Journal of Crustacean Biology Advance Access published 19 July 2018

The Crustacean Society

Journal of Crustacean Biology

Journal of Crustacean Biology 38(4), 475–482, 2018. doi:10.1093/jcbiol/ruy047

Effects of specific light wavelengths on osmotic stress in the ornamental cleaner shrimp *Lysmata amboinensis* (De Man, 1888) (Decapoda: Caridea: Lysmatidae)

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(Received 12 January 2018; accepted 11 May 2018)

ABSTRACT

We investigated the effects of particular wavelengths of visible light on salt-induced stress in the ornamental cleaner shrimp *Lysmata amboinensis* (De Man, 1888). Na^+/K^+-ATPase (NKA) activity and levels in tissues (gill, hepatopancreas, and muscle) of heat shock protein 70 (HSP70) and levels of glucose in whole-body fluids were measured for three days after the shrimp were exposed to red (630 nm), green (520 nm), and blue (455 nm) wavelengths of light at two intensities (0.5 and 1.0 W m^{-2}) while exposed to various salinities (25, 30, 35, and 40 PSU). The osmolality of whole-body fluids and the nuclear damage in hepatopancreas tissues were also measured. Although all experimental groups showed negative responses to changes in salinity, the groups exposed to green and red wavelengths of light showed milder negative effects than the groups exposed to blue and fluorescent light. There was no difference in the effect of different intensities of light, but green and red light effectively reduced the osmotic stress caused by salinity.

Key Words: heat shock protein, Na^+/K^+-ATPase, physiology, salinity, stress response

INTRODUCTION

Marine ornamental shrimps, which inhabit tropical coral reefs, are economically important in the aquarium industry (Sanjeevi *et al.*, 2016) and an important source of income in the Indo-West Pacific region (Calado *et al.*, 2009). *Lysmata amboinensis* (De Man, 1888) is a popular ornamental shrimp known for its bright colors and striking patterns (Calado *et al.*, 2003). This species inhabits temperate and sub-tropical waters and establishes a cleaning symbiotic relationship (Côté, 2000) with fishes, removing parasites, bacteria, and damaged tissues. Cleaner shrimps are expensive and they are primarily traded from naturally harvested stocks (Biondo, 2017), despite their ecological importance (Vaughan *et al.*, 2017). Studies on the physiology of *L. amboinensis*, however, have rarely been conducted.

Marine animals that are bred for ornamental purposes, such as cleaner shrimps, are likely to be exposed to a variety of changes in their environment due to inexperienced management of breeding aquaria. Salinity, one of the primary factors to consider when rearing marine organisms, influences the physiology of shrimps as well as their growth and survival (Liu *et al.*, 2006; Robles *et al.*, 2014). Ponce-Palafox *et al.* (1997) and Roy *et al.* (2007) reported the negative effects of rapid changes in salinity on growth, respiration rate, and survival in shrimps. Although studies on the physiological responses to temperature changes (Rui *et al.*, 2014), starvation (Calado *et al.*, 2009), and diet (Calado *et al.*, 2003) have been conducted in *L. amboinensis*, few have investigated the effects of rapid changes in salinity.

Aquatic organisms exposed to sudden changes in salinity generally undergo metabolic responses to maintain ion homeostasis (Choi & An, 2008). Osmotic pressure and ion homeostasis in crustaceans are based on ion transport (mainly Na^+ and Cl^- absorption or secretion), and Na^+/K^+-ATPase (NKA) is highly influential in ion transport (Chaudhari *et al.*, 2015). NKA plays an important role in maintaining intracellular homeostasisas well as in facilitating most transport systems in various osmotic epithelial cells, including in the gills (McCormick, 1995; Li *et al.*, 2015).

Glucose is used as an energy source for tissue damaged by environmental stress, and its concentration in the body is used as a biomarker that reflects the degree of external environmental stress (Hall & van Ham. 1998; Lorenzon, 2005). Glucose secretion in crustaceans is controlled by the crustacean hyperglycemic hormone (cHH), which is secreted from the eyestalk (Kamemoto & Oyama, 1985; Webster, 2015)

Heat shock protein (HSP) is one of the chaperone proteins involved in refolding the denatured proteins to protect the structural integrity of intracellular substances (Kregel, 2002). HSP was initially discovered in fruit flies exposed to thermal shock

environments and the protein became known as a heat-tolerant gene (Ritossa, 1962). HSP was subsequently reported to be involved in stress responses, the prevention of cell damage due to environmental pollutants, and the maintenance of cellular homeostasis, and was consequently used as an important *in vivo* stress indicator in other animals (Mudagandur *et al.*, 2016).

