of clock genes were significantly higher at daytime than at night-time. Furthermore, at the red and green wavelengths, the difference in clock gene mRNA levels between day and night was significantly higher than that observed under white fluorescent light. Melatonin levels showed the opposite trend. Collectively, our findings demonstrate that the photoperiod regulates the biological rhythm of the cleaner shrimp and that the irradiation wavelength affects the biological rhythm. In particular, the red and green wavelengths appeared to be linked to the clock gene rhythm and melatonin levels.

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KEYWORDS
Circadian rhythm; cleaner shrimp; clock gene; light emitting diode; melatonin; photoperiod

1. Introduction

Light is an important environmental factor that regulates various physiological phenomena in organisms. In particular, in organisms living in underwater environments that transmit the light spectrum to limited depths, according to the Lambert-Beer law (Weinberg 1976) physiological aspects such as the reproductive cycle and growth, as well as behavioural aspects such as avoiding predators and searching food, are greatly affected by light wavelength. The circadian clocks (biorhythm) of aquatic organisms follow a 24-hour cycle. Thus, light can induce or suppress hormone secretion in these biorhythms, affecting reproductive ability, growth, and behaviour (King and Takahashi 2000; Pierce et al. 2008).

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Organisms have various clock genes, also called pacemakers that regulate the rhythms of this physiological, biochemical, and behavioural phenomena *in vivo* (Nanako et al. 2012). Representative clock genes are period (*Per*) and cryptochrome (*Cry*), which are regulated by light and help organisms recognize the 24-hour cycle (Albrecht and Ripperger 2009). *Per* regulates the transcriptional activation/suppression of clock genes, acting as a negative feedback loop by forming a heterodimer with *Timeless*, which can bind to regulatory DNA and effectively regulate its own expression (Landskron et al. 2009). *Cry1* is a rapidly induced gene that is stimulated by light in circadian rhythm oscillators (Cermakian et al. 2002; Besharse et al. 2004). In addition, the Cry1 protein absorbs the energy of blue light due to its flavin-adenine-dinucleotide/pterin content. Thus, Cry1 regulates of the circadian rhythm in response to light stimulus by causing structural alteration and autophosphorylation of Timeless via direct interaction, as well as plays an important role in regulating and resetting the biorhythm according to the change in the light environment through direct interaction with the Per/Timeless heterodimer (Ceriani et al. 1999).

In addition to clock genes, melatonin is a well-known factor controlling the circadian rhythm. Melatonin is synthesized from 5-hydroxytryptamine, a neurotransmitter, by arylalkylamine N-acetyltransferase, which is inhibited by light, and is known as a "night hormone" because it is released into the plasma at night (Iuvone et al. 2005; Klein 2007). Generally, in mammals and vertebrates, it is mainly produced in the pineal gland and retina and functions via receptors belonging to the G-protein-coupled receptor superfamily (Klein et al. 2002). In crustaceans, melatonin is produced in optic lobes (lamina ganglionalis, medulla externa, medulla intema, and medulla terminalis) in the eyestalk (Withyachumnarnkul et al. 1995). Melatonin not only plays a role in neuroendocrine transduction regulating the biorhythm (Falcón et al. 2007), but is also involved in immune regulation (Petrovsky 2001) and antioxidant defence (Maciel et al. 2010). It also plays a role in moulting (Sainath and Reddy 2010) and limb regeneration (Tilden et al. 1997), especially in crustaceans.

Many physiological studies relating to the response to light have been conducted on fish; however, the molecular mechanisms underlying the circadian rhythm as well as its associated genes in the shrimp remain to be elucidated (Fanjul-Moles et al. 2004).

