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EFFECTS OF SPECIFIC WAVELENGTH SPECTRA ON ANTIOXIDANT STRESS AND CELL DAMAGE OF THE ORNAMENTAL CLEANER SHRIMP *LYSMATA AMBOINENSIS* (DE MAN, 1888) (DECAPODA, CARIDEA, LYSMATIDAE) EXPOSED TO CHANGING SALINE ENVIRONMENTS

BY

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ABSTRACT

A variety of environmental factors have a major influence on the survival of aquatic organisms, particularly light and salinity, changes in which lead to a range of physiological changes. In the present study, we investigated the levels of stress caused by changes in salinity in the ornamental cleaner shrimp, *Lysmata amboinensis*, as well as the effect of specific light wavelengths following stress to changes in salinity. We measured the activity of superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LPO) in the tissues (gill, hepatopancreas, and muscle) for three days after irradiating shrimp with specific wavelengths of light [red (630 nm), green (520 nm), and blue (455 nm)] at two intensities (0.5 and 1.0 W/m^2) following exposure to different saline environments [25 practical salinity units (psu), 30 psu, 35 psu (normal seawater), and 40 psu]. DNA damage was measured using comet assays. Although all of the experimental groups exhibited negative results to changes in salinity, the green and red light groups exhibited positive effects when compared to the other wavelengths and fluorescent light. The effect of wavelength was not influenced by the intensity. In conclusion, the light of green and red wavelengths effectively reduces antioxidant stress and cell damage in cleaner shrimp.

Key words. — Antioxidant stress, cell damage, cleaner shrimp, specific-wavelength spectra

RÉSUMÉ

Certains facteurs environnementaux ont une grande influence sur la survie des organismes aquatiques, en particulier les changements de lumière et de salinité qui conduisent à des changements physiologiques. Dans cette étude, nous avons examiné les niveaux de stress causés par des changements de salinité chez la crevette nettoyeuse ornementale, *Lysmata amboinensis*, ainsi que les effets de longueurs d'onde spécifiques après un stress de changement de salinité. Nous avons mesuré l'activité de la super oxyde dismutase (SOD), de la catalase (CAT) et la péroxydation des lipides

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(LPO) dans les tissus (branchies, hépatopancréas et muscles) pendant trois jours après avoir irradié les crevettes avec des longueurs d'onde spécifiques de lumière [rouge (630 nm), verte (520 nm) et bleue (455 nm)], à deux intensités (0.5 et 1.0 W/m^2) à la suite d'une exposition à différentes salinités (25, 30, 35 et 40 psu). Les lésions à l'ADN ont été mesurées par le test des comètes. Bien que tous les groupes expérimentaux montrent des résultats négatifs aux changements de salinité, les groupes lumière verte et rouge montrent des effets positifs par rapport aux autres longueurs d'onde. Les effets de la longueur d'onde n'ont pas été influencés par l'intensité. En conclusion, les longueurs d'onde rouge et verte réduisent effectivement le stress antioxydant et les lésions cellulaires chez une crevette nettoyeuse.

Mots clés. — Stress antioxydant, lésion cellulaire, crevette nettoyeuse, longueur d'onde spécifique

INTRODUCTION

Marine ornamental shrimps are decapod crustaceans that live in tropical coral reefs or small rocky caves. The cleaner shrimp *Lysmata amboinensis* (De Man, 1888) is one of many types of marine ornamental shrimp that inhabit temperate and subtropical waters. These shrimp have a well-established symbiotic relationship with host fish that inhabit nearby ecosystems, removing damaged skin, gills, and parasites from these host fish (Côté, 2000; Becker et al., 2005).

At present, these cleaner shrimp are obtained by collecting them from the ocean, rather than through aquaculture (Martinelli et al., 2008; Biondo, 2017). Furthermore, even though it is a commercially important species (Vaughan et al., 2017), research remains limited on the physiological aspects of this shrimp. To optimize aquaculture technology in order to facilitate the rapidly growing demand of the ornamental ocean life market, it is necessary to combine technology with scientific studies, including the physiological studies of organisms (Wabnitz et al., 2003).

Salinity is one of the environmental factors that must be considered when rearing marine life, as it has a major influence on the physiological changes of shrimp, as well as on their growth and survival (Liu et al., 2006; Robles et al., 2014). In general, changes in salinity that occur in fish also cause stress, leading to a physiological stress reaction, in parallel to negatively influencing resistance to disease, growth, and breeding success (Barton et al., 1991; Huang et al., 2015). In the case of shrimps, only basic studies have reported how changes in salinity affect growth, respiration, and survival rates (Ponce-Palafox et al., 1997; Roy et al., 2007). No studies have been conducted on shrimp subjected to physiological stress.

