Thesis for the Degree of Doctor of Philosophy

Molecular and Endocrinological Study on Environmental and Maturation Responses in Cleaner Shrimp *Lysmata amboinensis*

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List of Abbreviations

ANOVA	analysis of variance
ACTH	adrenocorticotropic hormone
BM	body mass
cDNAs	complementary DNAs
CAT	catalase
CHH	crustacean hyperglycemic hormone
CRH	corticotropin-releasing hormone
CRY	cryptochrome
Ct	cycle threshold
DD	continuous dark
DNA	deoxyriboNucleic Acid
E_2	estradiol
ELISA	enzyme-linked immunosorbent assay
FP	female phase
GTH	gonadotropin
HPG	hypothalamus–pituitary–gonad
HPI	hypothalamus–pituitary–interrenal
HSP	heat shock protein
LD	general photoperiod (12 h light : 12 h dark)
LH	luteinizing hormone
LL	continuous light
M-MLV	moloney-murine leukemia virus reverse transcriptase
MP	male phase
NKA	Na^+/K^+-ATPase
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
PER	preriod gene

PSH	protandric simultaneous hermaphroditism
PSU	practical salinity unit
ROS	reactive oxygen species
RT	reverse transcription
SE	standard error
SOD	superoxide dismutase
VTG	vitellogenin
VTG-R	vitellogenin receptor

클리너새우 *Lysmata amboinensis*의 환경 및 성숙 반응에 관한 분자내분비학적 연구

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요 약

클리너새우는 열대 산호초 해역에 서식하는 십각목에 속하는 종으로, 붉은 색상의 아름다운 줄무늬 패턴으로 인하여 해양생물 수집가들에게 인기 있는 관상용 새우이다. 본 종은 거의 대부분을 자연 채집에 의존하고 있는 고가의 관상용 새우이기 때문에 산업적 가치가 클 뿐 아니라, 호스트 어류의 기생충이나 박테리아와 손상된 조직을 제거하는 등 특이한 공생관계를 갖는 특성을 보이기 때문에 생태학적 측면에서도 연구 가치가 큰 종이라 판단된다.

그러나 아직까지 클리너새우에 관한 연구는 거의 수행된 적이 없을 뿐만 아니라, 앞으로 해양생명자원의 관리 및 종 보존 측면의 연구를 수행하기 위해서는 본 종의 생리학적 특성에 관한 연구가 반드시 필요하다. 최근에는 어류의 성장, 성숙 측면의 생리학적 특성을 조절하여 안정적인 양식생물의 대량 생산체제를 구축하기 위하여 특정 파장대의 빛을 활용한 긍정적인 연구 결과가 발표되고 있다. 이러한 연구결과가 관상 가치가 높은 클리너새우의 양식 기술 개발에 보다 용이하게 활용되기 위해서는 특정 파장대의 빛에 의한 클리너새우의 생리 대사 조절 가능성을 확인하고, 빛 파장대별로 클리너새우에 미치는 영향을 파악하는 것이 필요하다. 하지만 갑각류인 클리너새우에서의 경우에는 광반응과 성숙을 포함한 분자 메커니즘 측면의 기본적인 정보조차 찾아보기 어려운 실정이다. 따라서 본 연구는 특정 빛 파장에 따른 클리너새우의 생리학적 반응을 조사하고 급격한 환경 변화에 노출된 클리너새우의

스트레스 반응 조절에 미치는 특정 빛 파장의 효과를 확인하기 위하여 수행되었다. 또한, 특정 빛 파장이 갑각류의 주요 기관 중의 하나인 안병에서 성 성숙에 영향을 미치는 호르몬의 분비를 조절하는지에 대해서도 조사하였다.

1. 클리너새우의 일주기리듬에 미치는 다양한 광주기 및 특정 빛 파장의 효과

일반적으로 새우류는 야행성 종임에도 불구하고 클리너새우는 호스트 어류와 공생관계를 이루기 위해서 불가피하게 낮 시간에 활동하는 것으로 보이며, 특히 480～540 nm 범위의 파장대의 빛에 대해 반응한다고 알려져 있다. 그러나 행동학적 측면에서 본 종이 특정 파장대의 빛에 반응한다고만 알려져 있을 뿐, 광반응과 관련된 생리적 특성과 관련된 연구는 거의 찾아볼 수 없다. 따라서 본 연구에서는 우선 다양한 광주기(24시간 동안 지속적으로 빛을 조사한 실험구, LL; 12시간만 빛을 조사한 실험구, LD; 24시간 동안 빛의 조사가 없는 실험구, DD)와 특정 파장의 빛(적색, 630 nm; 녹색, 520 nm; 및 청색, 455 nm)이 클리너새우의 일주기리듬에 어떠한 영향을 미치는지에 대해서 조사하였다. 일주기/생체리듬을 조절하는 대표적인 시계유전자로 알려진 cryptochrome1 (Cry1)과 period2 (Per2) mRNA의 발현량 변화와 밤의 호르몬이라고 알려져 있는 melatonin의 농도 변화를 정량화하여 클리너새우의 일주기리듬을 확인하였다. 그 결과, 시계유전자인 Cry1과 Per2 mRNA의 발현량은 밤 시간대에 비하여 낮 시간대에서 유의적으로 높게 나타났다. 광주기별 시계유전자 mRNA의 발현량을 비교한 결과, LL 환경에서는 시계유전자 mRNA의 발현량이 높고 DD 환경에서는 낮은 발현량을 보였으며, LL 환경과 DD 환경에서 시계유전자의 발현량 변화 폭은 LD 환경에 비해 감소된 것을 확인하였다. 또한, 광주기 변화에 따른 melatonin 농도의 일주기 변화 패턴은 시계유전자 mRNA의 발현량 패턴과는 다르게 낮 시간대에 비하여 밤 시간대에 유의하게 높게 나타났다. Melatonin 농도는 LL 환경에서 낮게 나타났고 DD 환경에서 높게 나타났으며, LL 환경에서 melatonin 농도는 LD와 DD 환경에 비하여 주·야간에 따른 농도 변화의 폭이 감소한 것을 확인하였다.

또한, 적색과 녹색 파장 빛 실험구의 클리너새우에서 관찰된 Cry1과 Per2 mRNA의 발현 폭은 대조구로 사용된 형광등 실험구의 클리너새우에서 관찰된 발현 폭에 비하여 유의하게 높게 나타났다. 결론적으로 본 연구에서는 광주기와

특정 빛 파장이 다른 종에서와 같이 클리너새우의 일주기리듬과 melatonin의 농도 조절에 영향을 미친 것으로 판단되며, 특히 적색과 녹색 파장은 다른 빛 파장에 비하여 효과적으로 작용하고 있는 것으로 판단된다.

2. 염분변화 환경에 의해서 클리너새우에서 유도된 스트레스 반응 및 핵 DNA 손상에 미치는 특정 광 파장의 영향

수생생물은 서식 환경요인의 변화에 따라 다양한 생리학적 반응을 보이며, 자연 환경에 서식하는 경우에 비하여 관상용으로 소형 실내 수조에서 인위적으로 사육되는 경우에는 수분의 증발 등 급격한 염분변화를 포함한 다양한 환경변화에 빈번하게 노출되기 쉽다. 다양한 환경변화에 노출된 개체의 체내에서는 스트레스 반응이 유발되는데, 이를 조절하지 못하는 경우에는 폐사에 이르게 된다. 따라서 본 연구에서는 급격한 염분변화 환경에 노출된 클리너새우의 체내에서 유발된 스트레스가 특정 파장 빛의 조사로 인하여 조절될 수 있는지 여부와 그 영향을 조사하였다.

클리너새우를 일반적인 해수(35 psu)와 저염분 해수 (25 및 30 psu) 그리고 고염분 해수(40 및 45 psu) 환경에 각각 3일 동안 노출시킨 실험구에 다양한 파장대의 빛(적색, 630 nm; 녹색, 520 nm; 청색, 455 nm)을 실험구별로 조사하면서 클리너새우의 아가미 조직 내에서 Na+/K+-ATPase (NKA) 활성과 체액 내 glucose 농도를 측정하였다. 또한, 생리학적 스트레스 반응을 확인하기 위하여 아가미, 간췌장과 근육에서 heat shock protein 70 (HSP70) 농도를 측정하였으며, 산화스트레스 반응을 확인하기 위하여 대표적인 항산화효소인 superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO) 농도를 측정하고, comet assay 분석을 통하여 핵 DNA의 손상 정도를 측정하였다. 그 결과, 45 psu 고염분 실험구의 클리너새우는 하루 만에 모두 폐사하였다. 그러나 다른 모든 실험구의 클리너새우에서 측정된 NKA 활성과 glucose 농도뿐만 아니라, HSP70, SOD, CAT, LPO 농도와 핵 DNA의 손상 정도 값 모두 대조구(35 psu)에 비하여 유의적으로 높게 나타난 점으로 보아, 클리너새우는 염분변화에 의해서 (산화)스트레스를 받는 것을 확인할 수 있었다. 적색과 녹색 파장의 빛을 조사한 실험구의 클리너새우에서는 대조구와 청색 파장 실험구에 비하여 (산화)스트레스가 유의적으로 감소하였는데, 파장의

강도에 의한 영향은 거의 없는 것으로 확인되었다. 또한, 간췌장 세포 내에서 핵 DNA의 손상 정도를 확인한 결과, 25, 30 및 40 psu 실험구에서는 대조구에 비하여 tail length와 % DNA 값이 노출 시간이 경과함에 따라 유의적으로 증가하였다. Tail length와 % DNA 값 모두 적색과 녹색 파장에 노출된 실험구의 클리너새우 간췌장 세포에서는 유의적으로 낮게 나타난 반면, 청색 파장 실험구에서는 높은 값을 나타내었다.

결론적으로 적색과 녹색 파장의 빛은 클리너새우의 스트레스를 저감시킬 뿐만 아니라, 항산화 능력 또한 증강시켜 핵 DNA의 손상을 효과적으로 감소시킬 수 있는 것으로 분석되었다.

