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Effects of 17β-estradiol injection and red-spectrum light on eyestalk hormones and vitellogenesis of the ornamental cleaner shrimp *Lysmata amboinensis* (De Man, 1888) (Decapoda: Caridea: Lysmatidae)

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ABSTRACT

We analyzed the effects of 17β-estradiol (E_2) injection and red light on the sexual maturation and vitellogenesis of the cleaner shrimp *Lysmata amboinensis* (De Man, 1888) by measuring the change of mRNA expression levels of eyestalk and vitellogenesis-related hormones induced by both factors. We examined the eyestalk crustacean hyperglycemic hormone (CHH) and vitellogenesis-inhibiting hormone (VIH) genes. The E_2 injection did not affect changes of eyestalk hormones. The red light, however, caused a significant increase ($P < 0.05$) of CHH promoting maturity and a significant decrease of VIH, which suppressed vitellogenesis. The vitellogenin receptor (VTG-R) and mRNA expression (*in situ* hybridization) also caused the E_2 injection and red-wavelength irradiation to induce synthesis of vitellogenin (VTG). Red-light-induced gonadal maturation, promoting hormone and VTG synthesis, but E_2 did not change the eyestalk hormone. Red-light irradiation could thus be used as a novel, maturity-promoting alternative method in the culture of the species, which is different from the eyestalk-ablation method that has so far been used.

Key Words: cleaner shrimp, E_2 injection, eyestalk hormones, ornamental shrimps

INTRODUCTION

The cleaner shrimp *Lysmata amboinensis* (De Man, 1888), a tropical shrimp having bright colors and unusual patterns, is popular in the ornamental-shrimp trade (Calado *et al.*, 2003). This species maintains a unique symbiotic relationship with host fishes (Vaughan *et al.*, 2017). *Lysmata amboinensis* shows protandric simultaneous hermaphroditism (PSH), unlike most other shrimp species (Bauer & Holt, 1998). In PSH. individuals are transformed into the female phase (FP) through a male phase (MP) similar to general protandric crustaceans, but with the FP exhibiting both male and female functions (Bauer, 2006). PSH is the most common criterion for classifying the size of individuals to distinguish them between the MP and FP (Zhang & Lin, 2005; Tziouveli & Smith, 2009). Furthermore, when a shrimp is significantly larger than 34 mm, it is judged to be in the FP (Zhang & Lin, 2005; Tziouveli & Smith, 2009).

Factors controlling the reproduction and maturation of shrimps have been studied using various methods. In general, sexual maturation is controlled by various hormones such as vitellogenesis-inhibiting (VIH), crustacean-hyperglycemic (CHH), and molt-inhibiting hormones (MIH). All these hormones belong to the CHH family of peptides (Ollivaux *et al.*, 2006), and VIH is a major neuropeptide in the eyestalk that inhibits maturation of crustacean ovaries (Adiyodi & Adiyodi, 1970). A commonly used method to induce maturation and ovulation in shrimps is the removal of the eyestalk, which secretes gonadotropin-releasing hormone. This method induces sexual maturation, which is inhibited by X-organ/sinus gland complexes (XO-SG) in the eyestalk by blocking hormone production (Adiyodi & Subramoniam, 1983). The XO-SG secretes VIH as well as CHH and MIH, however. These two hormones are involved in glucose metabolism and inhibit molting. It is also known to regulate the synthesis of vitellogenin (VTG), an egg-yolk protein in decapods such as penaeid shrimps, lobsters, and brachyuran crabs (Gu *et al.*, 2002; Zmora *et al.*, 2009). Although eyestalk ablation in crustaceans may promote sexual maturation and ovulation by inhibiting VIH, it also

blocks the secretion of CHH and MIH, thus resulting in low-quality eggs (Kang *et al.*, 2014).

Sexual maturation of shrimps is generally known to be controlled by various eyestalk hormones, but recent studies indicate that vertebrate-type gonadotropic hormones such as 17β-estradiol (E_2) and progesterone are synthesized in the hepatopancreas and ovary (Subramoniam, 2000; Guan *et al.*, 2013; Sathapondecha *et al.*, 2015). No studies have so far investigated the function of each hormone in crustaceans (Subramoniam, 2017). E_2 is a sex steroid known to induce the synthesis of VTG, an egg-yolk protein (Wallace, 1985). Vertebrate-type sex steroids have also been shown to play a role in the induction of vitellogenesis in crustaceans (Subramoniam, 2016).