The solar light spectrum is absorbed by water molecules and the spectral composition decreases with depth. The optimal light spectrum for aquatic organisms, including crustaceans, thus varies with depth (Myrberg & Fuiman, 2002). Ornamental aquatic crustaceans, however, are often exposed to the artificial lighting installed in indoor aquaria. Light is received as an optical signal mainly via photoreceptors in the retina and induces physiological responses in the tissues when converted into electrical signals (Migaud *et al.*, 2006). Studies to reduce light-induced stress in aquatic animals have been conducted, specifically on the effect of particular wavelengths on stress regulation in fishes (Shin *et al.*, 2011; Kim *et al.*, 2014).

Crustaceans are more sensitive to weak light than fishes (Covich *et al.*, 2010). Studies on crustaceans have shown changes in growth (Guo *et al.*, 2011) and food intake (Xu *et al.*, 2003) when they are exposed to specific light wavelengths. Donohue *et al.* (2017) confirmed the presence of the photoreceptor opsin in the central nervous system (SCN) of the stomatopod *Neogonodactylus oerstedii* (Hansen, 1895). Caves *et al.* (2016) studied optical responses and sensitivity to light wavelengths in *L. amboinensis* and reported that this species had the highest sensitivity to green light (518 ± 5 nm). No research has yet examined the role of light of other wavelengths in the regulation of physiological responses in this species.

We investigated the effects of specific light wavelengths and intensities of light on the regulation of salinity stress in *L. amboinensis*. We exposed shrimp to three wavelengths (red (630 nm), green (520 nm), or blue (455 nm) light-emitting diodes (LEDs)) at two intensities (0.5, 1.0 W m^{-2}) for three days in four osmotic environments (25, 30, 35, and 40 PSU (practical salinity units)). We also investigated changes in homeostatic function, physiological stress hormone levels (NKA and HSP70), and glucose concentration, and compared degrees of nuclear DNA damage using comet assays.

MATERIALS AND METHODS

Experimental animals and laboratory conditions

Individuals of *Lysmata amboinensis* (N = 336; mean length, 42.8 ± 2.3 mm; mean weight 2.23 ± 0.15 g) were purchased from a commercial aquarium (Choryang, Busan, Korea) and allowed to acclimatize for two weeks in nine 300 l closed-circulation-filter tanks, each tank consisting of four mini tanks (45 cm × 45 cm × 45 cm), in the laboratory. Shrimp were reared using automatic temperature-regulation systems (JS-WBP-170RP; Johnsam, Seoul, Korea). The water conditions were maintained at 22 °C, pH 8.0, and 35 psu. Each filter tank (experimental groups at 25, 30, 35 (normal seawater), and 40 psu] consisted of 20 individuals. To minimize density stress between individuals, each mini tank was divided into five equal spaces, one per shrimp.

Shrimp in the control group were exposed to a white fluorescent bulb. Those in the experimental groups were exposed to either red (630 nm), green (520 nm), or blue (455 nm) LEDs (Daesin LED., Kyunggi, Korea) (Fig. 1). The LEDs were placed 20 cm above the water surface and the depth of the water was 40 cm. The irradiance level at the bottom layer of each tank with external light interception was maintained at approximately 0.5 W m^{-2} or 1.0 W m^{-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 photoperiod consisted of a 12 h light/dark cycle, with the photophase lasting from 0700 to 1900.

All experimental shrimp were fed with commercial feed (Tropical Tadeusz Ogrodnik, Chorzów, Poland) daily until the day prior to the experiments. The shrimp were anesthetized using 2-phenoxyethanol (Sigma, St. Louis, MO, USA) to minimize stress prior to tissue collection. Tissue was homogenized using 500 µl PBS (Sigma) and centrifuged (4 °C, 1000 × *g*, 15 min). We removed only the supernatant and stored it at –20 °C until analysis. The tissues were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

Analysis of NKA concentration in gill tissues

Gill tissues (20 mg) were immersed in SEI solution (200 mM sucrose, 5 mM Na_2 EDTA, and 100 mM imidazole-HCl buffer, pH 7.6) and then homogenized in 1 ml SEID solution (SEI + 0.1% sodium deoxycholate). Ten microliters of the supernatant obtained using centrifugation (4 °C, 2000 × g, 5 min) was added to reaction mixture A (125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, 5 mM Na_2 ATP, and 100 mM Imidazole-HCl buffer, pH 7.6) and 200 µl of reaction mixture B (mixture A + 10 mM ouabain). After incubation at 37 °C for 30 min, 50 µl of 30% trichloroacetic acid were added to stop the reaction. The content of inorganic phosphorus with and without ouabain was measured at 650 nm using a Phosphate Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). Protein content in tissues was measured using a Pierce BCA Protein Kit (Thermo, Waltham, MA, USA).