3. 17β-estradiol 및 적색 파장의 빛이 클리너새우의 성숙 관련 안병 호르몬 분비와 난황 형성에 미치는 영향

갑각류는 어류와는 달리 안병 내에 존재하는 X-Organ/sinus gland complex라는 특수한 조직에서 분비되는 다양한 호르몬을 통해서 성 성숙이 진행된다. 최근에는 갑각류의 흉부신경절과 뇌에서 척추동물에 존재하는 형태의 성 스테로이드 호르몬이 발견되면서 다양한 성 성숙 메커니즘 경로가 있는 것으로 알려지면서 갑각류의 X-Organ/sinus gland complex에서 분비되는 성 성숙 관련 호르몬과의 상호작용에 대한 연구의 필요성이 대두되고 있다. 따라서 본 연구에서는 갑각류에서 성 성숙을 유도하기 위하여 일반적으로 사용되는 안병 절제 방법의 문제점을 개선하고, 성 성숙과 빛의 상관관계를 조사하여 빛을 수용하는 기관인 안병을 절제하지 않고도 성 성숙을 유도할 수 있는 새로운 갑각류의 성 성숙 방법을 제시하고자 한다. 즉, 본 연구는 클리너새우의 성 성숙을 유도하기 위하여 17β-estradiol (E_2)를 주입하는 방법과 적색 파장 빛을 조사하는 방법이 안병에서 생성되는 성숙 관련 호르몬인 crustacean hyperglycemic hormone (CHH)과 vitellogenesis- inhibiting hormone (VIH)의 변화량에 미치는 영향을 확인하였다. 또한, in-situ hybridization 방법을 통하여 간췌장에서 vitellogenin (VTG) mRNA의 발현 변화를 직접 확인하였으며, 생식소에서는 vitellogenin 수용체 (VTG-R) mRNA의 발현 변화를 확인하여 E_2와 적색 파장의 빛이 클리너새우의 난황형성과정에 미치는 영향을 조사하였다. 그 결과, E_2 주입은 안병 호르몬의

변화에 영향을 미치지 않았지만, 적색 파장 빛의 조사는 갑각류의 성숙을 촉진하는 CHH mRNA의 발현을 유의적으로 증가시켰으며 동시에 성숙과 난황 형성을 억제하는 VIH mRNA의 발현까지도 유의적으로 감소시켰다. 또한, E_2의 주입 및 적색 파장 빛의 조사로 인하여 VTG 및 VTG-R mRNA의 발현이 증가된 것으로 보아, E_2와 적색 파장의 빛은 클리너새우의 VTG 합성을 유도하는데 효과적임을 확인할 수 있었다. 결론적으로, 적색 파장의 빛은 성숙 촉진 호르몬의 유도와 난황형성을 촉진하는 파장대의 빛으로 사료되며, E2는 성숙을 촉진하는 안병 호르몬에 영향을 미치지 않는다는 사실을 확인하였다. 이러한 연구결과는 갑각류의 성 성숙을 유도할 때 안병을 절제하는 기존의 방법과는 달리, 적색 파장 빛을 조사하는 방법을 사용하게 된다면 안병의 절제로 인하여 관상용 클리너새우의 미관을 해치게 될 수도 있는 단점을 극복할 수 있을 뿐만 아니라 갑각류의 성 성숙을 위한 효과적인 방법으로 활용될 수 있을 것으로 기대된다.

본 연구는 최근에 알려지기 시작한 특정 파장대의 빛이 어류의 성장, 면역 및 성숙에 긍정적인 영향을 미친다는 연구결과를 대중성이 높은 갑각류인 클리너 새우에 접목하여 다양한 파장대의 빛이 클리너새우의 생리학적 측면에 미치는 영향에 대한 중요한 정보를 제공하고 있다. 특히, 적색 파장대의 빛은 시계유전자의 일주기 리듬을 조절할 뿐만 아니라 급격한 환경 변화에 노출된 클리너새우의 스트레스 반응 조절 그리고 성 성숙에 영향을 미치는 안병호르몬의 조절에도 효과적인 것을 확인하였다. 따라서 본 연구 결과는 중요한 양식 대상 종인 클리너새우에 대한 기본적인 분자내분비학적 정보를 제공할 수 있을 뿐만 아니라 안정적인 양식생산에 필요한 기초 데이터로도 활용될 수 있을 것으로 기대된다.

Molecular and Endocrinological Study on Environmental and Maturation Responses in Cleaner Shrimp *Lysmata amboinensis*

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Abstract

Cleaner shrimp *Lysmata amboinensis* is a decapod crustacean inhabiting the tropical coral reefs. It is a tropical shrimp that is popular among marine species collectors because of very beautiful stripe pattern of red color. In addition, the species maintains a unique symbiotic relationship with host fishes, especially moray eel. It occupies an important position not only in the ecological aspect of the tank but also in the commercial aspect. In particular, it has been termed as "cleaner shrimp" owing to its maintenance of an unusual symbiotic cleaning relationship that eliminates parasites, bacteria, and damaged tissues in host fishes.

Despite its high commercial position, however, there is a lack of knowledge about molecular mechanisms involved in photoreactions as well as fundamental physiological responses to these species. Therefore, this study was carried out to clarify the effects of specific wavelengths on the physiological response and environment of cleaner shrimp by light, and also to investigate whether regulation of sex-related hormones secreted from the eyestalk, an important organ in crustaceans.

1. Effects of various photoperiods and specific wavelength lights on circadian rhythm in cleaner shrimp *Lysmata amboinensis*

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. In order to establish a symbiotic relationship with host fishes, the cleaner shrimp inevitably work during the daytime despite being nocturnal, and is 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, I investigated effect of photoperiod and specific wavelength (red, 630 nm; green, 520 nm; 및 blue, 455 nm) on the circadian rhythm of cleaner shrimp. Circadian rhythm was evaluated by quantifying the mRNA expression of clock genes such as *lysmata amboinensis* cryptochrome1 (*la*Cry1) and *lysmata amboinensis* period 2 (*la*Per2) 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, the 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.

2. Effects of specific wavelength lights on stress responses and cell damage in cleaner shrimp *Lysmata amboinensis* exposed to various salinity conditions

Living creatures in the aquatic environment exhibit a variety of physiological responses due to environmental factors, especially cleaner shrimp, which are more often breed as ornamental species than when they are inhabited in nature, are often exposed to various salinity condition by inexperienced administrators. It is a stress response factor and eventually causes mortality. Therefore, in this study, I

investigated the effects of specific wavelengths of light on various salinity condition in order to reduce salinity stress through improvement of rearing device.

Cleaner shrimp were exposed to low salt environment (25 and 30 psu) and high salinity environment (40 and 45 psu) for 3 days based on normal sea salt (35 psu), while irradiating specific wavelength light. Na^+/K^+-ATPase (NKA) activity in gills and glucose levels in whole-body fluids were measured as ionic homeostasis reactions, and the heat shock protein 70 (HSP70) concentration in tissues (gill, hepatopancreas and muscle) was measured as physiological stress reaction, and the activity of superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO) in tissues (gill, hepatopancreas and muscle) and the nuclear damage in hepatopancreas were measured by the oxidative stress reaction. As a result, all shrimp exposed to 45 psu were dead within a day of exposure. 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. In conclusion, there was no difference in the effect of different intensities of light, but green and red light effectively reduced the osmotic stress and cell damage caused by salinity.

3. Effects of E_2 injection and red wavelength light on eyestalk hormones of cleaner shrimp *Lysmata amboinensis*

The objectives of this study were to test the effects of 17β-estradiol (E_2) injection and red light on sex maturation and vitellogeneisis of cleaner shrimp *Lysmata amboinensis*. Therefore, we mesured the change of mRNA expressions of the eyestalk hormones and vitellogenesis-related hormone according to E_2 injection and red light irradiation. We used eyestalk genes such as crustacean hyperglycemic hormone (CHH) and vitellogenesis inhibiting hormone (VIH). E_2 injection did not affect the changes of eyestalk hormones. However the red light caused a significant increase of CHH promoting maturity and a significant decrease of VIH which suppressed vitellogenesis. In addition, as result of vitellogenin receptor (VTG-R)

and vitellogenin (VTG) mRNA expression (in-situ hybridization), E_2 injection as well as red wavelength irradiation were shown to induce the synthesis of VTG. In conclusion, red light induce gonadal maturation promoting hormone and VTG synthesis. However, E_2 does not change the eyestalk hormone. Therefore, it is considered that red light irradiation can be used as a new mature promotion alternative method which is different from the eyestalk ablation method which has been used at the culture field.

Chapter 1.

General Introduction

The cleaner shrimp *Lysmata amboinensis*, a decapod crustacean inhabiting the tropical coral reef area. This species is an economically important species for aquarium industry that is popular with marine species collectors because of very beautiful stripe pattern of red color (Calado et al., 2003), and known to be an important source of income in the Indo-West Pacific region (Calado et al., 2009). In addition, this 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). Also, since there is no form technology, cleaner shrimps are expensive and they are traded from only naturally harvested stocks (Biondo, 2017). This species has ecological importance establishing a cleaning symbiotic relationship (Côté, 2000) with fishes, removing parasites, bacteria, and damaged tissues, so it is called “cleaner shrimp”. Due to this symbiotic relationship, unlike other nocturnal crustaceans, the activity of this species occurs during the daytime when the host fish is active, and for that reason it is known to be very sensitive to light (Wicksten, 2009; Esaka et al., 2016). Therefore, the cleaner shrimp are suitable for carrying out photoreactive molecular endocrinological studies of crustaceans. In addition, cleaner shrimp *L. amboinensis* lives in a unique reproductive mechanisms, which has a general sexually mature developmental stage, but unlike pure male mature species, it has a unique sexual maturation system called protandric simultaneous hermaphroditism (PSH) (Bauer and Holt, 1998). PSH has a sex system in which an individual is transformed into a female phase (FP) through a male phase (MP) like a general protastric crustacean,

but at the FP stage, It is characterized by having both functions (Bauer, 2006). According to many studies, it is the most common method to classify the size of an individual as a criterion that distinguishes between the MP and FP stages, and when it is statistically greater than 34 mm, it is judged to be the FP stage (Zhang and Lin, 2005; Tziouveli and Smith, 2009). However, despite their economical, ecological and physiological importance (Vaughan et al., 2017), studies on *L. amboinensis*, however, have rarely been conducted.