Environments with rapid changes in salinity cause oxidative stress in aquatic organisms (Bagnyukova et al., 2006). In general, fish have a defensive system that enhances the activity of antioxidant enzymes in the body to protect them from reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide

(H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$), with this response being accompanied by increased oxidative stress (Roch, 1999). Representative antioxidant enzymes that are activated include superoxide dismutase (SOD), which converts O_2^- to H_2O_2, and catalase (CAT), the H_2O_2 then being converted to non-toxic H_2O and O_2 to prevent the accumulation of H_2O_2 in cells and tissues (Basha & Rani, 2003). However, reactive oxygen species are created in the body as a result of stress factors in the external environment that cause the degeneration of nucleic acid and protein structures, as well as a serious loss of function, reducing the resistance of organisms to disease and limiting reproductive output (Oldham & Bowen, 1998; Pandey et al., 2003).

Light is another important environmental factor that has a large impact on the endocrine system of almost all organisms on Earth (Jin et al., 2009). Light is generally accepted by the light source in the form of photoreceptors in the retina, and is converted to electrical signals in the body to elicit physiological responses from each tissue (Migaud et al., 2006). Recent studies have investigated how light causes physiological changes to organisms, such as the promotion of maturity by short wavelengths and the reduction of stress-related factors, using light-emitting diodes (LED) that only emit light of specific wavelengths (Shin et al., 2011). Studies have also investigated how to minimize oxidative stress in aquatic organisms. For instance, specific wavelengths promote an effective reduction of oxidative stress in fish (Shin et al., 2011; Kim et al., 2014). Furthermore, results showed that light from specific wavelengths has positive effects on the physiology of fish, including the reduction of stress and enhancing immune control (Shin et al., 2011; Choi et al., 2015).

Among ornamental organisms, crustaceans are sensitive to light (Covich et al., 2010). However, photoreaction studies of shrimp have only reported a degree of sensitivity to light of green wavelengths by cleaner shrimp (518 $\pm$ 5 nm) (Caves et al., 2016). To date, no studies have been performed on how the physiological responses of shrimp differ in relation to irradiation at different wavelengths.

Therefore, this study aimed to investigate how specific wavelengths and intensities of light regulate changes in salinity stress in the cleaner shrimp, *L. amboinensis*. We exposed cleaner shrimp to four salinity environments [(25, 30, 35, and 40 practical salinity units (psu)] at three different wavelengths (red, 630 nm; green, 520 nm; blue 455 nm) and two intensities (0.5 and 1.0 W/m^2) for three days. Subsequently, we analysed the oxidative stress response (SOD, CAT) and lipid peroxidation (LPO) activity in the tissues. The effect of stress on the extent of cell damage at different light wavelengths was confirmed by comet assays through evaluating the degree of damage to nuclear DNA. Our results are expected to provide new insights in the optimal aquaculture rearing conditions of shrimp to meet the demands of the ornamental marine life market.

MATERIAL AND METHODS

Experimental species and conditions

For each experiment, cleaner shrimp *Lysmata amboinensis* [total number of shrimp used in the experiment: $n = 560$; length, 42.8 ± 2.3 mm; weight, 2.23 ± 0.15 g] were purchased from a commercial aquarium (Choryang, Busan, Korea). The shrimp were allowed to acclimate for two weeks in nine 300-L circulation filter tanks [a tank consists of four mini tanks (size: 45 cm × 45 cm × 45 cm)] in the laboratory. The shrimp were reared using automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea). Each filter tank (each experimental group) contained 20 individuals (each mini tank contained five individuals) and water conditions were maintained at 22°C, pH 8.0 and 35 psu, with exposure to a white fluorescent bulb.

During the experimental period, the shrimp were transferred to each filter tank [each experimental group: 25, 30, 35 (normal seawater) and 40 psu], with a total of 20 individuals in each tank (each mini tank contained five individuals).

The shrimp in the control group were exposed to a white fluorescent bulb. For the experimental groups, the shrimp were exposed to red (630 nm), green (520 nm), or blue (455 nm) LEDs (Daesin LED Co., Kyunggi, Korea) (fig. 1). The LEDs were placed 20 cm above the water surface and the depth of the bottom water layer was 40 cm. The irradiance level at the bottom layer of tanks with external light interception was maintained at approximately 0.5 or 1.0 W/m^2 using a spectrometer (MR-16; Rainbow Light Technology Co. Ltd., Taoyuan, Taiwan) and a photo-radiometer (HD 2102.1; Delta OMH CO., Caselle di Selvazzano, Italy).