Sexual maturation of crustaceans is also controlled by various external environmental factors, among which is light. Light wavelengths transmit a limited spectrum depending on water depth, and light affects physiological functions such as the reproductive cycle and growth as well as behaviors such as avoidance and search for food (Weinberg, 1976). The effects of light on crustaceans have been studied with varying results depending on species and wavelength. The penaeid shrimp *Fenneropenaeus chinensis* (Osbeck, 1765) showed rapidly growth and molting cycles under green and blue wavelengths (Wang *et al.*, 2003). The ovarian maturation of the shrimps *Litopenaeus setiferus* (Linnaeus, 1767) and *Farfantepenaeus duorarum* (Burkenroad, 1939) was promoted under blue and green lights, and the number of eggs produced increased (Caillouet, 1973; Wurts & Stickney, 1984). *Macrobrachium nipponense* (De Haan, 1849) showed the highest light sensitivity and food intake under red light (Wang *et al.*, 2003). In the case of *L. amboinensis*, red light effectively reduced the stress of individuals exposed to rapid osmotic changes, enhancing immunity (Choe *et al.*, 2018; Choi *et al.*, 2018). Compared with fishes, however, knowledge of the molecular mechanism related to photoreaction in crustaceans is limited.

We sought to find a new method to determine the correlation between sexual maturity and light to address the disadvantages of ocular resection, which involves removing the eyestalk tissue that processes light for sexual maturation in crustaceans. To this end, we investigated the effects of E_2 and red-wavelength light on maturation and vitellogenesis. Both factors have been reported to effectively control positive physiological responses to stress and enhance immunity (Choi *et al.*, 2018). We specifically measured the mRNA expression levels of the estrogen receptor (ER), CHH, VIH, and VTG receptor (VTG-R). The mRNA expression of VTG in hepatopancreas injected with E_2 at two concentrations or exposed to red-wavelength (630 nm) irradiation was also examined using *in situ* hybridization.

MATERIALS AND METHODS

Experimental shrimp

For each experiment, cleaner shrimp *L. amboinensis* (N = 540; length, 39.2 ± 3.6 mm; weight, 1.78 ± 0.43 g) were purchased from a commercial aquarium (Choryang, Busan, Korea) and allowed to acclimate for two weeks in 600 l circulation-filter tanks. Each tank consisted of four 45 cm × 45 cm × 45 cm mini tanks, each containing five individuals. The 540 individuals were used for 32 experiments, which involved the following treatment groups: Control, E_2 0.5, and 1.0 μg g^{-1} injections and red-light-emitting diode (LED) exposure for 0, 3, 6, 12, 18, 24, 48, 96, and 144 h. Five individuals were used in each case and the experiments were conducted in triplicate. The shrimp were reared in automatic temperature regulation systems (JS-WBP-170RP; Johnsam, Seoul, Korea) under a white fluorescent bulb (lights on at 0700, off at 1900). The water conditions were maintained at 26 °C, pH 8.0, and 35 psu. The shrimp were fed a commercial feed twice daily (0900 h and 1700 h).

Light conditions and E_2 injection

The experimental specimens were divided into two groups (the E_2 injected and red LED-exposed). E_2 was intraperitoneally injected at two concentrations (0.5 and 1.0 μg g^{-1} body weight) under a white fluorescent bulb (0.5 W m^{-2} at the bottom of the aquarium); the red LED group was exposed to 630 nm (0.5 W m^{-2} at the bottom of the aquarium), and the control group was exposed only to the white fluorescent bulb (no E_2 treatment) (Fig. 1). Sampling was conducted 0, 3, 6, 9, 12, 18, 24, 48, 96, and 144 h after E_2 injections, red LED exposure, and no treatment (as control group). The irradiance level at the bottom of each tank with external light interception was maintained at approximately 0.5 W m^{-2} using a spectrophotometer (MR-16; Rainbow Light Technology, Taoyuan, Taiwan) and a photo-radiometer (HD 2102.1; Delta OMH., Caselle di Selvazzano, Italy).

The shrimp were anaesthetized using 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) and the eyestalk, hepatopancreas, and gonad tissues collected under dim light using an attenuated white fluorescent bulb to minimize stress, immediately frozen in liquid nitrogen, and then stored at −80 °C until analysis.