Therefore, this study was conducted to investigate the physiological changes of cleaner shrimp *L. amboinensis* caused by external environmental factors. As an external environmental factor, 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). Many physiological studies relating to the response to light have been conducted on fish. In few study, crustaceans are more sensitive to weak light than fishes (Covich et al., 2010), and have shown changes in growth (Guo et al., 2011) and food intake (Xu et al., 2003) when they are exposed to specific light wavelengths. However, the molecular mechanisms underlying the circadian rhythm as well as its associated genes in the crustacean remain to be elucidated (Fanjul-Moles et al., 2004).

As another environmental factor, the effect of salinity change on the physiological changes of *L. amboinensis* was confirmed. 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 and 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). In addition, exposure to saline causes 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 nontoxic H_2O and O_2 to prevent the accumulation of H_2O_2 in cells and tissues (Basha and 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 and Bowen, 1998; Pandey et al., 2003).

Therefore, this study was conducted to identify and evaluate the overall physiological mechanisms of cleaner shrimp *L. amboinensis*, which occupies an ecologically, economically and physiologically important position. In order to accomplish this, I focused on the circadian rhythm analysis to measure the response to light, the analysis of the stress response according to salinity among the external environment, the sex-maturation effect and vitellogenesis of specific wavelength lights.

Chapter 2.

Effects of various photoperiods and specific wavelength lights on circadian rhythm in cleaner shrimp *Lysmata amboinensis*

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).

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 *L. amboinensis*, a decapod crustacean inhabiting the tropical coral reef area, is an economically important species for aquarium industry that is popular with marine species collectors because of very beautiful stripe pattern of red color (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

crustacean 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*. I 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 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 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.

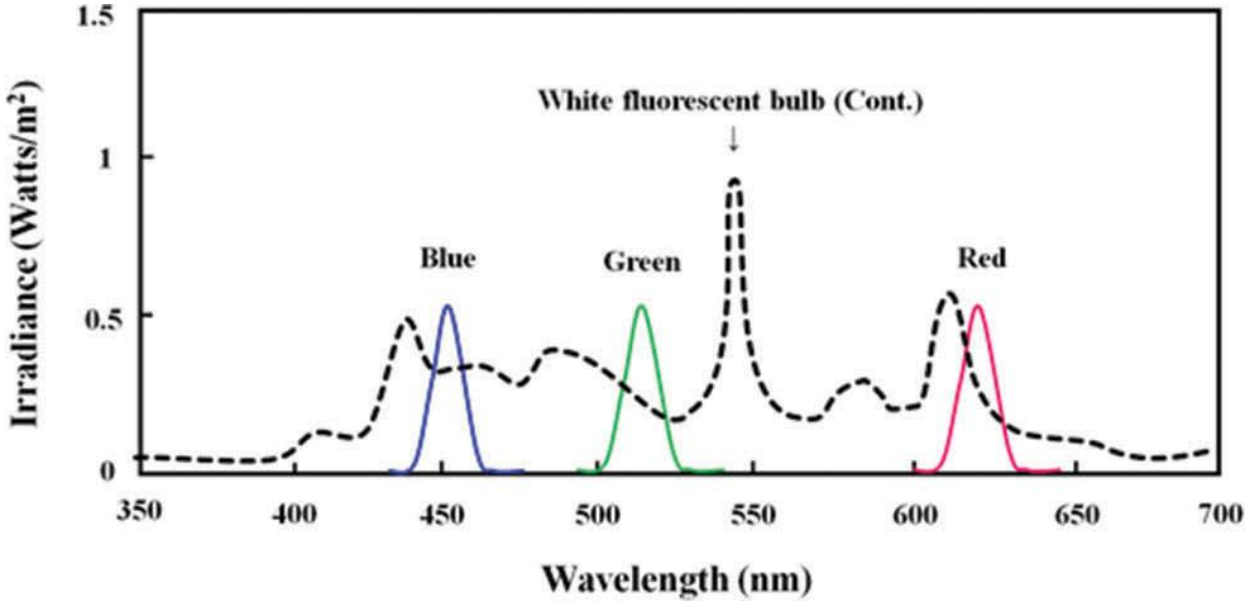
1.5
1
0.5
0
Irradiance (Watts/m²)
White fluorescent bulb (Cont.)
Blue
Green
Red
350
400
450
500
550
600
650
700
Wavelength (nm)

Fig. 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. Quantitative PCR (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 ΔΔCt = $2^{-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})}$.

2.4. Analysis of melatonin in eyestalk

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. Change in clock gene mRNA expression in the photoperiod group

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 nighttime. 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 (Fig. 2).

3.2. Change in melatonin level in the photoperiod group

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 (Fig. 3).

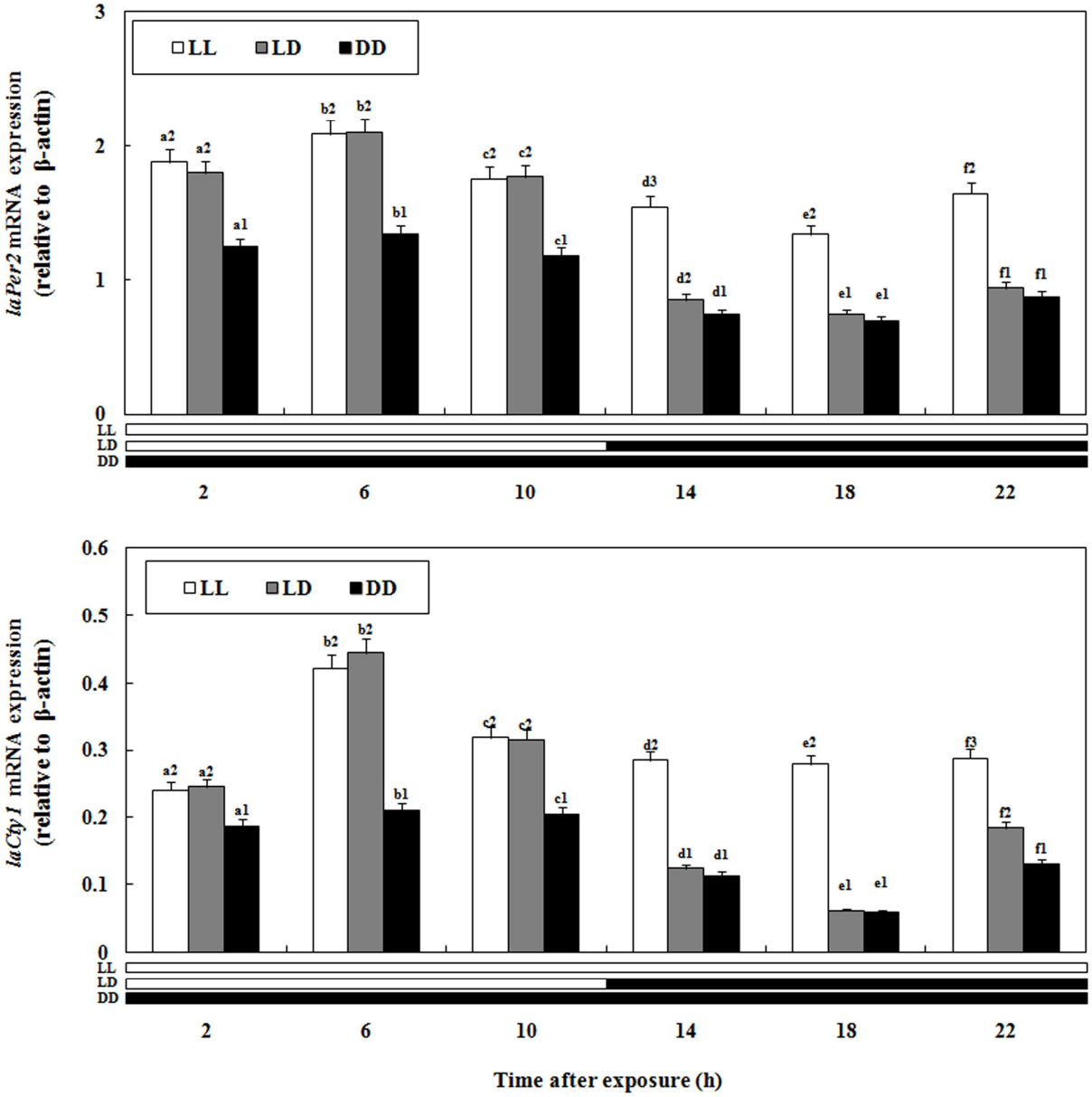
laPer2 mRNA expression
(relative to β-actin)
LL
LD
DD
a2 a2 a1
b2 b2 b1
c2 c2 c1
d3 d2 d1
e2 e1 e1
f2 f1 f1
0
1
2
3
2
6
10
14
18
22
laCry1 mRNA expression
(relative to β-actin)
a2 a2 a1
b2 b2 b1
c2 c2 c1
d2 d1 d1
e2 e1 e1
f3 f2 f1
0
0.1
0.2
0.3
0.4
0.5
0.6
Time after exposure (h)

Fig. 2. Changes of the laCry1 and laPer2 mRNA expressions in the eyestalk in *L. amboinensis* under different photoperiod conditions [LD, 12h light:12h dark; LL, continuous light; and DD, continuous darkness]. The tissues 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 photoperiods. The white bar represents the photophase and the black bar represents the scotophase. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in the same photoperiods and exposure time ($P < 0.05$). All values are means ± SE (n=3).