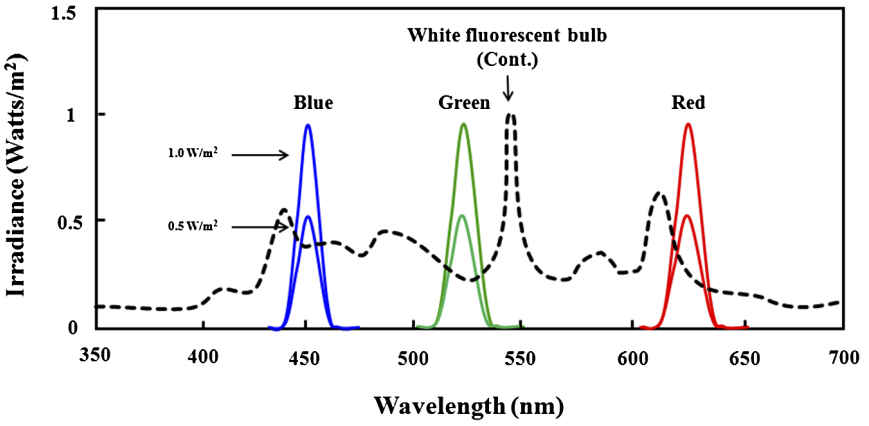

Fig. 1. Spectral profiles of the red, green and blue light emitting diodes (LEDs) and white fluorescent bulb (Control) used in this study. Different light intensities (0.5 and 1.0 W/m^2) were used for each type of LED in this study.

The photoperiod consisted of a 12-h light /12-h dark cycle, with the photophase lasting from 07:00 to 19:00 h (the lights were turned on at 07:00 and turned off at 19:00).

For all experiments, the shrimp were given commercial feed (Topmarine, Gyeongna, Tabia, Korea) daily until the day before sampling. The shrimp were anaesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, U.S.A.) to minimize stress before collecting the tissues [gill, hepatopancreas, and muscle (abdominal muscle)]. The tissues were homogenated with 500 μL PBS (Sigma) and centrifuged (4°C, 1000× g, 15 min.). Then, the supernatant was removed and the samples were stored at −20°C until analysis. The tissues were removed from the shrimp, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Analysis of SOD and CAT concentrations

SOD analysis of cleaner shrimp tissues was performed by homogenizing the tissues at a ratio of 300 mg/1 ml (1× PBS, pH 7.3) and separating the supernatant through centrifugation (14 000× g, 20 min., 4°C). After each SOD reagent was dispensed into each well, standard Cu/Zn SOD solution and the samples were dispensed into each well. Then, xanthine oxidase solution [xanthine oxidase 2.0 ml (Sigma) + ice-cold 2 mol/L ammonium sulphate (Sigma)] was added and the mixture was incubated in a water bath at 25°C for 20 min. The $CuCl_2$ solution was removed to terminate the reaction, and the remaining mixture was allowed to incubate for 20 min. The SOD concentration was measured at 560 nm and the concentration was measured using a standard curve.

The CAT concentration in each tissue was measured using the assay kit [CAT (catalogue no. 707002; Cayman, Ann Arbor, Michigan, U.S.A.)]. Each tissue was homogenized with a buffer as recommended in the experimental method, and the supernatant was separated through centrifugation. CAT analysis was carried out using the experimental method given by the company. The concentration was measured at 455 nm and the activity was calculated.

Analysis of LPO concentration

The LPO concentration in each tissue was measured using the assay kit [LPO (catalogue no. 10009055; Cayman)]. Each tissue was homogenized with a buffer as recommended in the experimental method, and the supernatant was separated through a centrifuge. LPO analysis was carried out using the experimental method given by the company. The concentration was measured at 500 nm and the activity was calculated.

Comet assays

The comet assay is a relatively simple and sensitive technique used to measure DNA damage to eukaryotic cells quantitatively (Bajpayee et al., 2005). Hepatopancreas cells (1×10^5 cells/mL) were examined using a CometAssay Reagent kit with single-cell gel electrophoresis assays (Trevigen Inc, Gaithersburg, MD, U.S.A.), according to the method described by Singh et al. (1988), with some modifications. Cells were immobilized in agarose gels on CometAssay comet slides and immersed in freshly prepared alkaline unwinding solution for 20 min. Next, slides were electrophoresed at 18 V for 30 min. The samples were stained with SYBR Green (Trevigen Inc.) for 30 min. in the dark and then read using a fluorescence microscope (excitation filter 465-495 nm; Eclipse Ci, Nikon, Japan). At least 100 cells from each slide were analysed. To quantify the comet assay results, we analysed the tail length (distance of DNA migration from the head), percentage of DNA in the tail (tail intensity/total intensity in tail), and tail moment (amount of DNA damage, product of tail length and percentage of DNA in tail) using comet assay IV image analysis software (version 4.3.2; Perceptive Instruments Ltd., U.K.).