Total RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from eyestalks, hepatopancreas, and gonads (for the analysis of hormones, the ER, and VTG-R, respectively) using TRI reagent (Molecular Research Center,

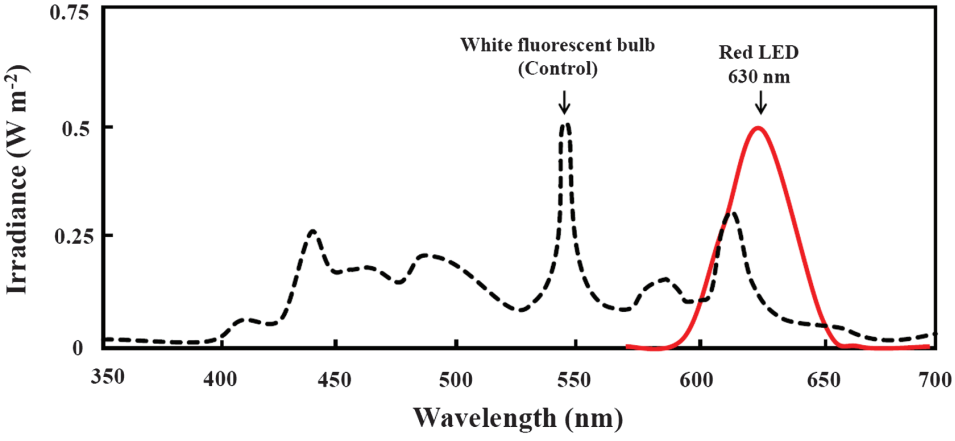

Figure 1. Spectral profiles of red-light-emitting diodes (LEDs) and white fluorescent bulb (Control) used in this study. The light intensity of 0.5 W m^{-2} was used for each type of LED and white fluorescent bulb.

Cincinnati, OH, USA) according to the manufacturer's instructions. Subsequently, 2 μg 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 complementary DNA (cDNA) was diluted and stored at 4 °C for use in polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) analyses.

Real-time quantitative polymerase chain reaction (qPCR)

The qPCR was conducted to determine the relative expression of ER, CHH, VIH, and VTG-R mRNA using total RNA extracted from the hepatopancreas and eyestalks of *L. amboinensis*. Each gene sequence was identified using a next-generation sequencing (NGS) technique and they showed more than 80% homology with the sequences of other crustaceans. The following primers for the qPCR were designed with reference to the NGS results (GenBank accession numbers: ER, MN119281; CHH, MN119282; VIH, MN119280; VTG-R, MN119285; and β-actin, MN119284): ER forward (5′-GCT GCC AGA TCC AAC TGT TT-3′) and reverse (5′-GCA TGG AGC GAT AGG CTA AT-3′); CHH forward (5′-TCC AAC AGC ATC CCA GCA AT-3′) and reverse (5′- ACG GAG TTC TTG TCG CTG TA -3′); VIH forward (5′-CAC GCC CAC ACG AAG TCC A-3′) and reverse (5′-CCT GTC CAG CTT GAA GA-3′); VTG-R forward (5′-AGT TGT CCT TCC CAG AG-3′) and reverse (5′-GAA TCT GTA CCA CTC ACCM AC-3′); 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 Laboratories, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR was performed using the following schedule: one denaturation cycle at 95 °C for 5 min, 30 denaturation cycles at 95 °C for 20 s, and annealing at 55 °C for 20 s. Each experimental group was run in triplicate to confirm consistency. Experiments were duplicated using β-actin as an internal control. The efficiencies of the reactions were determined by performing the 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 $\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$.

Tissue sections

We used shrimp one day after observed holding eggs. Fresh hepatopancreas tissues were sampled at 0, 18, and 144 h after exposure in each experiment. The freshly sampled tissue was washed in phosphate-buffered saline (PBS), placed in 4% paraformaldehyde (PFA) for 4 h at 4 °C, and subsequently immersed in 30% sucrose solution until they sank. The sunken tissues were removed from the 30% sucrose, rapidly frozen in optimal cutting temperature (OCT) compound using dry-ice, and cut into 6 μm thick sections using a freezing microtome (CM1510 S Cryostat, Leica, Wetzlar, Germany). All samples were processed quickly to avoid degradation of the RNA.