Analysis of glucose levels

Body fluids from whole bodies of shrimp were used as it was difficult to separate the hemolymph. Tissue preparation followed the

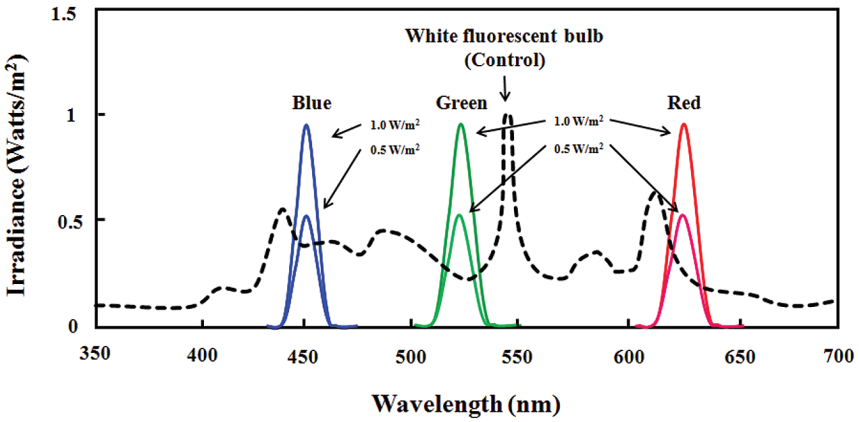

Figure 1. Spectral profiles of the red, green, and blue light-emitting diodes (LEDs) and white fluorescent bulb (control) used in the study. Two different light intensities (0.5 and 1.0 W m^{-2}) were used for each type of LED. This figure is available in colour at *Journal of Crustacean Biology* online.

method of Njemini *et al.* (2005) with modifications. The whole body was homogenized using 500 µl PBS (Sigma) and centrifuged (4 °C, 1000 × g, 15 min). We used the supernatant for glucose measurement using a dry multiplayer analytic-slide method using a biochemistry auto analyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

Analysis of HSP concentration in tissues

The HSP concentration in the tissues was analyzed using an ELISA Starter Accessory Kit (E101; Bethyl, Montgomery, TX, USA) following a modified method of Njemini *et al.* (2005). After homogenization, we added 100 mg of tissue to 1 × PBS and centrifuged (4 °C, 5000 × g for 5 min) to separate the supernatant. Standards and samples were dispensed and coated at 4 °C for at least 14 h. The coated wells were washed with washing buffer and treated with blocking buffer, primary antibody (1:4000), and secondary antibody (1: 4000). Washing was performed after each reaction using a washing buffer, and 100 µl of TMB substrate was dispensed into each well, reacted at room temperature for 30 min at 250 rpm, and stopped by adding 100 µl of stop solution. The final HSP activity was expressed as µM Pi^{-1} mg $protein^{-1}$ h^{-1}. The HSP concentration was measured at 450 nm absorbance and the concentration was expressed as ng mg^{-1} total protein using a standard curve.

Comet assays

The comet assay is a relatively simple and sensitive technique for quantitatively measuring DNA damage in eukaryotic cells (Bajpayee *et al.*, 2005). Hepatopancreas cells (1 × 10^5 $cells^{-ml}$) were examined using a CometAssay Reagent kit and single-cell gel electrophoresis (Trevigen, Gaithersburg, MD, USA) 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. The slides were then electrophoresed at 18 V for 30 min. The samples were stained using SYBR Green (Trevigen) for 30 min in the dark and then examined using a fluorescence microscope (Eclipse C*i*, excitation filter 465–495 nm; Nikon, Tokyo, Japan). At least 100 cells from each slide were analyzed. To quantify the comet assay results, we analyzed the tail length (distance of DNA migration from the head), % DNA in the tail (tail intensity/total intensity in tail), and tail moment (amount of DNA damage, product of tail length and % DNA in tail) using comet assay IV image analysis software (version 4.3.2; Perceptive Instruments, Bury St Edmunds, UK).