Statistical analysis

All data were analysed using the SPSS statistical package (version 19.0; SPSS Inc., U.S.A.). 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 means $\pm$ standard error (SE). The n represents the number of shrimp used in the statistical analysis.

RESULTS

Changes to SOD and CAT concentrations

Compared to the SOD and CAT concentrations of the shrimp, *Lysmata amboinensis*, in the group at 35 psu (normal seawater), the SOD and CAT concentrations of the antioxidative enzymes in all the tissues (gill, hepatopancreas and muscle) of the experimental groups (25, 30 and 40 psu) significantly increased with increasing exposure time (figs. 2 and 3). The highest SOD and CAT levels were obtained at 25 psu. When the light source was present, SOD and CAT levels significantly declined at red and green LED wavelengths when compared with the control group. In comparison, SOD and CAT concentrations generally increased significantly in the blue LED group. Thus, there was no difference in the intensity of light or of the pattern between the tissues with respect to SOD and CAT concentrations; however, the highest SOD and CAT concentrations were detected in the hepatopancreas.

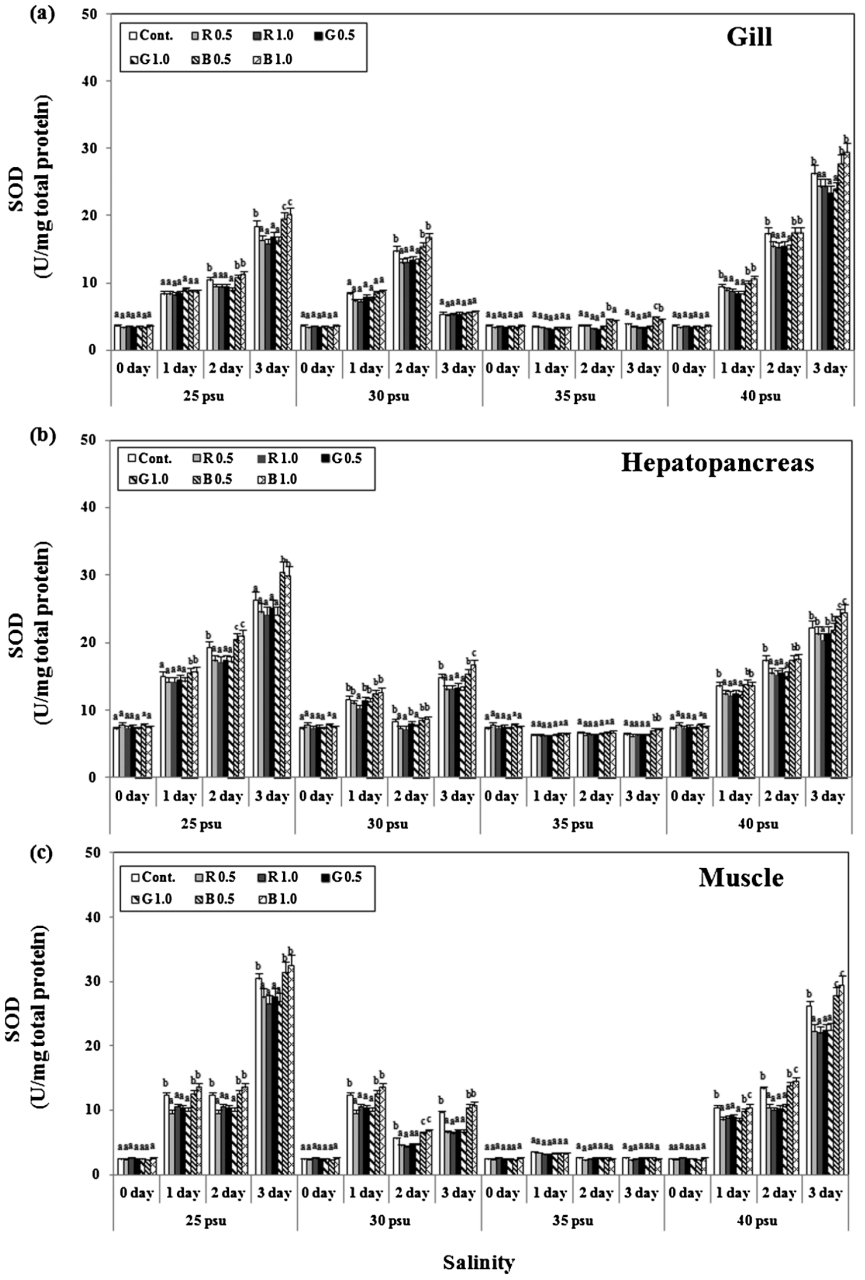

Fig. 2. Concentrations of SOD in, a, the gill; b, hepatopancreas; and, c, muscle of *Lysmata amboinensis* (De Man, 1888) exposed to environments of different salinities under different light conditions [red (R), green (G), and blue (B) LED with irradiance at approximately 0.5 and 1.0 W/m^2 and a white fluorescent bulb (Cont.)]. SOD was analysed with a plate reader. Values with letters indicate significant differences among lights of different wavelengths over the same salinity and period ($P < 0.05$). All values are means ± SE ($n = 5$).

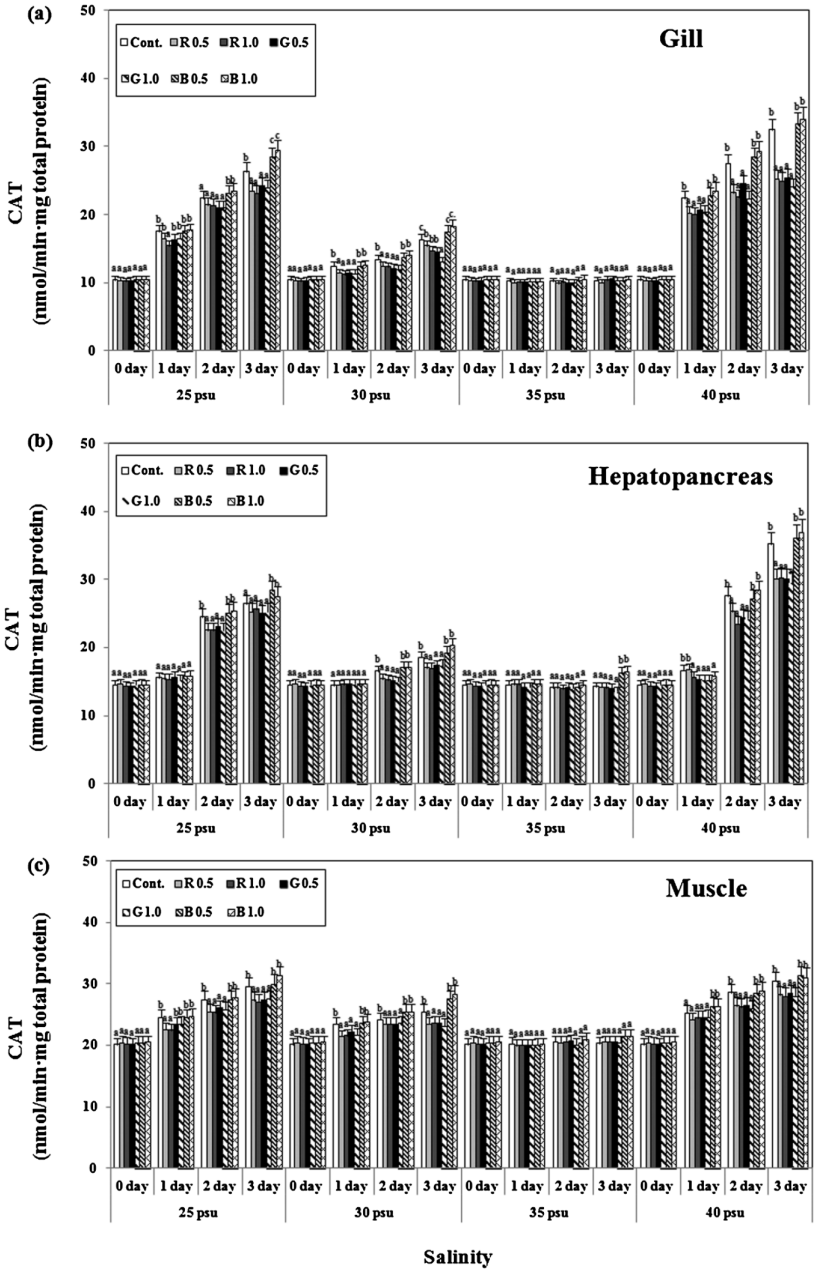

Fig. 3. Concentrations of CAT in, a, the gill; b, hepatopancreas; and, c, muscle of *Lysmata amboinensis* (De Man, 1888) exposed to environments of different salinities under different light conditions [red (R), green (G) and blue (B) LED with irradiance at approximately 0.5 and 1.0 W/m^2 and a white fluorescent bulb (Cont.)]. CAT was analysed with a plate reader. Values with letters indicate significant differences among lights of different wavelengths over the same salinity and period ($P < 0.05$). All values are means ± SE ($n = 5$).