In situ hybridization

We modified the previously known *in situ* technique (Sumi *et al.*, 2018). The VTG sequence was obtained using the NGS technique. The template sequence for the *in situ* hybridization probe was designed (MN119283) based on the 3′-UTR and CDS (497 bp) of VTG identified by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) system (mRNA 73% homology with *Pandalopsis japonica* Balss, 1914, VTG, KF731996),amplified using PCR, and then ligated to TOPO-TA vector (Invitrogen, Carlsbad CA, USA).Primers for in situ hybridization were sense (5′-TCT TCG GAT CTA CCC GAA GA-3′) and anti-sense (5′- GTA GCA CTT ATA TCT TCA CCA G -3′). Furthermore, we confirmed the anti-sense sequence and amplified plasmid DNA through PCR using the antisense and T7 primers. Digoxigenin (DIG)-labeled probes were created by DIG RNA Labeling Mix (Roche, Germany) and the PCR product using the anti-sense primer and T7 RNA polymerase was used as the antisense labeling probes.

Fresh hepatopancreas tissue sections were hybridized with hybridization buffer (5 mL deionized formamide, 2.5 ml of 20× saline sodium citrate [SSC], 100 μl 0.1% Tween-20, 92 μl 1 M citric acid [pH 6.0], and DEPC-H_2O up to a total volume of 20 ml), yeast total RNA (50 μl), and the RNA probe overnight at 65 °C. Subsequently, the hybridized sections were thoroughly washed with 75% hybridization buffer plus 25% 2× SSC (10 min, 65 °C), 50% hybridization buffer plus 50% 2× SSC (10 min, 65 °C), 25% hybridization buffer plus 75% 2× SSC (10 min, 65 °C), and 0.2× SSC (two times, 30 min) followed by 75% 0.2× SSC plus 25% PBS with Tween-20 (PBST, 5 min, 22 °C), 50% 0.2× SSC plus 50% PBST (5 min, room temperature), 25% 0.2× SSC plus 75% PBST (5 min, room temperature), and PBST (5 min, room temperature).

For hybridization signal detection, tissue sections were first incubated with a blocking solution (containing 10% calf serum in PBST) for 1 h at room temperature, followed by overnight incubation at 4 °C with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000 in blocking solution, Roche). After a series of washing steps (6 × 15 min each, in PBST at room temperature) and rinsing in alkaline Tris buffer consisting of 1 M Tris at pH 9.5, 1 M $MgCl_2$, 5 M NaCl, and 10% Tween-20 (3 × 5 min each at room temperature), color imagining was performed using a labeling mix (1 mL alkaline Tris buffer, 4.5 mL nitroblue tetrazolium, and 3.5 μl 5-bromo-4-chloro-3- indolyl phosphate disodium salt), which was sprayed over the sections.

Then, the sections were kept in a dark and humid chamber for at least 8 h to develop the color. The slides were washed with PBST, fixed with 4% PFA for 1 h, mounted with Aquamount (Aqua Polymount, Polisciences, Warrington, PA, USA), and coverslipped. A stereomicroscope (Eclipse Ci; Nikon, Tokyo, Japan) was used to capture the images. For the quality check of the probe, gill tissue and non-treated hepatopancreas (negative control) were similarly analyzed.

Statistical analysis

All data were analyzed using the statistical package for the social sciences (SPSS) software program (version 19.0; www.ibs.com/products/spss-statistics). A one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was used to compare differences ($P < 0.05$). The values are expressed as the means ± standard error (SE).

RESULTS

Changes in ER mRNA expression

The ER mRNA expression was increased in all experimental groups injected with E_2, but the time of significant increase was dependent on the E_2 concentration. At 1.0 μg g^{-1}, a significant increase was observed between 0 and 6 h, which continued to increase until 48 h and then decreased. At 0.5 μg g^{-1}, the levels slightly increased from 0 to 18 h, but were significant between 18 and 24 h, after which they then decreased. A significant increase was only observed between 96 and 144 h in the red LED group (Fig. 2).

Change in CHH mRNA expression

We measured changes in CHH, an ocular sex hormone known to promote reproduction, to investigate the effects of E_2 injection and red LED on sexual maturation in *L. amboinensis*.CHH mRNA expression tended to decrease with increasing E_2 concentration in the group injected with E_2, but there was no significant change. The levels nevertheless increased from 0 to 18 h under red light and remained high for up to 144 h (Fig. 3).

Change in VIH mRNA expression

We measured changes in VIH, an ocular sex hormone known to suppress reproduction to investigate the effects of E_2 injection and red LED on sexual maturation. The results contrasted with those of the CHH analysis. In the group injected with E_2, the VIH mRNA expression showed no significant change. The levels remained stable, however, until 24 h under red LED and then decreased from 48 to 144 h (Fig. 4).