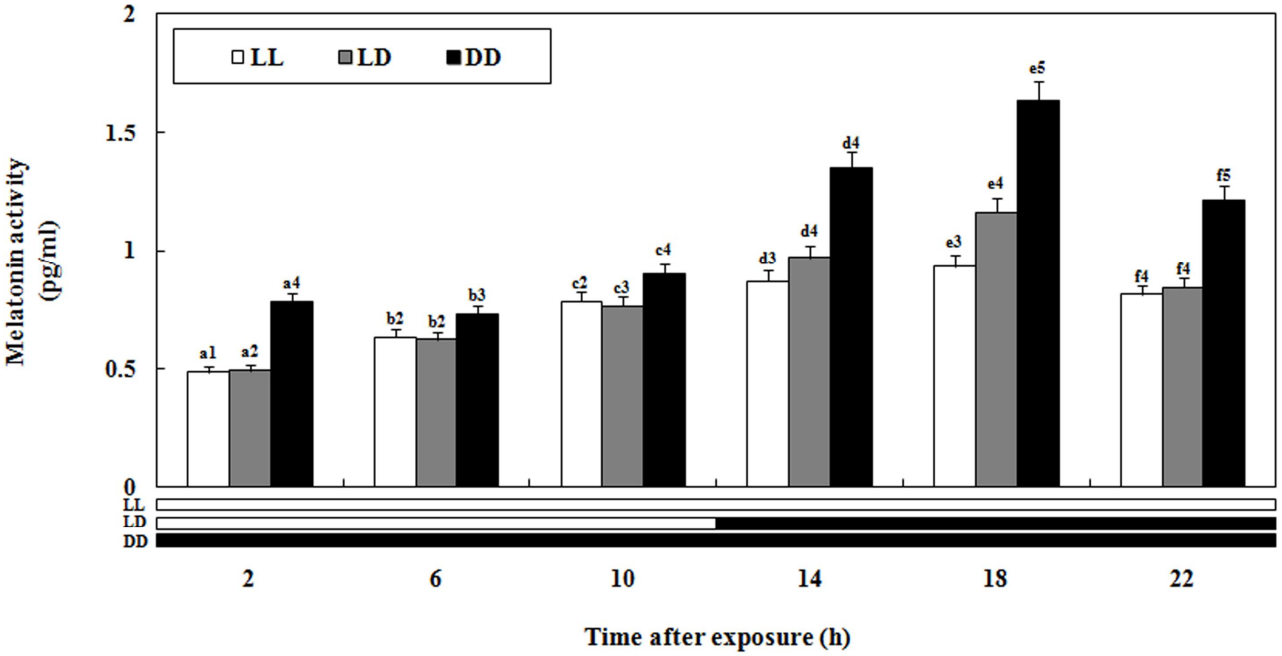
LL
LD
DD
Melatonin activity (pg/ml)
2
1.5
1
0.5
0
a1
a2
a4
b2
b2
b3
c2
c3
c4
d3
d4
d4
e3
e4
e5
f4
f4
f5
LL
LD
DD
2
6
10
14
18
22
Time after exposure (h)

Fig. 3. Changes of melatonin activity in the eyestalk in *L. amboinensis* under different photoperiod conditions [LD, 12 h light:12 h dark; LL, continuous light; and DD, continuous darkness]. The tissues 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 photoperiods. The white bar represents the photophase and the black bar represents the scotophase. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in the same photoperiods and exposure time ($P < 0.05$). All values are means ± SE (n=3).

3.3. Change in clock gene mRNA levels in the LED group

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 (Fig. 4).

3.4. Change in melatonin level in the LED group

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 (Fig. 5).

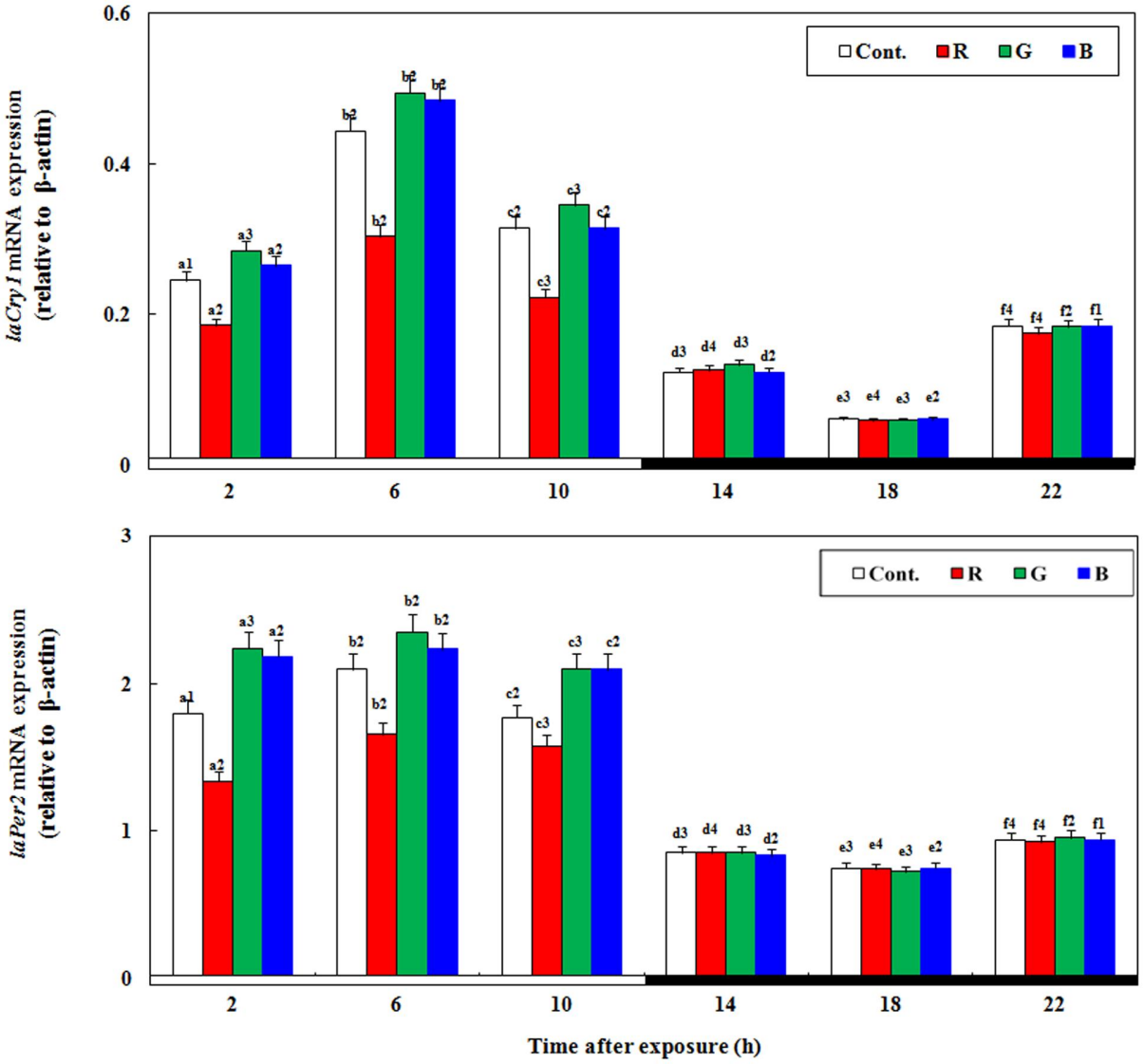
laCry1 mRNA expression (relative to β-actin)
Cont.
R
G
B
0.6
0.4
0.2
0
2
6
10
14
18
22
laPer2 mRNA expression (relative to β-actin)
3
2
1
0
Time after exposure (h)

Fig. 4. Changes of the laCry1 and laPer2 mRNA expressions in the eyestalk in *L. amboinensis* under different wavelength light [red LED, 630 nm; green LED, 520 nm; blue LED, 455 nm; and used white fluorescent bulb as a control light source (Cont.)] of two intensity (0.5 and 1.0 W/m^2 at bottom of aquarium). The tissues 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 photoperiods. The white bar represents the photophase and the black bar represents the scotophase. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in the same photoperiods and exposure time ($P < 0.05$). All values are means ± SE (n=3).

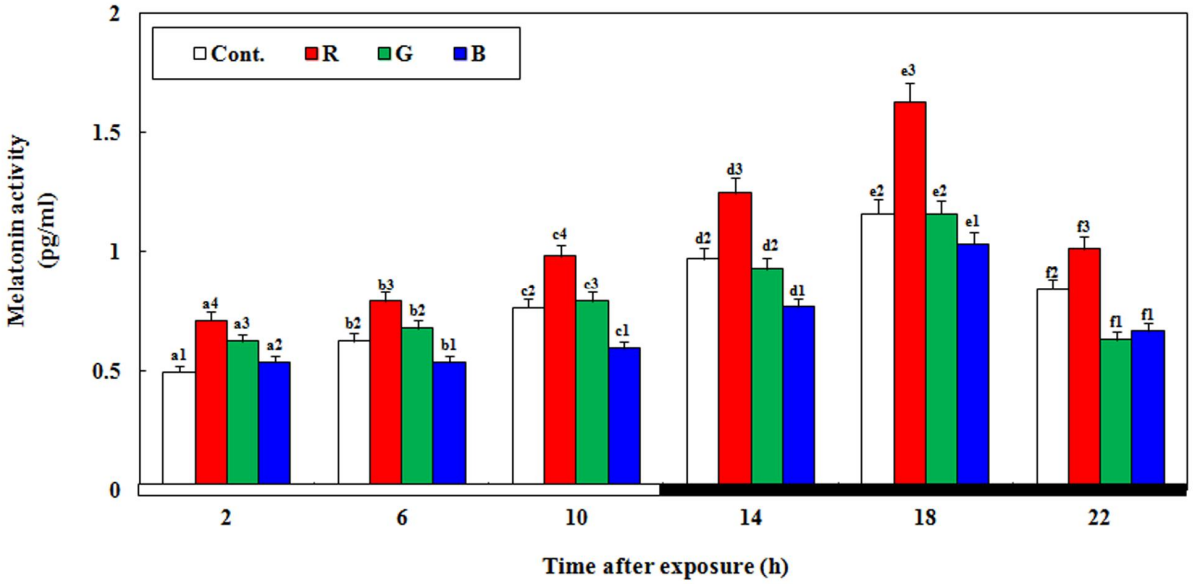
Cont.
R
G
B
Melatonin activity (pg/ml)
2
1.5
1
0.5
0
a1
a4
a3
a2
b2
b3
b2
b1
c2
c4
c3
c1
d2
d3
d2
d1
e2
e3
e2
e1
f2
f3
f1
f1
2
6
10
14
18
22
Time after exposure (h)

Fig. 5. Changes of melatonin activity in the eyestalk in *L. amboinensis* under different wavelength light [red LfED, 630 nm; green LED, 520 nm; blue LED, 455 nm; and used white fluorescent bulb as a control light source (Cont.)] of two intensity (0.5 and 1.0 W/m2 at bottom of aquarium). The tissues 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 photoperiods. The white bar represents the photophase and the black bar represents the scotophase. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in the same photoperiods and exposure time ($P < 0.05$). All values are means ± SE (n=3).