Statistical analysis

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

RESULTS

Change in NKA activity

NKA activity in the gill tissues of the experimental groups (25, 30, and 40 psu) on day 1 was higher than on day 0, and then decreased significantly with time (from day 0 to day 3) in all light groups except the blue LED group (Fig. 2). The lowest NKA activity was measured at 40 psu. NKA activity decreased more with time under red and green LED wavelengths than it did in the control group (white fluorescent bulb). Conversely, NKA activity was maintained over time under the blue LED wavelength. The responses were not different between the two light intensities.

Changes in glucose levels

Glucose levels in whole-body samples from the experimental groups (25, 30, and 40 psu) on day 1 were higher than they were on day 0, and then increased significantly with time (from day 0 to day 3) in all light groups (Fig. 3). The highest glucose level was measured at 25 psu. When the light source was included as a factor, glucose levels increased less with time under red and green LED wavelengths than they did under the control group's white fluorescent bulb. Conversely, glucose levels increased more with time under the blue LED wavelength. The responses also showed no differences between the two light intensities.

Changes in HSP70 concentration

HSP70 concentration in each of the tissues (gill, hepatopancreas, and muscle) on day 1 was higher than it was on day 0, and then increased significantly with time (from day 0 to day 3) in all light groups (Fig. 4). The highest HSP70 concentration was measured at 25 psu. When the light source was included as a factor, the HSP70 concentration increased less with time under red and green LED

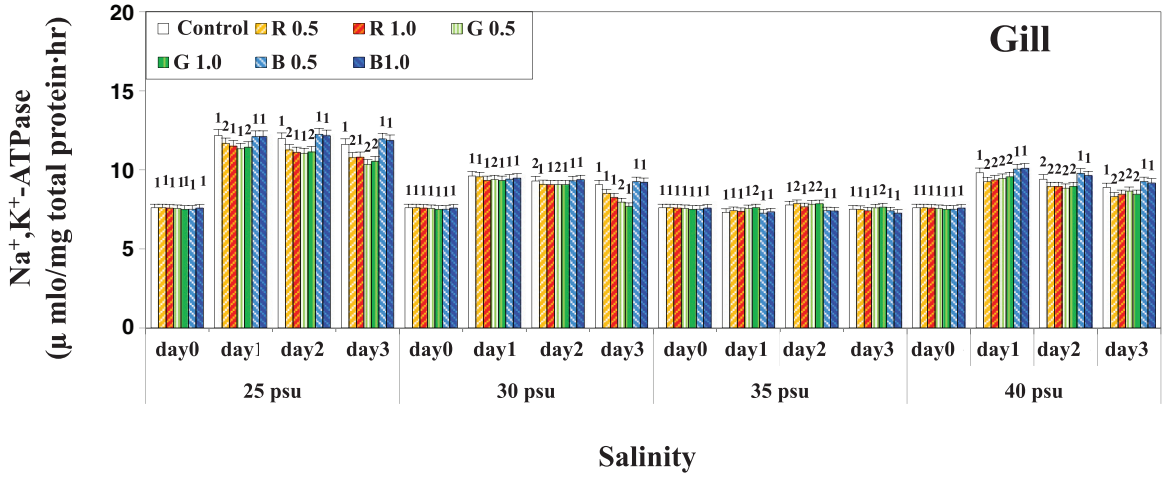

Figure 2. Concentration of Na^+-K^+-ATPase (NKA) in the gills of *Lysmata amboinensis* exposed to various salinity environments and lighting: white fluorescent bulb (control), red (R), green (G), and blue (B). NKA activity was analyzed using a plate reader. Bars with different numbers indicate significant differences between each experimental group within the same salinity and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$). This figure is available in colour at *Journal of Crustacean Biology* online.