The cleaner shrimp *Lysmata amboinensis*, a decapod crustacean inhabiting the tropical coral reef area, is an economically important aquarium industry ornamental shrimp that is popular with marine creature collectors due to its bright colour and unique patterns (Calado et al. 2003). Cleaner shrimps are ornamental and are mainly imported and exported through natural collection (Biondo 2017). However, they are also important in terms of ecology due to their peculiar symbiotic relationship with host fishes (Vaughan et al. 2017). In particular, it is called "cleaner shrimp" because it maintains a unique symbiotic relationship in which it removes parasites, bacteria, and damaged tissues from host fishes (Côté 2000). Thus, while most crustaceans are nocturnal, the cleaner shrimp is highly sensitive to light and maintains relatively high activity during the daytime when the host fishes are active (Wicksten 2009; Esaka et al. 2016). Cleaner shrimp has been reported to recognize various colouring patterns of host fishes and is particularly sensitive to light in the 480–540 nm range (Caves et al. 2016). However, few studies have been conducted on the physiological aspects of this species, and unlike other crustaceans, it is considered to be a suitable species for conducting photoreactive molecular endocrinological studies because it is a relatively unknown species with characteristic daytime activity levels that match that of host fish.

Therefore, in this study, we investigated the effect of photoperiod and specific wavelength on the biological rhythm of the ornamental shrimp *L. amboinensis*. We measured the changes in the levels of *Per2* and *Cry1* mRNA and melatonin levels in the eyestalk of shrimp exposed to various photoperiods [LD, 12 h light:12 h dark; LL, continuous light; and DD, continuous darkness] and various wavelengths of LED (light-emitting diode) (red, green, blue LED).

2. Materials and methods

2.1. *Experimental species and light conditions*

For each experiment, cleaner shrimp *L. amboinensis* (length, 43.0 ± 2.5 mm; 2.28 ± 0.13 g) were purchased from the commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in 18 tanks (6 tanks for photoperiod experiment; 12 tanks for wavelength experiment; each tank consist of 4 mini tanks). For each experiment, cleaner shrimp used 30 individuals, and each expriment was conducted in triplicate. The shrimps were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) under white fluorescent bulb (light on at 07:00, light off at 19:00). The water conditions were maintained to 22°C, pH 8.0 and 35 psu.

After completion of the run, the experiment was divided into two experiments (the photoperiod group and LED group). The photoperiod group was exposed to three photoperiods [LD, 12 h light:12 h dark; LL, continuous light; and DD, continuous darkness] and the wavelength group was exposed to lights of three wavelengths [red LED, 630 nm; green LED, 520 nm; blue LED, 455 nm; white fluorescent bulb as a control light source (Cont.)] at an intensity of 0.5 W/m^2 at the bottom of aquarium (Figure 1) for a day [sampled at 2 (09:00), 6 (13:00), 10 (17:00), 14 (21:00), 18 (01:00), and 22 (05:00) hours after exposure; light on at 07:00, light off at 19:00 in LD and wavelength groups)]. The irradiance level at the bottom layer of each tank with external light interception was maintained at approximately 0.5 Wm^{-2} using a spectrometer (MR-16; Rainbow Light Technology, Taoyuan, Taiwan) and a photo-radiometer (HD 2102.1; Delta OMH CO., Caselle di Selvazzano, Italy).

The shrimps were anaesthetized using 2-phenoxyethanol (Sigma, St. Louis, MO, USA) to collect the tissue under dim light using an attenuated white fluorescent bulb to minimize stress prior to eyestalk collection and were stored at −80°C until analysis. The eyestalk was removed from the shrimp, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

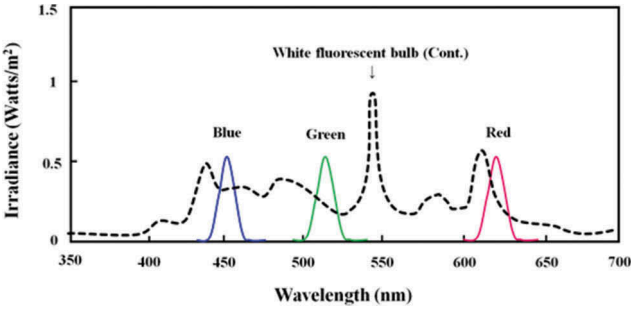

Figure 1. Spectral profiles of red, green, and blue light-emitting diodes (LEDs) and the white fluorescent bulb (Cont.) used in this study. The same light intensities (0.5 W/m^2) were used for each type of LED.