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 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, I 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, I 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, I 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, I 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 a uatic organisms recognize the red light as dark environments dueto 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, I 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. I 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.

Chapter 3.

Effects of specific wavelength lights on stress responses and cell damage in the cleaner shrimp *Lysmata amboinensis* exposed to various salinity conditions

1. 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 (Cote, 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 rnamental 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 and 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 and van Ham, 1998; Lorenzon, 2005). Glucose secretion in crustaceans is controlled by the crustacean hyperglycemic hormone (CHH), which is secreted from the eyestalk (Kamemoto and 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).

In additional, the 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 nontoxic H_2O and O_2 to prevent the accumulation of H_2O_2 in cells and tissues (Basha and 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 and Bowen, 1998; Pandey et al., 2003).

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 and 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.

Therefore, I investigated the effects of specific light wavelengths and intensities of light on the regulation of salinity stress in *L. amboinensis*. I exposed shrimp to three wavelength lights [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)). I also investigated changes in homeostatic function, physiological stress hormone levels (NKA and HSP70) and glucose concentration, and analysed the oxidative stress response (SOD, CAT) and lipid peroxidation (LPO) activity in the tissues and compared degrees of nuclear DNA damage using comet assays.

2. Materials and methods

2.1. Experimental animals and laboratory conditions

Individuals of *Lysmata amboinensis* [N = 896 (used in physiological stress expriment: 336 individuals and in oxidative stress experiment: 560 individuals); 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. 6). 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 07:00 to 19:00.

All experimental shrimp were fed with commercial feed (Tropical Tadeusz Ogrodnik, Chorzow, 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, 1,000 × g, 15 min). I removed only the supernatant and stored it at –20°C until analysis. The tissues were immediately

frozen in liquid nitrogen andstored at –80°C until analysis.

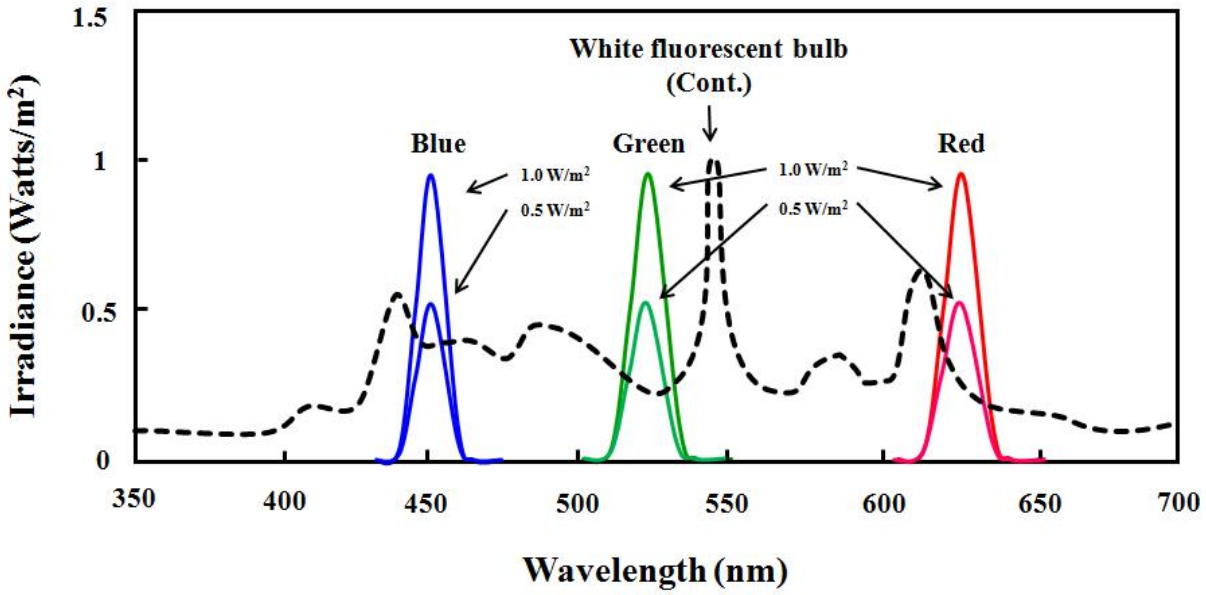
1.5
1
0.5
0
Irradiance (Watts/m²)
White fluorescent bulb
(Cont.)
Blue
Green
Red
1.0 W/m²
0.5 W/m²
1.0 W/m²
0.5 W/m²
350
400
450
500
550
600
650
700
Wavelength (nm)

Fig. 6. Spectral profiles of 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.

2.2. Analysis of NKA concentration in gill tissue

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, 2,000 × 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).

2.3. Analysis of glucose level in whole body

Body fluids from whole bodies of shrimp were used as it was difficult to separate the hemolymph. Tissue preparation followed the method of Njemini et al. (2005) with modifications. The whole body was homogenized using 500 μL PBS (Sigma) and centrifuged (4 °C, 1,000 × g, 15 min). I 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).

2.4. 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, I added 100 mg of tissue to PBS and centrifuged (4 °C, 5,000 × 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 (1:4,000), and secondary antibody (1:4,000). 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·mg protein·h. The HSP concentration was measured at 450 nm absorbance and the concentration was expressed as ng mg– total protein using a standard curve.

2.5. Analysis of SOD and CAT concentrations in tissues

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.

2.6. Analysis of LPO concentration in tissues

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.

2.7. Analysis of 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, USA), according to the method described by Singh et al. (1988), with some modifications. Cells were immobilized in agarose gels on Comet Assay 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, I analysed the tail length (distance of DNA migration from the head), percentage of DNA in the tail (tail intensity/total intensity in tail), and ail 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.).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). One-way analysis of variance followed by Tukey's post hoc test was used to test for significant differences in the data ($P < 0.05$). Values are expressed as mean ± SE.

3. Results

3.1. 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. 7). 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.

3.2. Change in glucose level

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. 8). 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 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.

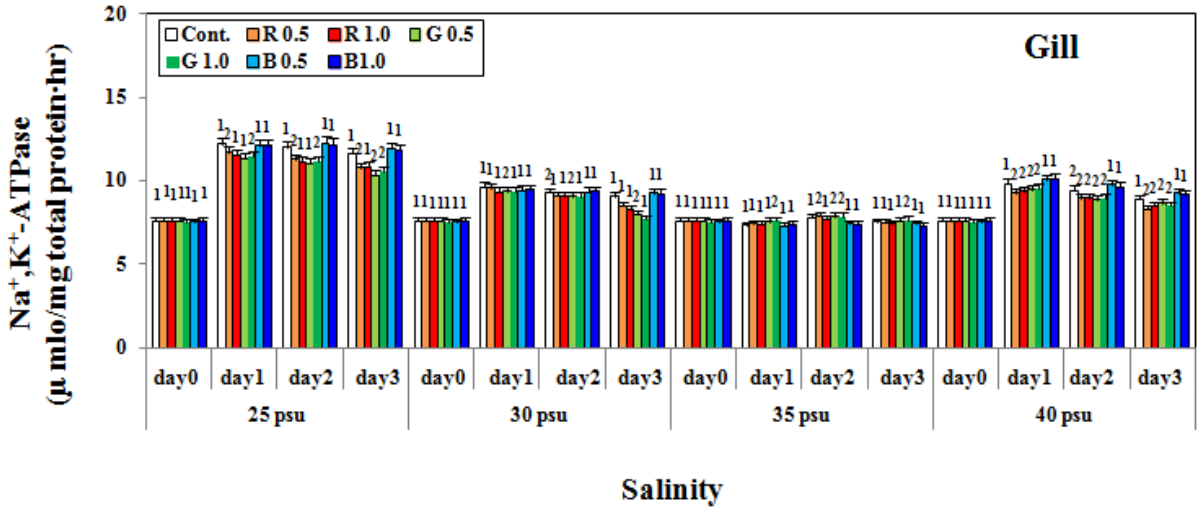
Cont.
R 0.5
R 1.0
G 0.5
G 1.0
B 0.5
B1.0
Gill
Na+,K+-ATPase
(μ mlo/mg total protein·hr)
20
15
10
5
0
day0
day1
day2
day3
25 psu
30 psu
35 psu
40 psu
Salinity

Fig. 7. Concentration of Na^+/K^+-ATPase (NKA) in the gill of cleaner shrimp, *Lysmata amboinensis* exposed to various salinity environments and different light [white fluorescent bulb (Cont.), red (R), green (G), blue (B)]. NKA activity was analyzed using a plate reader. Bars with different number indicate significant difference between each experiment group in the same salinity and exposure time ($P < 0.05$). All values are means ± SE ($n = 5$).