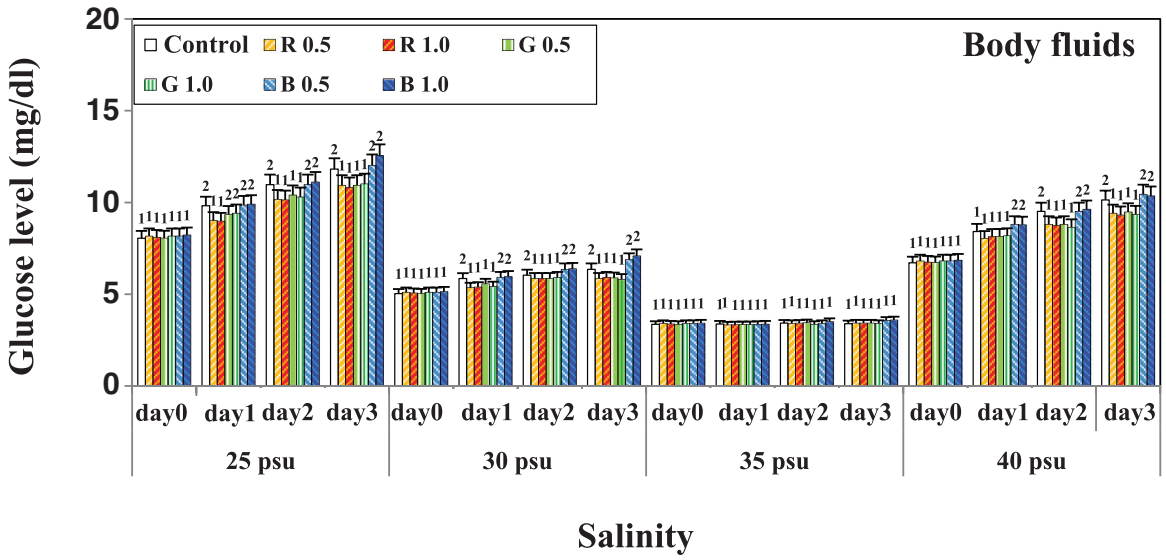

Figure 3. The level of glucose in the whole-body fluids of *Lysmata amboinensis* exposed to various salinity environments and lighting: white fluorescent bulb (control), red (R), green (G), and blue (B). Bars with different numbers indicate significant differences between each experimental group within the same salinity and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$). This figure is available in colour at *Journal of Crustacean Biology* online.

wavelengths than it did in the control group (white fluorescent bulb). Conversely, HSP70 concentration increased more with time under the blue LED wavelength. There were no differences in the responses between the two light intensities.

Analysis of DNA damage

Hepatopancreas tissue DNA damage following a three-day exposure to various salinities (25, 30, 35, and 40 psu) was analyzed using 100 randomly selected cells. For the comet assay, we analyzed the tail length and % DNA in the tail. The DNA content in the tail and tail length in the experimental groups (25, 30, and 40 psu) increased significantly with exposure time, compared with the 35 psu group (normal seawater) (Fig. 5). When the light source was included as a factor, the % DNA in the tail and the tail length in the 25 and 40 psu samples were significantly lower in the groups exposed to red and green LED wavelengths, than those in the control group. Conversely, % DNA in the tail and tail length in the 25 and 40 psu samples was significantly higher in the blue LED group than in the controls and the 30 psu groups.

DISCUSSION

Although *L. amboinensis* is an important commodity in the trade of ornamental fishes, most studies have focused on sexual maturity or ecology (Tziouveli *et al.*, 2011; Rui *et al.*, 2014), rather than on physiology despite its sensitivity to changes in light, salinity, temperature, pH, and other environmental factors.

Although aquatic animals commonly adapt to changes in their environment and/or move to a more suitable environment, ornamental species are unable to move from their aquaria so responses to stress by environmental changes must take place *in situ*. NKA activity, one such response to maintain ion homeostasis, is increased to regulate Na^+ and K^+ concentrations in the body. NKA also promotes the synthesis of glucose, a metabolic energy source needed to restore tissue damaged by stress. NKA activity in *L. amboinensis* was higher in all the salinity experimental groups (25, 30, and 40 psu) than it in the control group (35 psu, normal sea water), and it decreased significantly under all light sources, except the blue LED, as the exposure time increased.

Liu *et al.* (2014) examined NKA activity in the whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), after exposure to a high osmotic environment and found that NKA activity significantly increased and played an important role in the osmotic reaction that regulated the glucose synthesis required for the metabolism of NKA activity. Pan *et al.* (2014) investigated NKA activity in *L. vannamei* exposed to a low-salinity (21 psu) environment for nine days and showed that NKA increased compared to that in animals exposed to normal salinity (31 psu). Furthermore, NKA activity gradually decreased over time and adapted to the change in the osmotic environment.

We investigated the possibility of reducing stress in ornamental animals that are particularly sensitive to environmental changes, such as rapid changes in salinity, by using the artificial light (wavelengths), which are almost always installed in indoor aquaria. Studies on the effects of specific light wavelengths on the physiological responses in crustaceans have been rare until recently, with studies focusing on growth, the endocrine system, and reproduction in fishes (Ruchin, 2004; Karakatsouli *et al.*, 2008). Exposure to green wavelengths, for instance, was reported to reduce the synthesis of the stress indicators cortisol and glucose in the goldfish (*Carassius auratus* (Linnaeus, 1758)) and the rock bream (*Oplegnathus fasciatus* (Temminck & Schlegel, 1844)) exposed to high temperature (30 °C) (Jung *et al.*, 2016; Choe *et al.*, 2017).