Changes in LPO concentrations

Compared to the 35 psu (normal seawater) group, the LPO concentration of the antioxidative enzyme in all of the tissues (gill, hepatopancreas, and muscle) of the experimental groups (25, 30, and 40 psu) significantly increased with exposure time (as compared to normal seawater) (fig. 4). As a result, mRNA and LPO levels increased significantly with changes in salinity in all experimental groups. The mRNA and LPO levels in the red and green LED irradiation groups were significantly lower than those of the control groups with white fluorescent bulbs. There were no significant differences either in mRNA and LPO levels between 0.5 and 1.0 W/m^2 of the red and green LED irradiation groups. However, the mRNA and LPO levels in the blue LED groups significantly increased with increasing light intensity ($0.5 \rightarrow 1.0$ W/m^2).

Analysis of DNA damage

DNA damage to hepatopancreas tissue following 3 days of exposure to various salinities (25, 30, 35, and 40 psu) was analysed using 100 randomly selected cells. Compared to the 35 psu (normal seawater) group, the DNA content of the tail and the length of the tail in the experimental groups (25, 30, and 40 psu) increased significantly with increasing exposure time (fig. 5). When the light source was observed, DNA damage was significantly lower in the group exposed to red and green LED wavelengths when compared to the control group. In comparison, DNA damage tended to increase significantly in the blue LED group.

DISCUSSION

Although the cleaner shrimp, *Lysmata amboinensis*, is commercially important in the ornamental fish industry, only a few studies have been conducted on their sexual maturity and ecology (Tziouveli et al., 2011; Rui et al., 2014). Cleaner shrimp are usually raised indoors as ornamental animals, resulting in their exhibiting sensitive responses to changes in the environment (light, salinity, temperature, pH, etc.). Therefore, it is important to conduct studies on the physiological responses of shrimp to various breeding environments.

Our study showed that SOD and CAT activity in the gills, hepatopancreas, and muscle of shrimp exposed to various salinity environments (25, 30, 35, and 40 psu) was significantly higher in the experimental groups (25, 30, and 40 psu) compared to the control group (35 psu). In particular, SOD and CAT activity increased significantly with increasing exposure time. In general, animals exposed to oxidative stress produce reactive oxygen species (ROS), which cause fatal