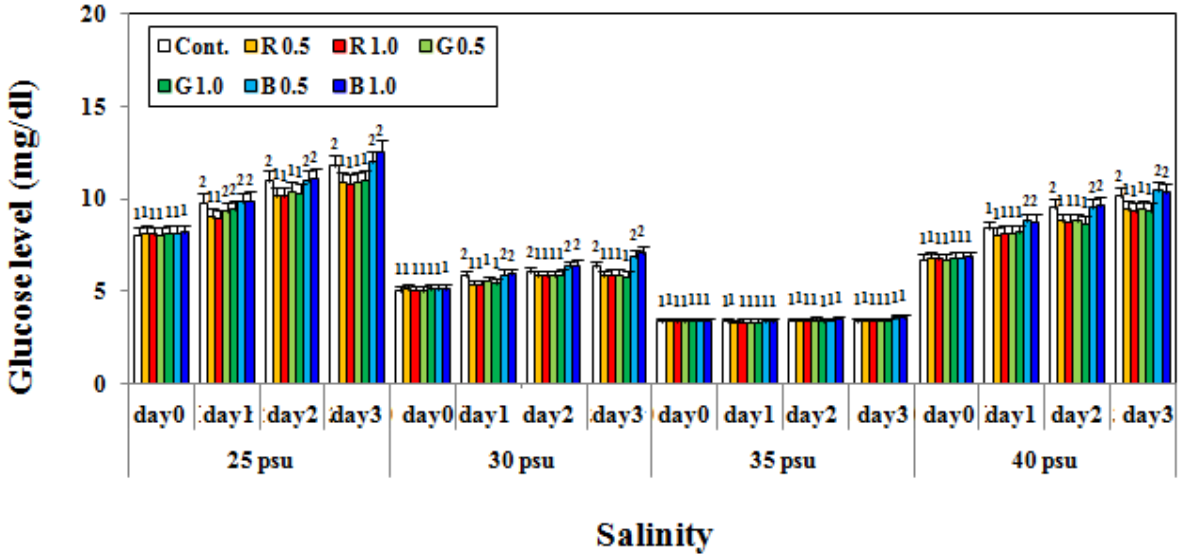
Glucose level (mg/dl)
20
15
10
5
0
Cont.
R 0.5
R 1.0
G 0.5
G 1.0
B 0.5
B 1.0
day0
day1
day2
day3
25 psu
30 psu
35 psu
40 psu
Salinity

Fig. 8. The level of glucose in whole body fluids of cleaner shrimp, *Lysmata amboinensis* exposed to various salinity environments and different light [white fluorescent bulb (Cont.), red (R), green (G), blue (B)]. Bars with different number indicate significant difference between each experiment group in the same salinity and exposure time ($P < 0.05$). All values are means ± SE ($n = 5$).

3.3. Change 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. 9). 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 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.

3.4. Changes in SOD and CAT concentrations

Compared to the SOD and CAT concentrations of the shrimp, *L. 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. 10 and 11). 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.

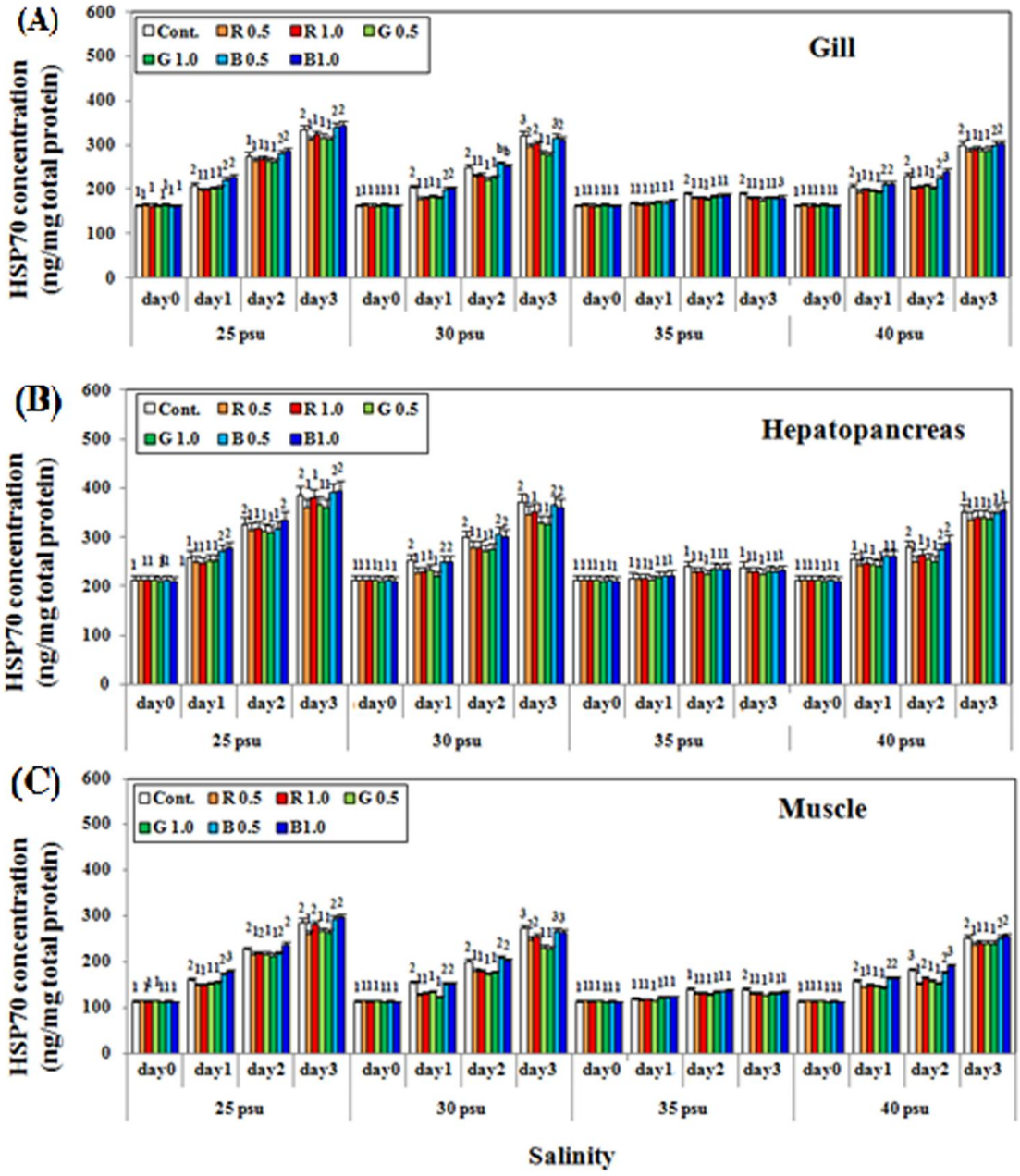
(A)
Gill
(B)
Hepatopancreas
(C)
Muscle
HSP70 concentration (ng/mg total protein)
Cont.
R 0.5
R 1.0
G 0.5
G 1.0
B 0.5
B1.0
day0
day1
day2
day3
25 psu
30 psu
35 psu
40 psu
Salinity

Fig. 9. Concentration of heat shock protein 70 (HSP70) in the gill (A), hepatopancreas (B), and muscle (C) of cleaner shrimp, *Lysmata amboinensis* exposed to various salinity environments and different light [white fluorescent bulb (Cont.), red (R), green (G), blue (B)]. HSP70 was analyzed using a plate reader. Bars with different number indicate significant difference between each experiment group in the same salinity and exposure time ($P < 0.05$). All values are means ± SE (n =5).

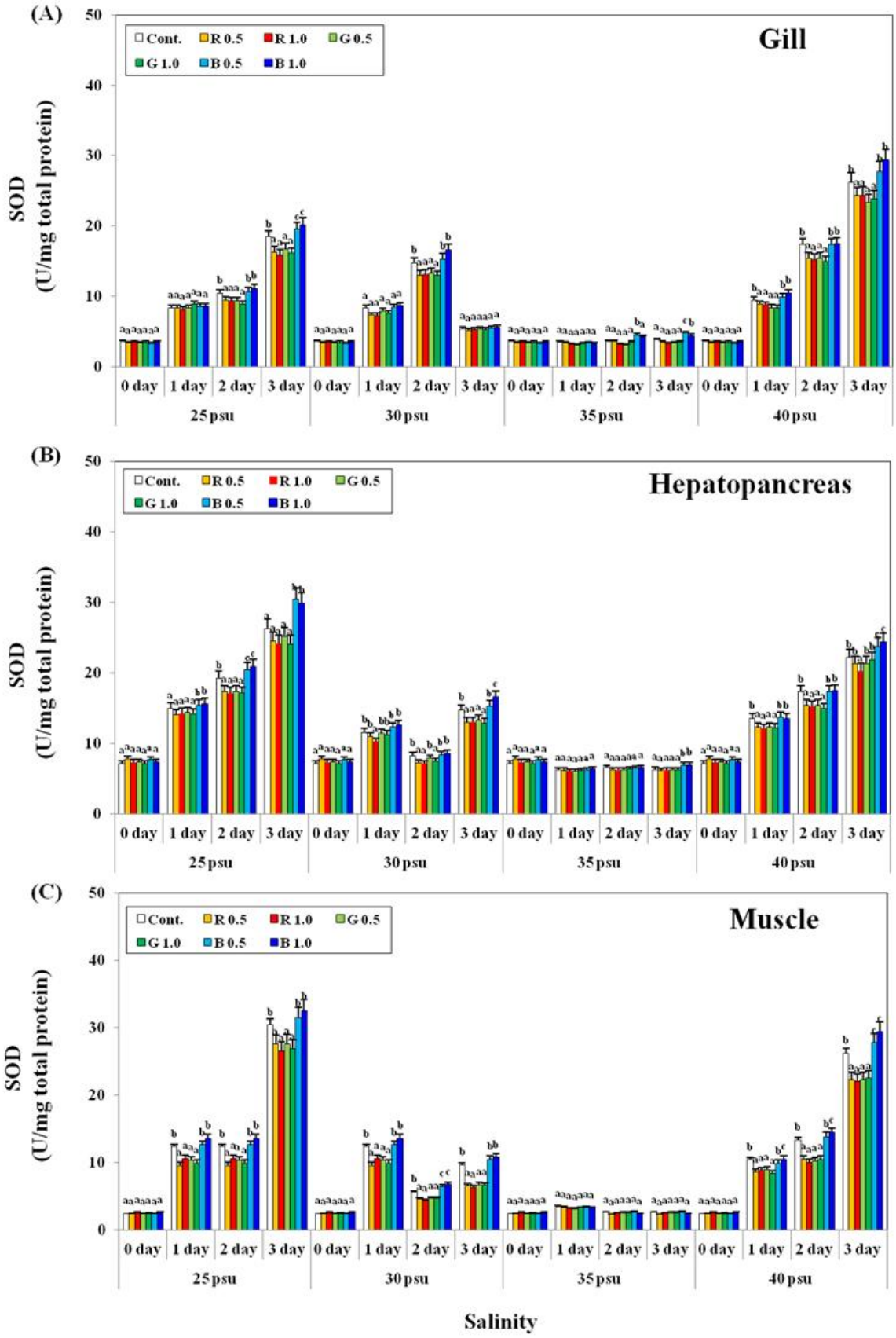
(A)
Gill
Cont.
R 0.5
R 1.0
G 0.5
G 1.0
B 0.5
B 1.0
SOD
(U/mg total protein)
0 day
1 day
2 day
3 day
25psu
30psu
35psu
40psu
(B)
Hepatopancreas
(C)
Muscle
Salinity

Fig. 10. Concentrations of SOD in the gill (A), hepatopancreas (B), and muscle (C) 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 analyzed with a plate reader. Values with letters indicate significant differences a mong lights of different wave lengths over the same salinity and period ($P < 0.05$). All values are means ± SE ($n = 5$).