Green and red light wavelengths significantly decreased NKA activity and glucose concentration in *L. amboinensis*. We conclude that these two light wavelengths were effective in reducing osmotic stress in this species as they reduced NKA activity and glucose concentration to steady states (Liew *et al.*, 2015).

In studies of light wavelengths and fishes, red light has been reported as having a negative effect on the stress response (Choi *et al.*, 2015b; Choe *et al.*, 2017). Notwithstanding, Caves *et al.* (2016) studied the regions of perceived light (350–600 nm) in *L. amboinensis* and showed that the species is not very sensitive to red light. The stress response in red light, however, was kept at a low level compared to the control, and the red wavelength also positively influenced adaptation to changing salinity by promoting a lower level of NKA activity. Their results of stress response were also similar to the green wavelength. Because studies on the photoreactions of *L. amboinensis* are limited, it will be necessary to

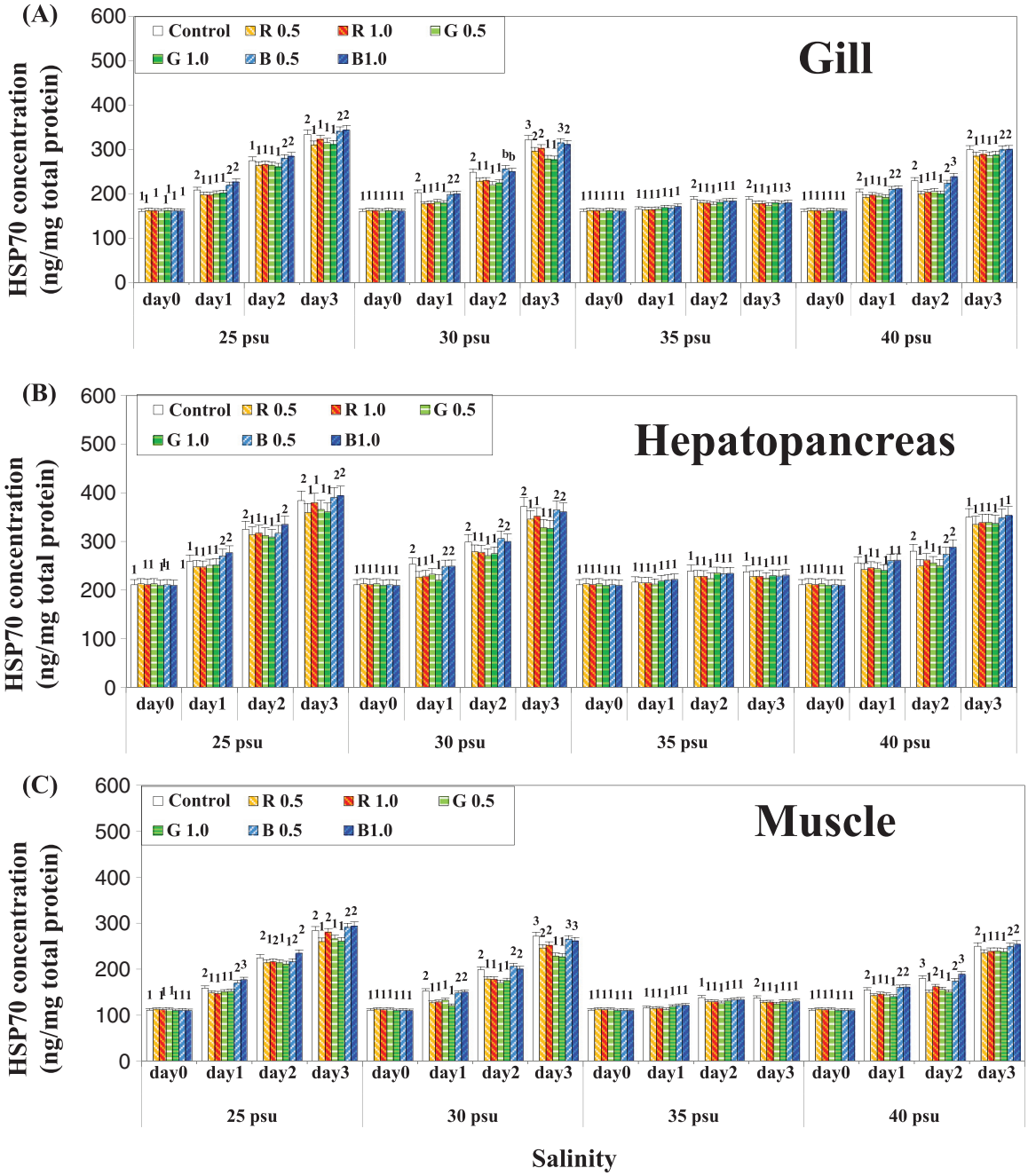

Figure 4. Concentration of heat shock protein 70 (HSP70) in the gill (**A**), hepatopancreas (**B**), and muscle (**C**) of *Lysmata amboinensis* exposed to various salinity environments and lighting: white fluorescent bulb (control), red (R), green (G), and blue (B). HSP70 was analyzed using a plate reader. Bars with different numbers indicate significant differences between each experiment group within the same salinity and exposure time ($P < 0.05$). All values are means ± SE ($N = 5$). This figure is available in colour at *Journal of Crustacean Biology* online.