2.2. *Total RNA extraction and complementary DNA (cDNA) synthesis*

Total RNA was extracted from each sample using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. Then, 2 µg of total RNA was reverse-transcribed in a total reaction volume of 20 µL using an oligo-(dT)15 anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4°C for use in polymerase chain reaction (PCR) and quantitative PCR (qPCR) analysis.

2.3. *qPCR*

qPCR was conducted to determine the relative expression of *laPer* and *laCry* mRNA using total RNA extracted from the eyestalk of *L. amboinensis*, respectively. The following primers were designed with reference to known sequences: *laPer* forward (5′-CTC TGA AGT TGC ACG ACA CT-3′) and reverse (5′-CTG AAG CTG CTC ATG GAT GG-3′) primers; *laCry* forward (5′-CTG CTG CGA CAA ATA ACC CA–3′) and reverse (5′-ACC TTC ATG CCT TCT TCC CA–3′) primers; and *β-actin* forward (5′-TCG AGC ACG GTA TTG TGA CC-3′) and reverse (5′-GAC CCA GAT CAT GTT CGA GA-3′). PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. qPCR was performed as follows: 1 cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. Each experimental group was run in triplicate to confirm consistency. As an internal control, the experiments were duplicated using β-actin. The efficiencies of the reactions were determined by qPCR. All data were expressed as change with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (*β-actin*) was calculated as $\Delta\Delta Ct = 2\text{^-}(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

2.4. *Melatonin analysis*

Melatonin levels were analysed using the immunoassay technique. Melatonin was extracted using the modified methods of Tilden et al. (1997) and Pape et al. (2008). For the ethanol–chloroform method, EtOH and deionized water were added to eyestalk material in a test tube. The mixture was then sonicated, and melatonin extraction was performed as described above. The chloroform phase was separated and dried under a stream of nitrogen. The residue was dissolved in methanol (MeOH) and was separated into an aliquot of 10% for direct melatonin ELISA determination. Finally, the melatonin level was measured using direct ELISA (EK-DSM, Buhlmann Laboratories AG, Schonenbuch, Switzerland).

2.5. *Statistical analysis*

All data were analysed using the SPSS statistical package (version 19.0; SPSS Inc., Chicago, USA). A one-way ANOVA followed by Tukey's post hoc test was used to compare differences in the data ($p < 0.05$). The values are expressed as the mean ± standard error (SE).

3. Results

3.1. *Changes in clock gene mRNA expression in the photoperiod groups*

The mRNA level of *laPer* in the daytime was not significantly different between the LL experimental group and the LD experimental group. However, the level of *laPer* mRNA was significantly higher in the LL group than in the LD group in the night-time. In DD group, mRNA level of *laPer* was significantly lower in day/night-time than in LD. The periodic pattern of *laPer* was also observed in the expression of *laCry* mRNA (Figure 2).