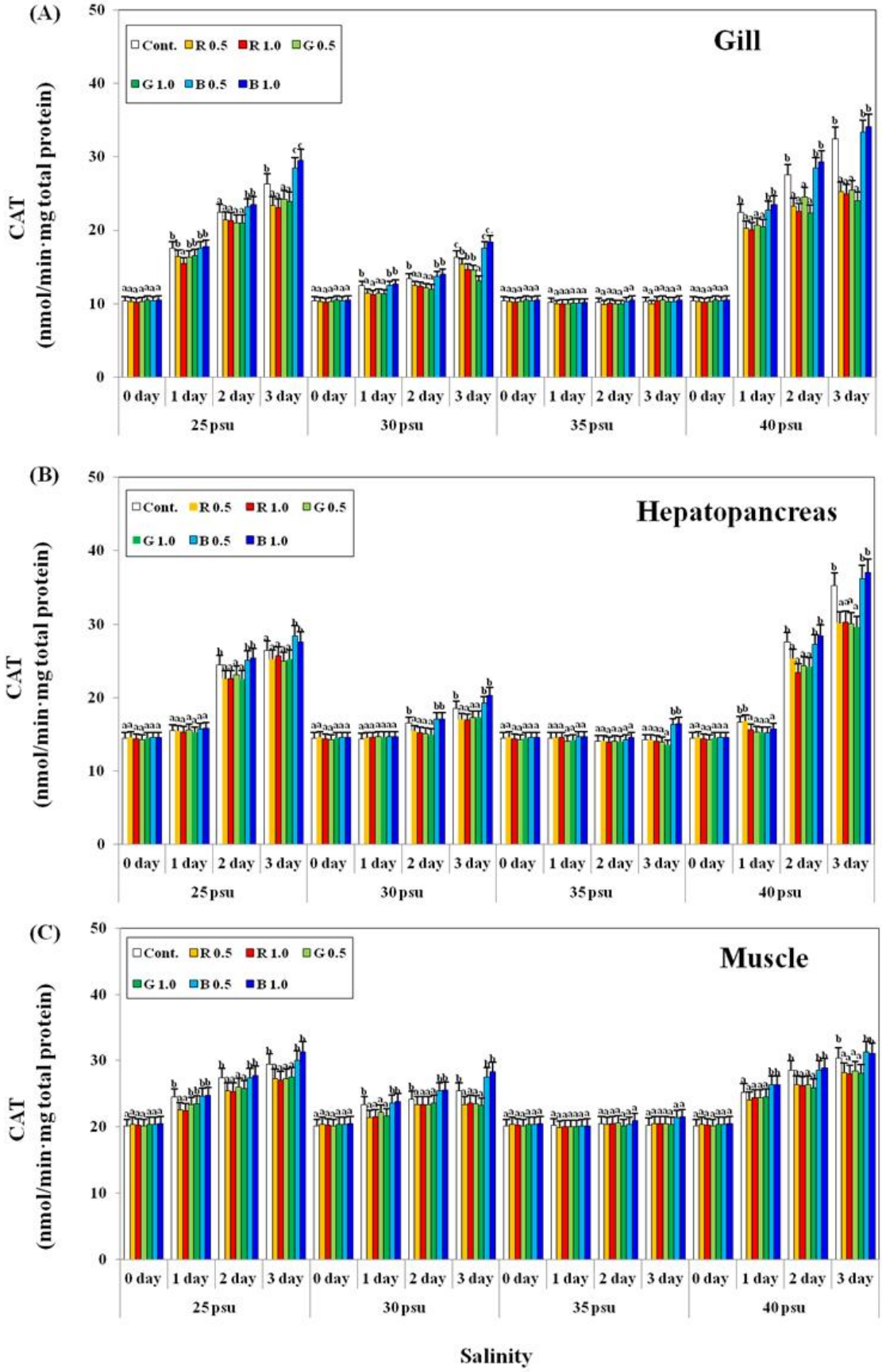
(A)
Gill
Cont. R 0.5 R 1.0 G 0.5
G 1.0 B 0.5 B 1.0
CAT
(nmol/min·mg total protein)
0 day 1 day 2 day 3 day
25 psu
30 psu
35 psu
40 psu
(B)
Hepatopancreas
(C)
Muscle
Salinity

Fig. 11. Concentrations of CAT in the gill (A), hepatopancreas (B), and muscle (C) 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 analyzed with a plate reader. Values with letters indicate significant differences a mong lights of different wave lengths over the same salinity and period ($P < 0.05$). All values are means ± SE ($n = 5$).

3.5. Change in LPO concentration

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. 12). 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→1.0 W/m^2).

3.6. 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. 13). 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.

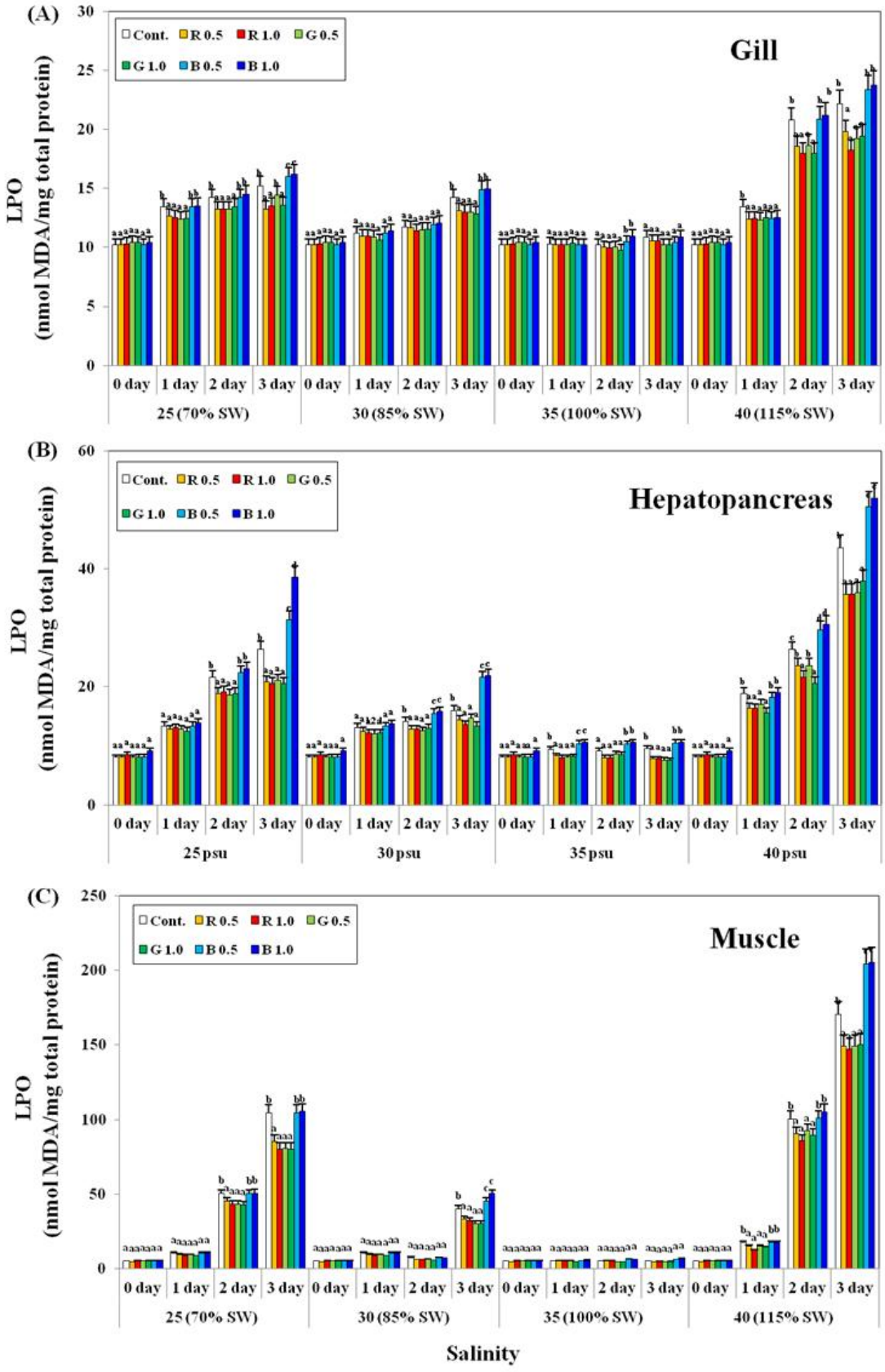
(A)
Gill
Cont. R 0.5 R 1.0 G 0.5 G 1.0 B 0.5 B 1.0
LPO (nmol MDA/mg total protein)
0 day 1 day 2 day 3 day
25 (70% SW)
30 (85% SW)
35 (100% SW)
40 (115% SW)
(B)
Hepatopancreas
25 psu
30 psu
35 psu
40 psu
(C)
Muscle
Salinity

Fig. 12. Concentrations of LPO in the gill (A), hepatopancreas (B), and muscle (C) of *Lysmata amboinensis* 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.)]. LPO was analyzed with a plate reader. Values with letters indicate significant differences a mong lights of different wave lengths over the same salinity and period ($P < 0.05$). All values are means ± SE ($n = 5$).