study the range of optically recognized wavelengths and the photoreactive mechanisms in the species.

We found that the activity of HSP70, which was used as a stress index for gill, hepatopancreas, and muscle tissues, was significantly higher in shrimp exposed to 25, 30, and 40 psu, than it was in the controls. It also increased significantly with increasing exposure time (from day 0 to day 5). In general, the protein-synthesis system is disrupted when a subject is exposed to salinity stress (Somero & Yancey, 1997; Deane *et al.*, 2002). The expression of HSP is thus increased in order to restore the protein structure to its original state through refolding (see Kregel, 2002; Dong *et al.*, 2008). We hypothesize that the activity of HSP70 was increased as it plays an important role in preventing damage to cells and in cell homeostasis. Sun *et al.* (2013) reported that HSP70 concentrations increased when juvenile Chinese mitten crabs *Eriocheir sinensis* (H. Milne Edwards, 1853) were exposed to various salinities (1, 2, 10, 15 psu) in a river (5 psu). Our results showed that HSP70 concentration was significantly lower after exposure to red and green wavelengths, but higher after exposure to blue light. These data suggest that the green wavelength reduces environmental stress, and that the stress-reducing effect of green wavelengths also applies to crustaceans. Green light is assumed to reduce HSP70 activity in *L. amboinensis* by reducing the stress induced by an abrupt change in salinity. From similar studies in fishes (see Choi *et al.*, 2015a, 2016), we conclude that salinity changes increased the secretion of glucose and HSP70 in the tissues of *L. amboinensis*. and that red- and green-wavelength