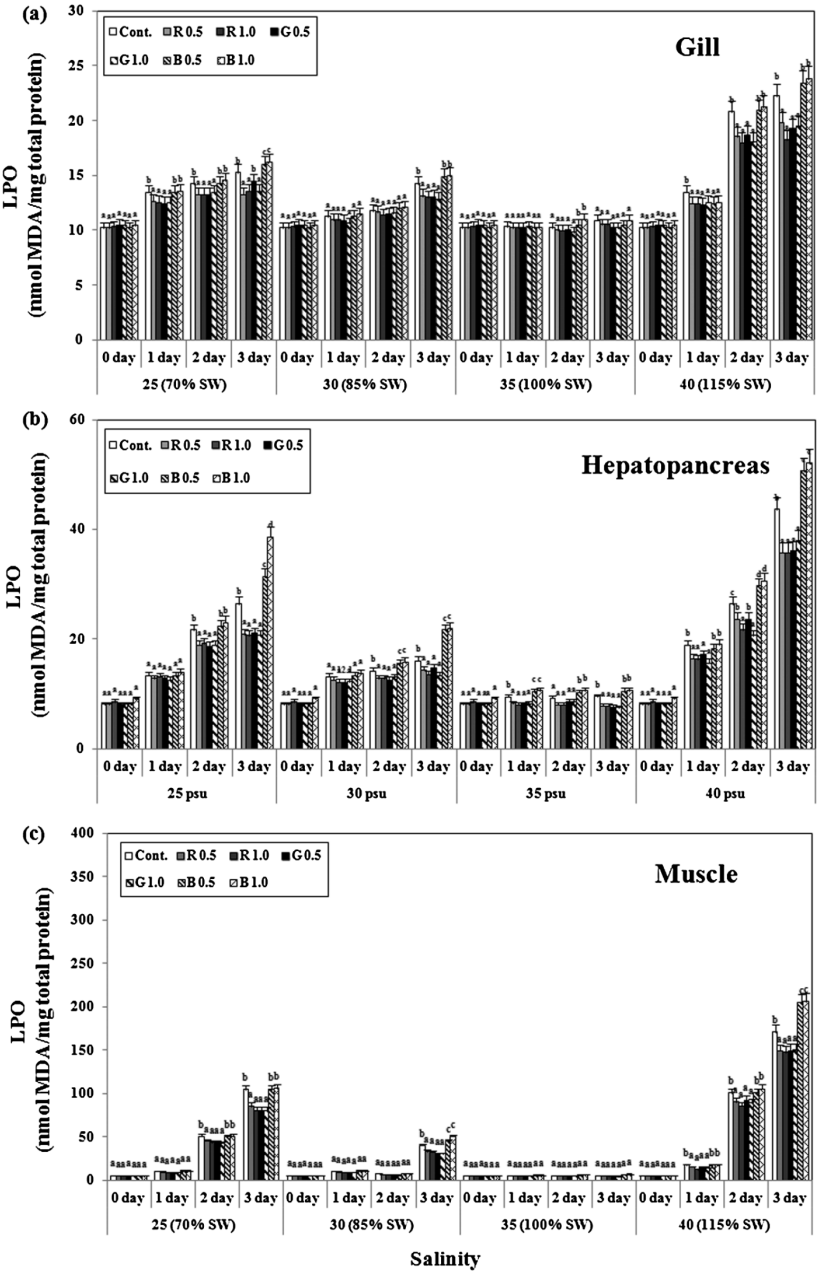

Fig. 4. Concentrations of LPO in, a, the gill; b, hepatopancreas; and, c, muscle of *Lysmata amboinensis* (De Man, 1888) exposed to environments of different salinities under different light conditions [red (R), green (G), blue (B) LED, with irradiance at approximately 0.5 and 1.0 W/m^2 and a white fluorescent bulb (Cont.)]. LPO was analysed with a plate reader. Values with letters indicate significant differences among lights of different wavelengths over the same salinity and period ($P < 0.05$). All values are means $\pm$ SE ($n = 5$).

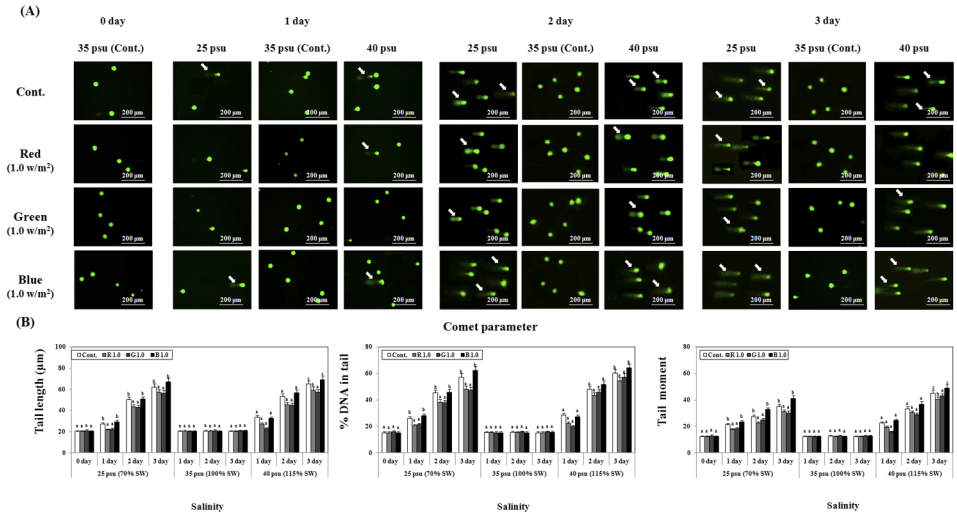

Fig. 5. Comet assay images following initial conditions (at day 0) and different salinity conditions (25, 30, 35, and 40 psu after 1, 2, and 3 days) under different light conditions [white fluorescent bulb (Cont.), red (R), green (G), blue (B); 1.0 W/m^2] in the hepatopancreas cells of *Lysmata amboinensis* (De Man, 1888). White arrows in (A) indicate damaged nuclear DNA (DNA breaks) in the hepatopancreas cells, which were stained using SYBR Green. Scale bars = 200 μm. Lower-case letters in (B) represent significant differences within the same experimental group and within the same salinity treatment ($P<0.05$).