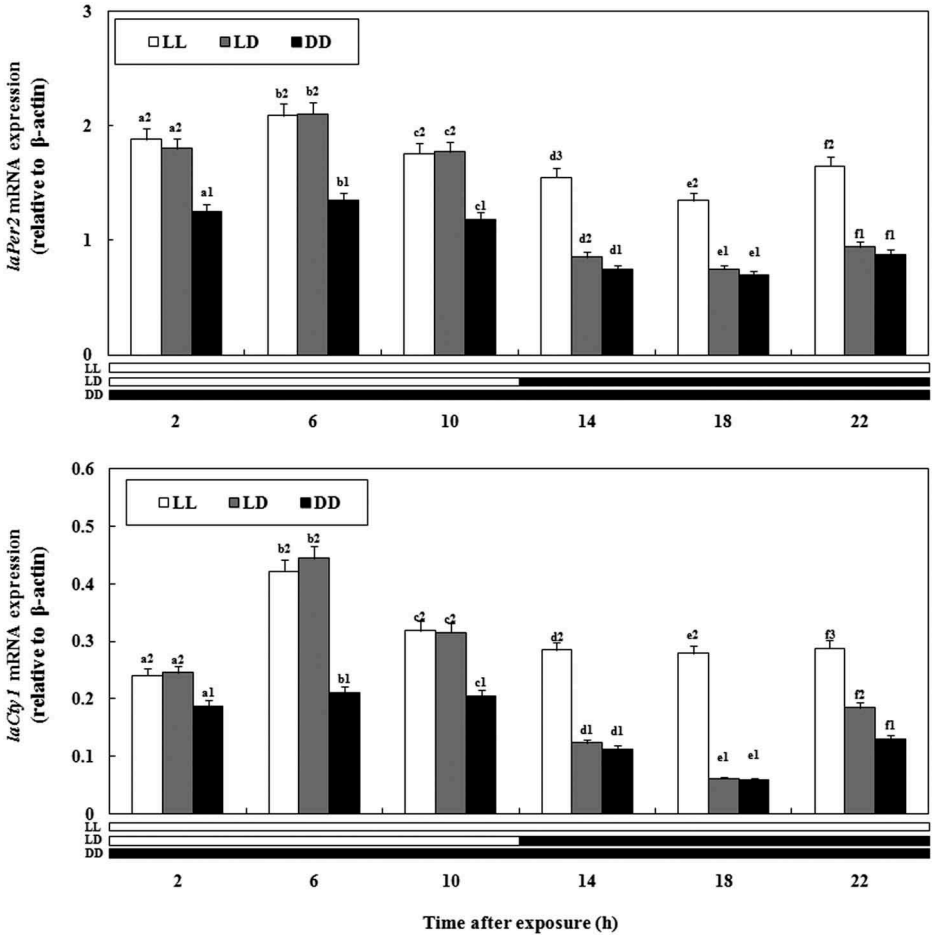

Figure 2. Changes in *laCry1* and *laPer2* mRNA expression in the eyestalk of *L. amboinensis* under different photoperiod conditions [LD, 12 h light:12 h dark; LL, continuous light; and DD, continuous darkness]. The tissue samples were taken at 2 (09:00), 6 (13:00), 10 (17:00), 14 (21:00), 18 (01:00), and 22 (05:00) h after exposure to the various photoperiods. The white bar represents the photophase and the black bar represents the scotophase. Different lowercase letters indicate significant differences at different exposure times. Different numbers indicate significant differences between the experiment groups for the same photoperiod and exposure time ($p < 0.05$). All values are mean ± SE ($n = 5$).

3.2. *Changes in melatonin level in the photoperiod groups*

The melatonin level in the daytime was not significantly different between the LL experimental group and the LD experimental group. However, it was significantly lower in the LL group than in the LD group in the night-time. In the DD group, melatonin was significantly higher in day/night-time than in the LD. Melatonin concentration was the highest at 18 h (Figure 3).

3.3. *Changes in clock gene mRNA levels in the LED groups*

The mRNA level of *laPer* during the daytime was significantly lower under red LEDs than under the white fluorescent bulb, while it was significantly higher under green and blue LEDs. At night-time, the mRNA level of *laPer* was not significantly different between the LED groups. The periodic pattern of *laPer* was also observed in the expression of *laCry* mRNA (Figure 4).

3.4. *Changes in melatonin level in the LED groups*

The concentrations of melatonin during the daytime period were significantly higher in the red LED group than in the fluorescent lamp group, while there was no significant difference between green and blue LED groups. At night-time, the concentrations of melatonin were not significantly different among the LED groups (Figure 5).