Change in VTG-R mRNA expression

Change in VTG-R, a known indicator of vitellogenesis, were used to investigate the effects of E_2 injection and red LED on vitellogenesis. Result showed that VTG-R mRNA expression was increased in both the E_2 injection and red-light-irradiation groups. The time when VTG-R mRNA expression increased was nevertheless different between both treatment groups, from 0 and 48 h in the E_2 and red LED irradiation groups, respectively. The E_2 injection groups showed a peak value at 18h, which then decreased (Fig. 5).

VTG mRNA expression using in situ hybridization

The gill tissue used for the quality check of the probe did not express any color, and the same results were obtained with the untreated hepatopancreas (Figs. 6A, 6B). *In situ* hybridization of VTG mRNA confirmed that the highest expression of VTG-R mRNA in each experiment occurred at 18 h and 144 h, compared to 3 h. The results indicated that the VTG mRNA expression

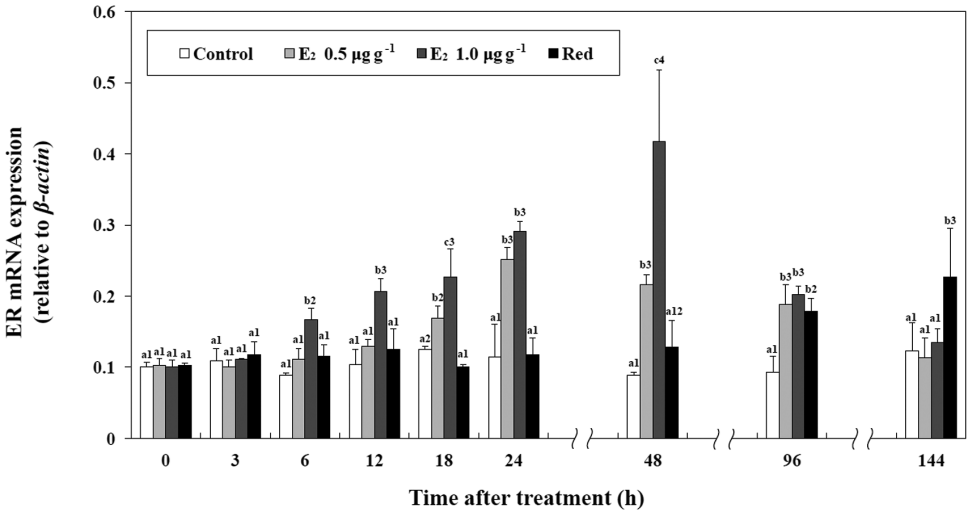

Figure 2. Change in estrogen receptor (ER) gene mRNA expressions in the hepatopancreas of *Lysmatas amboinensis*. Tissue samples were collected 0, 3, 6, 12, 18, 24, 48, 96, and 144 h after 17β-estradiol (E_2) injection or irradiation with red-light-emitting diode (LED). Different lower case letters indicate significant differences in exposure time. Different numbers indicate significant differences between experimental groups using same methods and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$).

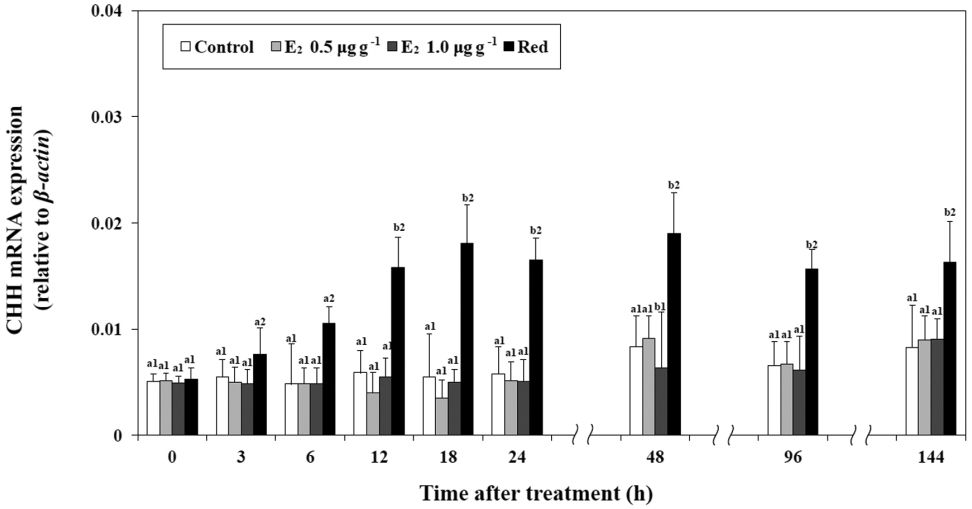

Figure 3. Change in crustacean hyperglycemic hormone (CHH) gene mRNA in eyestalk of *Lysmatas amboinensis*. Tissue samples were collected 0, 3, 6, 12, 18, 24, 48, 96, and 144 h after 17β-estradiol (E_2) injection or irradiation with red-light-emitting diode (LED). Different lower case letters indicate significant differences in exposure time. Different numbers indicate significant differences between experimental groups using same methods and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$).