damage to cells (Roch, 1999; Nordberg & Arner, 2001). Therefore, organisms protect themselves by secreting antioxidant enzymes into the body to prevent damage by ROS (Rui et al., 2014). Liu et al. (2007) confirmed that SOD and CAT activity increases when whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931) [currentlyalso, again, referred to as *Penaeus vannamei* Boone, 1931] are exposed to low salinity environments (5, 15 and 30 psu). Park et al. (2011) reported that H_2O_2, one of the ROS, significantly increases in the cinnamon clownfish *Amphiprion melanopus* Bleeker, 1852, a seawater aquarium fish, exposed to low salinity environments (17.5 psu), while SOD and CAT activity significantly increased to detoxify H_2O_2. In the present study, SOD and CAT activity was higher in the group of the experiment involving salinity change, whereas SOD and CAT activity was lower in the red and green wavelength groups. Thus, red and green wavelengths effectively reduce the oxidative stress in cleaner shrimp, regardless of the intensity of light.

When ROS is overproduced in vivo due to various environmental stress factors, it induces lipid peroxidation (LPO) in cells in order to inactivate cell membrane damage and enzymes. The oxidation of DNA bases also damages DNA and proteins, ultimately having a negative effect on cell survival (Sikka et al., 1995; Pandey et al., 2003). Rodrigues et al. (2012) recorded a significant increase in LPO

activity in the salinity change experiment group (8 psu) compared to the control group (14 psu) for the European green shore crab, *Carcinus maenas* (Linnaeus, 1758) after 7 days of exposure to a changing salinity environment. The authors confirmed that LPO was highly expressed in the salinity change experiment group. In the present study, LPO activity was higher in the salinity change experiment group, but was lower in the red and green wavelength groups. Unfortunately, no other studies have investigated the positive effects of light wavelengths on salinity change induced stress in crustaceans, with only reports on fish being available. In the current study, changes in salinity caused stress in *L. amboinensis*, indicating that SOD, CAT, and LPO activity are induced in the tissues of the cleaner shrimp. In addition, SOD, CAT, and LPO activity in cleaner shrimp declined under the red and green wavelengths of the control group in the salinity control experiment. Therefore, exposure of cleaner shrimp to red and green wavelengths seems to have a positive effect at reducing stress caused by changes in salinity.

To determine the effective light wavelength and intensity for controlling stress caused by changing salinity, the cleaner shrimp exposed to the salinity change environment were assessed at three wavelengths and two intensities for each wavelength. The degree of nuclear DNA damage in the hepatopancreas cells of the shrimp was observed following exposure. Similar to our study, Goedken et al. (2005) found that the exposure of the eastern oyster *Crassostrea virginica* (Gmelin, 1791) to low salinity groups (17.1 psu) increased the level of apoptosis when compared to the control group (28.4 psu). Kim et al. (2016) also confirmed that the expression level of caspase-3 mRNA, which is closely associated with apoptosis, increased when the olive flounder *Paralichthys olivaceus* (Temminck & Schlegel, 1846) was exposed to high temperatures (25 and 30°C) versus the control (20°C). For olive flounder exposed to high temperatures, green LED wavelengths caused the expression of caspase-3 mRNA to decrease. Similar to what was found in previous studies, cell damage increased as a result of exposing cleaner shrimp to changes in salinity in the current study. However, cell damage significantly decreased under the red and green LED wavelengths. Thus, red and green wavelengths might have the indirect function of suppressing the degree of nuclear DNA damage affecting the response of cells to damage. In general, oxidative stress induces apoptosis and promotes damage to DNA (Lesser et al., 2001). Thus, oxidative stress might be associated with DNA damage, with the increased oxidative stress of cleaner shrimp exposed to salinity change, inducing DNA damage.

In conclusion, the results of this study showed that light of red and green wavelengths reduces the oxidative stress of cleaner shrimp, and might be effective in increasing antioxidant levels and immunity at low light intensity (0.5 W/m^2). In comparison, the blue wavelength induced oxidative stress, which increased as

the intensity of light increased (0.5 → 1.0 W/m^2). Furthermore, red and green wavelengths inhibited apoptosis, whereas blue wavelengths might contribute to apoptosis. The results of this study revealed the photoreaction effect of crustaceans; however, the mechanisms of photochemical reactions (including the role of the red wavelength) in crustaceans require clarification in future studies. In conclusion, this study is the first to investigate the physiological effects of specific light wavelengths on cleaner shrimp exposed to salinity change environments. Our results are expected to provide baseline data on the photoreactions of crustaceans, which could be used to select the optimum wavelength and salt concentrations for the aquaculture of cleaner shrimp, in particular *Lysmata amboinensis*.

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