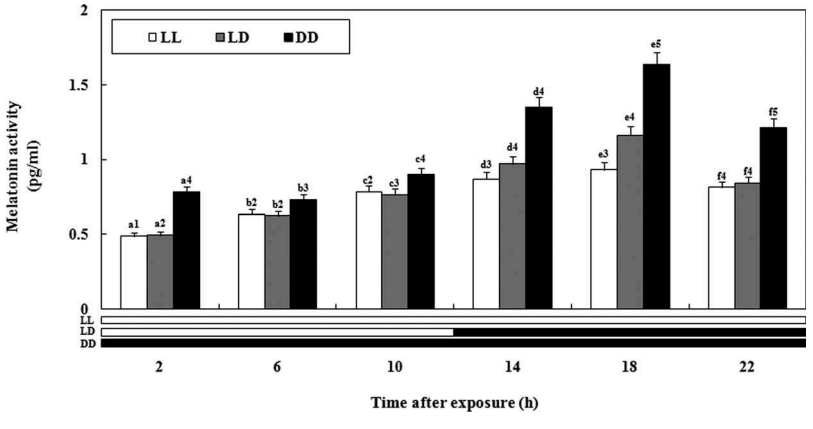

Figure 3. Changes in melatonin activity in the eyestalk of *Lysmata amboinensis* under different photoperiod conditions [LD, 12 h light:12 h dark; LL, continuous light; and DD, continuous darkness]. The tissue samples were taken at 2 (09:00), 6 (13:00), 10 (17:00), 14 (21:00), 18 (01:00), and 22 (05:00) h after exposure to the various photoperiods. The white bar represents the photophase and the black bar represents the scotophase. Different lowercase letters indicates significant differences at different exposure times. Different numbers indicate significant differences between the experiment groups for the same photoperiods and exposure time ($p < 0.05$). All values are mean ± SE ($n = 5$).

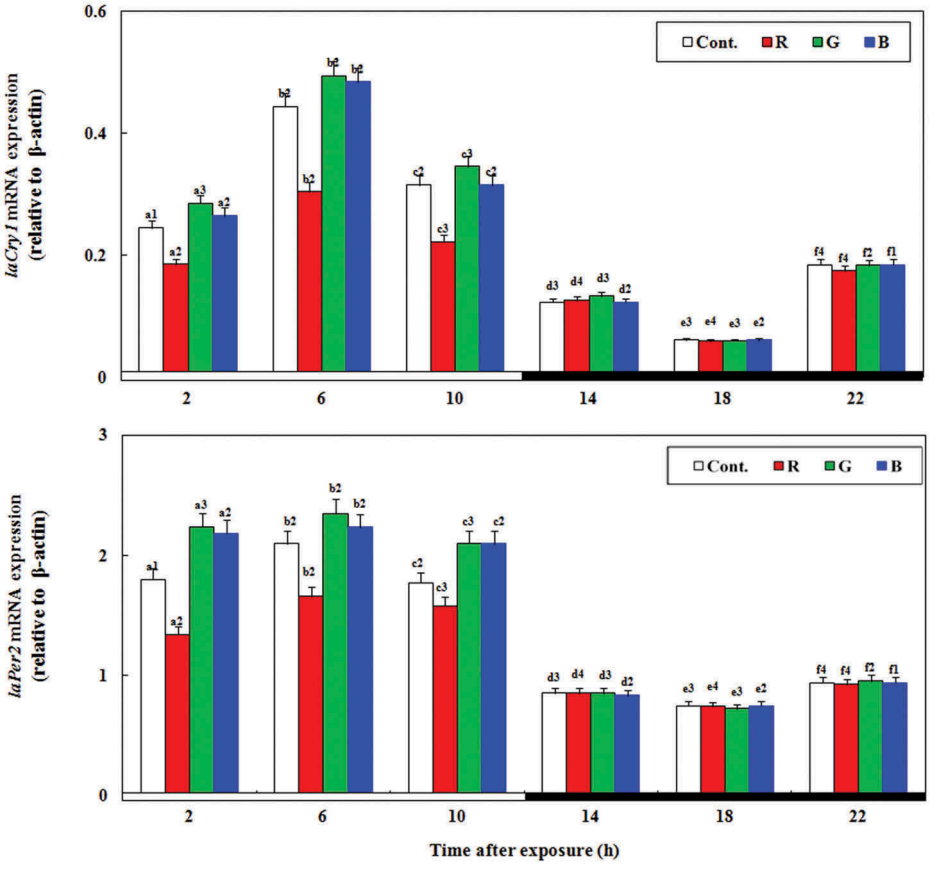

Figure 4. Changes in *laCry1* and *laPer2* mRNA expression in the eyestalk of *Lysmata amboinensis* under light of different wavelengths [red LED, 630 nm; green LED, 520 nm; blue LED, 455 nm; control, white fluorescent light (Cont.)] at the same intensity (0.5 W/m^2 at bottom of aquarium). The tissue samples were taken at 2 (09:00), 6 (13:00), 10 (17:00), 14 (21:00), 18 (01:00), and 22 (05:00) h after exposure to light. The white bar represents the photophase and the black bar represents the scotophase. Different lowercase letters indicate significant differences at the various exposure times. Different numbers indicate significant differences the experiment groups for the same exposure time ($p < 0.05$). All values are mean ± SE ($n = 5$).