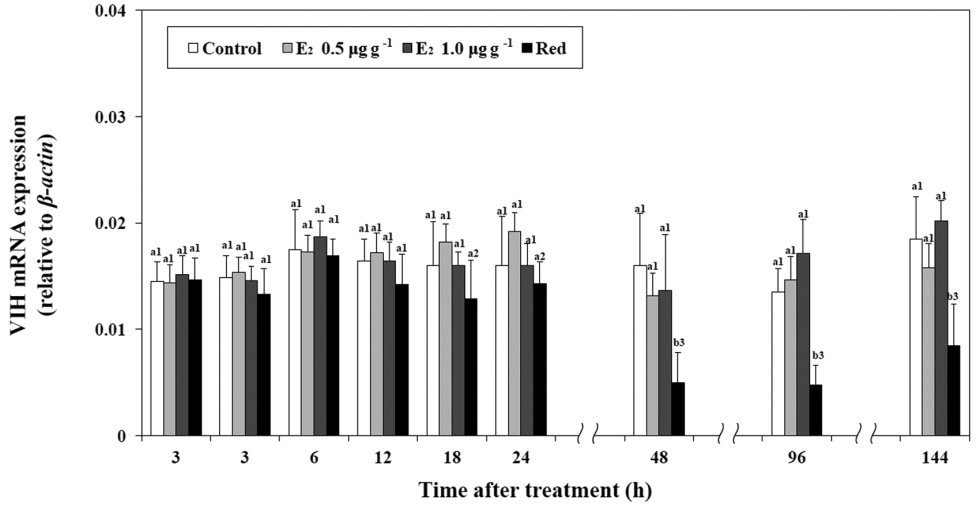

Figure 4. Change in vitellogenesis-inhibiting hormone (VIH) gene mRNA in eyestalk of *Lysmatas amboinensis*. Tissue samples were collected 0, 3, 6, 12, 18, 24, 48, 96, and 144 h after 17β-estradiol (E_2) injection or irradiation with red-light-emitting diode (LED). Different lower case letters indicate significant difference in exposure time. Different numbers indicate significant differences between experimental groups using same methods and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$).

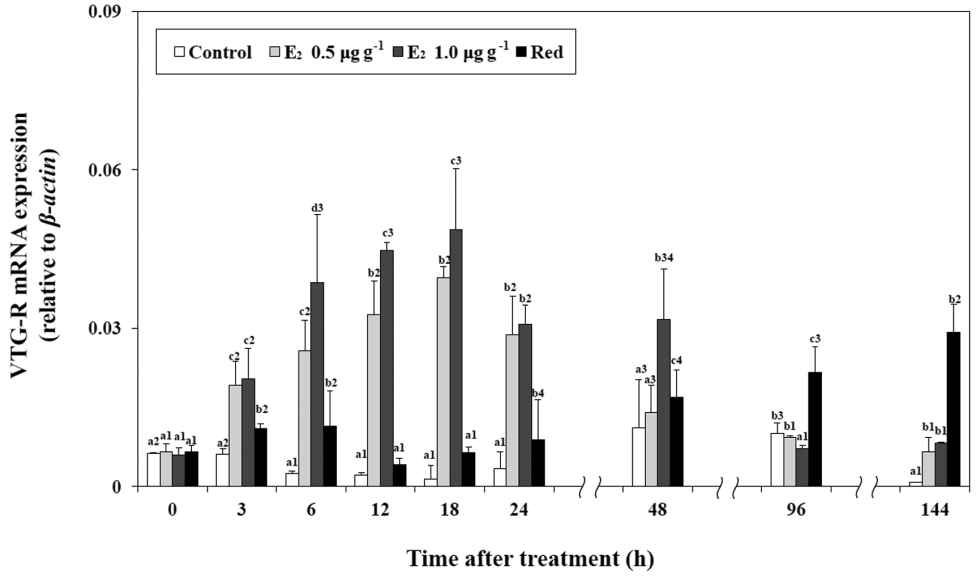

Figure 5. Change in vitellogenin receptor (*VTG-R*) gene mRNA in gonads of *Lysmata amboinensis*. Tissue samples were collected taken at 0, 3, 6, 12, 18, 24, 48, 96 and 144 h after 17β-estradiol (E_2) injection or irradiation with red-light-emitting diode (LED). Different lower case letters indicate significant differences in exposure time. Different numbers indicate significant differences between experimental groups using same methods and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$).

in hepatopancreas tissue was similar to that of VTG-R mRNA expression and was highest at 18 h in the E_2 injection group. A higher level was also observed in the red irradiation group after exposure for 144 h than levels in the E_2 injection group, which showed a tendency to decrease (Fig. 6C).