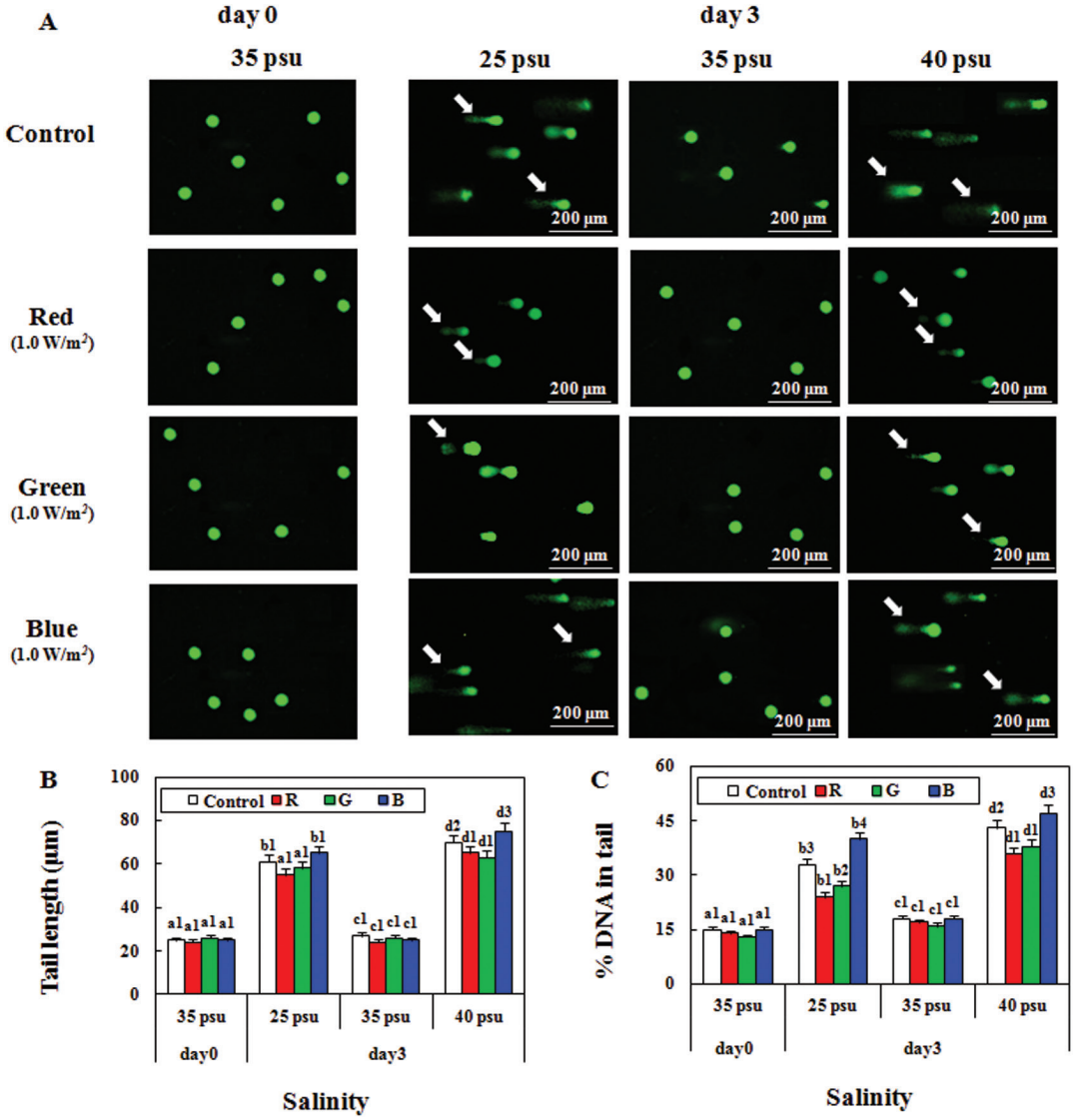

Figure 5. Comet-assay images (**A**) and comet-assay parameters for tail length (**B**) and % DNA in the tail (**C**) following initial conditions (at day 0) and various salinity conditions (25, 30, 35, and 40 psu after three days) under different lighting and white fluorescent bulb (control), red (R), green (G), and blue (B) (1.0 W m^{-2}), in hepatopancreas cells of *Lysmata amboinensis*. White arrows in (**A**) indicate damaged nuclear DNA (DNA breaks) in the hepatopancreas cells, which were stained using SYBR Green. Bars with different numbers indicate significant differences between each experiment group within the same salinity and exposure time ($P < 0.05$). The asterisks (*) indicate significant differences between different experimental groups within the same concentration ($P < 0.05$). Scale bars = 100 μm. This figure is available in colour at *Journal of Crustacean Biology* online.

light had a stress-reducing effect. We suggest additional research on the mechanisms of the stress-reducing effects of red-wavelength light in *L. amboinensis* and other shrimps.

Tail length and % DNA were significantly lower after exposure to red- and green-wavelength light, but were higher after exposure to blue-wavelength light. In general, when an organism is exposed to stress, free radicals (reactive oxygen species, or ROS) are produced that are lethal to cells (Roch, 1999; Nordberg & Arnér, 2001). ROS modify the DNA and degrade the ribose ring, resulting in strand breaks, and strand breakdown occurs before recovery (Hong *et al.*, 2006). DNA damage appears to be correlated with ROS, and DNA damage from the changes in salinity was due to degradation of the DNA strand from an overproduction of ROS over time.

We conclude that changes in salinity promoted NKA activity and glucose levels. As the exposure time increased, there was a gradual decrease in NKA activity and glucose levels, which appeared to be an adaption to the salinity. NKA and glucose could therefore be used as saline-stress biomarkers in shrimps. Blue LED light acted as a stress factor under osmotic conditions, which increased HSP70 concentration and caused DNA damage in the nuclei of hepatopancreas cells, and green and red light were effective wavelengths for decreasing stress as well as increasing the ability of *L. amboinensis* to control osmotic pressure. These results still present some difficulties in explaining detailed mechanisms, such as the positive effects of red wavelengths and of photo reactions and their effects in crustaceans. Our work aims to provide a basis for further research into light responses in crustaceans.

ACKNOWLEDGEMENTS

This research was supported by the project "Development and commercialization of high density low temperature plasma based seawater sterilization purification system" and by a grant from the National Research Foundation of Korea (NRF) of the Korean Government (MSIP) (2018R1A2B6002569). All authors thank the anonymous reviewers for their help with the manuscript. The authors declare that they have no conflicts of interest.

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