4. Discussion

Most organisms exposed to sunlight sense light via photoreceptors present in the retina. In particular, in the case of crustaceans, it not only receives light from the eyestalk, it but also secretes important hormones necessary for reproduction and growth (usually responsible for brain function in fish) (Aréchiga et al. 1985). In addition, changes to photoperiod affect the circadian rhythm of organisms naturally adapted to a 24-hour light cycle. Vertebrates, including fish, and non-vertebrates, including crustaceans, are known to have the same light-receiving mechanism (Strauss and Dircksen 2010).

Therefore, it can be assumed that the light reaction in vertebrates will be similar to that in the shrimp. In addition, while most crustaceans are nocturnal, *L. amboinensis* is active during the daytime to maintain its symbiotic relationship with host fishes (Strauss

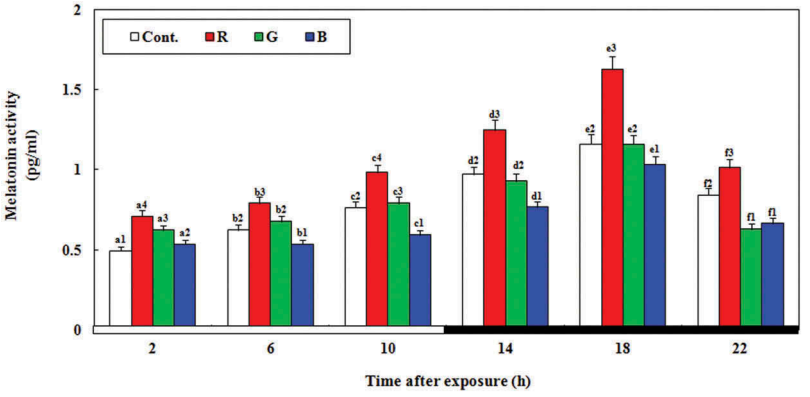

Figure 5. Changes in melatonin activity in the eyestalk of *Lysmata amboinensis* under light of different wavelengths [red LED, 630 nm; green LED, 520 nm; blue LED, 455 nm; control, white fluorescent light] of two intensities (0.5 W/m^2 at bottom of aquarium). The tissue samples were taken at 2 (09:00), 6 (13:00), 10 (17:00), 14 (21:00), 18 (01:00), and 22 (05:00) hours after exposure to LED. The white bar represents the photophase and the black bar represents the scotophase. Different lowercase letters indicates significant differences at the various exposure times. Different numbers indicate significant differences in the experiment groups for the same exposure time ($p < 0.05$). All values are mean ± SE ($n = 5$).

and Dircksen 2010). Therefore, this study was conducted to obtain basic data on the photoreaction in cleaner shrimp. Furthermore, since cleaner shrimp *L. amboinensis* is an ornamental shrimp that is typically reared in an aquarium, there is a high possibility of these shrimp being exposed to rapid and artificial photoperiod changes. Therefore, it is important to understand the effect of photoperiod changes on the circadian rhythm.

First, in order to investigate the effect of photoperiod on the circadian rhythm of the cleaner shrimp, we measured the changes in the levels of *laCry1* and *laPer2* mRNAs and melatonin in eyestalk under three light periods (LL, LD, and DD). The oscillation in the mRNA levels of clock genes between daytime and night-time was the largest in LL and the smallest in DD. Fanjul-Moles et al. (2004) reported that Cry concentration in crayfish *Procambarus clarkii* exposed to DD for 72 h was not significantly different from that in those exposed to LD conditions. In contrast, Zhang et al. (2013) reported that *Cry* and *Per* mRNA expression in the marine crustacean *Eurydice pulchra* was high at daytime rather than at night-time. Although this was not the case in all crustaceans, *Sigmaus guttatus*, a host fish of *L. amboinensis* with the same photoperiod, also maintained significantly higher *Per* mRNA expression during daytime exposure to LD, but the levels were higher under the LL than under LD condition (Park et al. 2007). Therefore, the expression patterns of clock genes may change according to photoperiod changes depending on species and habitat. Regardless, *L. amboinensis* has similar photoreaction mechanisms to vertebrates, including fish.