DISCUSSION

Unlike fishes, crustaceans are known to sexually mature by the activities of various hormones secreted by the XO-SG, a specialized tissue in the eyestalk (Nagaraju, 2011). The eventual discovery of vertebrate-type sex steroid hormones in the hepatopancreas and ovary (Subramoniam, 2000; Guan *et al.*, 2013) suggests that various sexual maturation mechanisms exist. These findings have encouraged further studies on the interaction between sex hormones associated with the sex-related XO-SG. We investigated whether the vertebrate-type sex steroid E_2 and red-light wavelength were effective in controlling eyestalk hormones during sexual maturation and vitellogenesis.

Our results on the effect of E_2 injection and red-wavelength irradiation on the expression of ER mRNA showed it was significantly different between a 0.5 μg g^{-1} treatment during 24 h and 1.0 μg g^{-1} during 6 h. Sexual maturation is known to be regulated by E_2 in vertebrates such as fishes (Pakdel *et al.*, 1991). It has been suggested that sexual maturity is induced by hormones secreted from the eyestalk in crustaceans (Yano *et al.*, 2000); the role of E_2 has not yet been reported in crustaceans. E_2 is closely related to changes in VTG during the ovarian cycle in *Pandalus kessleri* (Czerniavsky, 1878) and *Penaeus monodon* (Fabricius, 1798) (Quinitio *et al.*, 1991). The injection of E_2 in *P. monodon* also stimulated the synthesis of VTG in the ovary and hepatopancreas, and the