In addition, the melatonin cycle of *L. amboinensis* according to the photoperiodic environment was different from that of clock gene mRNAs. Han et al. (2018) analysed the diurnal pattern of melatonin in the eyestalk of the Chinese mitten crab, *Eriocheir sinensis*, and Chinese grass shrimp, *Palaemonetes sinensis*, in the intertidal zone, and both species showed two peaks during a daily cycle. They found that melatonin level was the highest

at 24:00 at night-time in *E. sinensis* and at 16:00 during daytime in *P. sinensis*. As such, the circadian pattern of melatonin in marine crustaceans is known to be different from that of crustaceans, depending on the various *in vivo* functions of melatonin and the complex actions associated with feedback control mechanisms and changes in the habitat (tide, light, depth). Maciel et al. (2008) measured the melatonin concentration in the optic lobes of the crab *Neohelice granulata* was exposed to various LL, LD, and DD conditions. The highest melatonin level was observed at 03:00 in LD and DD conditions. Furthermore, no significant difference in melatonin concentration was observed during a daily cycle in the LL condition. In contrast, in the present study, melatonin concentration in the LL condition was lower than that in the LD and DD conditions. Collectively, these findings suggest that the presence or absence of light and changes in the photoperiod affect melatonin secretion in *L. amboinensis* in a laboratory environment. Moreover, the physiological response could be controlled through melatonin, which has various effects *in vivo*.

In this study, to clarify the effect of light of a specific wavelength on the circadian rhythm of cleaner shrimp, we investigated the changes in *laCry1* and *laPer2* mRNA expression and the concentration of melatonin in cleaner shrimp exposed to light of three wavelengths (red, green, and blue). Caves et al. (2016) reported that the light wave range recognized by *L. amboinensis* is 380–540 nm, but the possibility that light of specific wavelengths may influence the physiological processes of crustaceans has not been studied. In this study, it was difficult to confirm the physiological response that light of a specific wavelength elicits from *L. amboinensis*, but based on the present results and previous research, we speculate the following: First, *Cry* is known to be sensitive to blue wavelengths, but it also regulates the expression of brain photoreceptors sensitive to green wavelengths that control the locomotion of crustaceans in terms of circadian rhythmicity (Sullivan et al. 2009). Therefore, because *L. amboinensis* inevitably needs to be active during the daytime, we concluded that the expression of *laCry* mRNA and *laPer* mRNA, which interacts with *laCry* for regulating the circadian rhythm, was increased significantly under the green and blue wavelengths. Second, the correlations between the red wavelength and the circadian rhythm were not studied. However, the expression of the clock gene and the periodic pattern oscillator was reduced in comparison with that in the LD fluorescence experimental condition, and the increase in melatonin activity during the daytime was similar to that in the DD condition. It has been reported that aquatic organisms recognize the red light as dark environments due to various reasons (low permeability to molecules of water, lack of visual perception, etc.) (Migaud et al. 2006). Although no other studies have examined the changes in circadian rhythm caused by the 630 nm (red wavelength) used in this study, the melatonin concentration and the melatonin receptor mRNA levels were significantly higher at red wavelength (630 nm) than under fluorescent light in the reef fish yellowtail clownfish *Amphiprion clarkii* (Choi et al. 2014). These results suggest that *L. aboinensis* recognizes the red wavelength as a dark environment during the daytime. Therefore, the levels of *laCry* and *laPer* mRNA, which are generally increased during daytime, decreased and melatonin activity significantly increased.

In conclusion, we demonstrated that unlike most crustaceans, which are mostly nocturnal, *L. amboinensis* is diurnal and has the same photoreaction mechanism and pattern as vertebrates due to its symbiotic relationship with host fishes. We also

confirmed that green light is effective for regulating the biological rhythm by altering the periodic pattern of the clock gene, whereas the red wavelength seems to be a dark environment, such as the DD environment, which can blunt the rhythm of the organism.

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