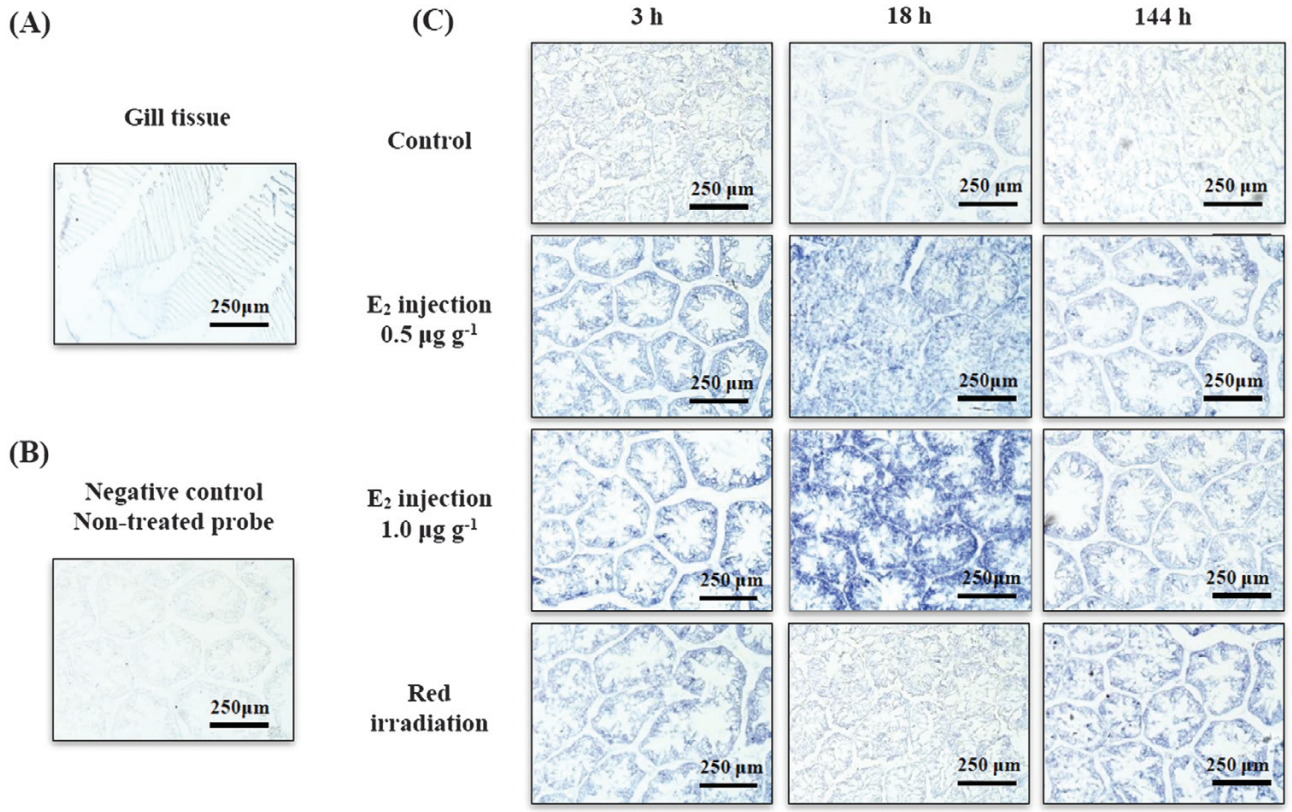

Figure 6. *In situ* hybridization images of vitellogenin mRNA in gill (**A**) and hepatopancreas (**B**, **C**). Gill tissue analysis for quality check of probe (**A**) and non-probe treatment results in hepatopancreas tissue (**B**). Tissue samples were collected 3, 18, and 144 h after E_2 injection or irradiation with red LED (**C**). *In situ* hybridization of vitellogenin mRNA cat 18 and 144h was confirmed to show highest expression of vitellogenin receptor (VTG-R) mRNA in each experiment, compared to 3 h. Scale bars = 250 μm.

ER level as an E_2 receptor was increased. This phenomenon is evidence of maturation (Merlin *et al.*, 2015). The increased ER mRNA in the E_2 injection group observed was considered to have been the result of sexual maturation induced by stimulating E_2. The E_2 injection did not affect the expression level of the hormone secreted by the XO-SG. The sex steroid hormone E_2 thus seems to induce sexual maturation of the hepatopancreas and ovary. The pathways involved in sexual maturation induction by sex steroids including E_2 and hormones secreted by the XO-SG of eyestalks are different for sexual maturation in crustaceans (Subramoniam, 2011). The results suggest that E_2 in *L. amboinensis* used in this study did not affect the expression of VIH and CHH mRNA, and that it directly influenced egg maturation.

The retina receives light signals, and its photoreceptors recognize the color and intensity. Crustaceans nevertheless perceive light from the eyestalk (Aréchiga, 1985), and the light signal is thought to have a significant effect on the synthesis and secretion of hormones secreted in the XO-SG. The red-wavelength light affected the stress reduction and immunity enhancement of *L. amboinensis*, thus enhancing the physiological capacity of the shrimp (Choi *et al.*, 2018). The red-wavelength light has so far been used to minimize stress during the dissection of crustacean samples (Calado, 2009). Our results showed that irradiation of *L. amboinensis* with red light changed the expression level of the hormone secreted from the XO-SG. We observed that from 3 h after irradiation, CHH mRNA tended to increase significantly and that of VIH, which inhibits maturation, tended to decrease significantly. Numerous studies have investigated the effects of CHH as triggers on the development of yolk protein and oocytes maturation in shrimps (De Kleijn *et al.*, 1995). Our study suggests that CHH, which triggers sexual maturation, is also a hormone induced by red wavelength, which is considered to promote sexual maturation. Furthermore, in the group irradiated with the red-wavelength light, the ER-expression level did not increase more rapidly than that of the group injected with E_2, but the ER-expression level increased significantly from 96 h (day 4) after irradiation of the red-wavelength light for up to 144 h (day 6).The increase in ER mRNA expression related to sexual maturation by red-wavelength light is considered to be mediated not only by secretion of the eyestalk hormone but also the secretion and action pathway of E_2 as a steroid hormone. The results of the VTG-R mRNA expression analysis and *in situ* hybridization to VTG mRNA also support the usefulness of E_2 injection and red irradiation as strategies for promoting egg maturation and vitellogenesis.

We suggest that the red-wavelength irradiation method effectively promoted vitellogenesis by regulating mRNA expression of VTG as well as VTG-R by regulating the mRNA expression of eyestalk hormones and ER during maturation. The red-wavelength irradiation method could thus be an efficient novel strategy to replace the maturation method using eyestalk ablation in *L. amboinensis*. It is also expected to be applicable as a technique to replace maturation methods in other shrimps.

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