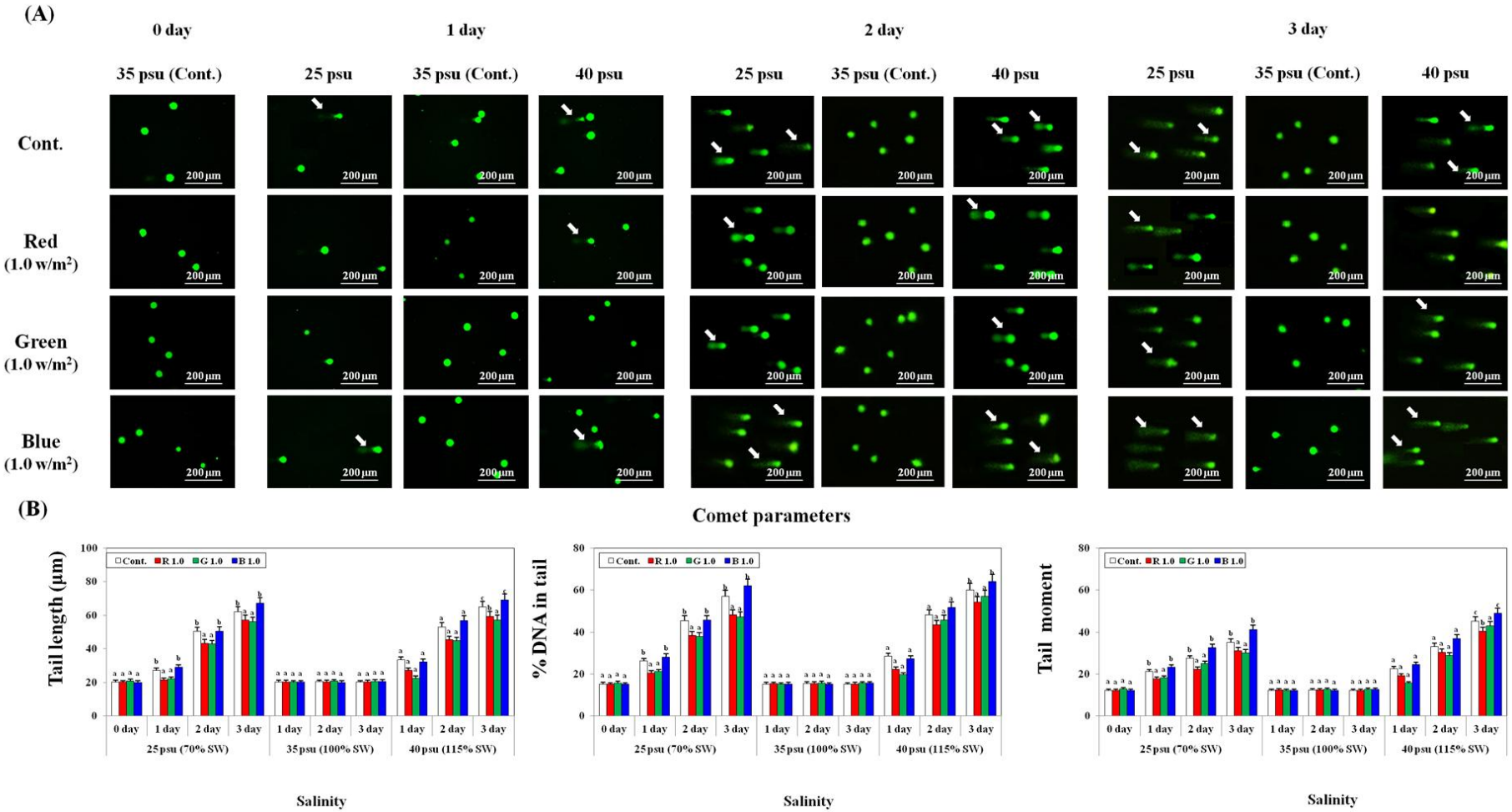
(A)
0 day
1 day
2 day
3 day
35 psu (Cont.)
25 psu
35 psu (Cont.)
40 psu
25 psu
35 psu (Cont.)
40 psu
25 psu
35 psu (Cont.)
40 psu
Cont.
Red (1.0 w/m²)
Green (1.0 w/m²)
Blue (1.0 w/m²)
200 μm
(B)
Comet parameters
Tail length (μm)
% DNA in tail
Tail moment
Cont.
R 1.0
G 1.0
B 1.0
0 day
1 day
2 day
3 day
25 psu (70% SW)
35 psu (100% SW)
40 psu (115% SW)
Salinity

Fig. 13. 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*. White arrows in (A) indicate damaged nuclear DNA (DNA breaks) in the hepatopancreas cells, which were stained using SYBR Green. Scale bars = 100 μm. Lowercase letters in (B) represent significant differences within the same experimental group and within the same salinity treatment ($P < 0.05$).

4. 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.

I 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 and 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*. I 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 (350–00 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 study the range of optically recognized wavelengths and the photoreactive mechanisms in the species.

I 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 and 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). I 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* (Milne Edwards, 1853) were exposed to various salinities (1, 2, 10, 15 psu) in a river (5 psu). This 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 stressreducing 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), I conclude that salinity changes increased the secretion of glucose and HSP70 in the tissues of *L. amboinensis*. and that red- and green-wavelength light had a stress-reducing effect. I suggest additional research on the mechanisms of the stress-reducing effects of red-wavelength light in *L. amboinensis* and other shrimps.

This study also 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 damage to cells (Roch, 1999; Nordberg and 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.

In previous studies, cell damage increased as a result of exposing cleaner shrimp to changes in salinity in the current study. 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 and Arner, 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. 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.

I 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. In addition, the light of red and green wavelengths reduces the oxidative stress of cleaner shrimp. 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. This work aims to provide a basis for further research into light responses in crustaceans.

Chapter 4.

Effects of E_2 injection and red wavelength light on eyestalk hormones and vitellogenesis of cleaner shrimp *Lysmata amboinensis*

1. Introduction

Cleaner shrimp *Lysmata amboinensis* (De Man, 1888) is a decapod crustacean that inhabits tropical coral reefs. It is a tropical shrimp known to be popular among marine creature collectors with bright colors and unusual patterns (Calado et al., 2003). In addition, this species maintains a unique symbiotic relationship with host fishes and occupies an important position in the ecology of tanks and also commercially (Vaughan et al., 2017). In particular, it has been termed a "cleaner shrimp" because it maintains an unusual cleaning symbiotic relationship that eliminates parasites, bacteria, and damaged tissues in host fishes (Côté, 2000). In addition, this species has a unique reproductive mechanism called protandric simultaneous hermaphroditism (PSH) unlike other pure male mature species (Bauer and Holt, 1998). PSH is a sex system where an individual organism is transformed into the female phase (FP) through a male phase (MP) similar to general protandric crustacean, but at the FP stage, it characteristically exhibits both male and female functions (Bauer, 2006). According to many studies, PSH is the most common criterion for classifying the size of an individual that distinguishes between the MP and FP stages (Zhang and Lin, 2005; Tziouveli and Smith, 2009). Furthermore, when the organism is significantly larger than 34 mm, it is judged to be in the FP stage (Zhang and Lin, 2005; Tziouveli and Smith, 2009).

Factors controlling the reproduction and maturation of shrimp have been studied

using various methods. In general, sexual maturation of shrimp is controlled by various hormones such as vitellogenesis-inhibiting hormone (VIH), crustacean hyperglycemic hormone (CHH), and molt-inhibiting hormone (MIH). All these hormones belong to the CHH family of peptides (Ollivaux, et al., 2006) and VIH, a major neuropeptide in the eyestalk that inhibits maturation of ovaries of crustaceans (Adiyodi and Adiyodi, 1970). To date, a commonly used method to induce maturation and ovulation in shrimp is the removal of the eyestalk that secretes gonadotropin-releasing hormone. This method induces sexual maturation, which is inhibited by X-organ/sinus gland complexes (XO-SG) in eyestalk by blocking hormone production (Adiyodi and Subramoniam, 1983). However, the XO-SG secretes VIH as well as CHH and MIH. These hormones are involved in glucose metabolism and inhibits molting. It is also known to regulate the synthesis of vitellogenin (VTG), an egg yolk protein, in a variety of decapods including lobster, penaeid shrimp, and brachyuran crab (Alcock, 1900; Gu et al., 2002; Zmora et al., 2009). Therefore, although eyestalk ablation in crustaceans may promote sexual maturation and ovulation since the secretion of CHH and MIH is blocked, it is not effective in producing high-quality eggs.

In addition, sexual maturation of shrimp is generally known to be controlled by various hormones in the eye, 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). However, to date, no research study has investigated the function of each hormone in crustaceans (Subramoniam, 2017). Sexual steroid hormones are known to be the only substances that synthesize VTG, an egg yolk protein (Wallace, 1985). In crustaceans, vertebrate-type sex steroids have also been shown to play a role in the induction of vitellogenesis (Subramoniam, 2016).

In addition, sexual maturation of crustaceans is controlled by various external environmental factors, among which light is important in regulating the physiology of crustaceans. In particular, light wavelengths transmit a limited spectrum depending on the water depth (Weinberg, 1976), which affects the functions of aquatic

organisms in such environments including their physiology such as reproductive cycle and growth, and behavior such as avoidance and search for food. The effects of light wavelengths on crustaceans have been studied with varying results depending on species and wavelength. Wang et al. (2003) reported that *Fenneropenaeus chinensis* (Osbeck, 1765) has rapid growth and molting cycles under green and blue wavelengths. In addition, the ovarian maturation of *Litopenaeus setiferus* (Linnaeus, 1767) and *Farfantepenaeus duorarum* (Burkenroad, 1939) was promoted under blue and green light conditions, and the number of eggs produced was also increased (Caillouet, 1973; Wurts and Stickney, 1984). Furthermore, Xu et al. (2003) reported that *Macrobrachium nipponense* (De Haan, 1849 [in De Haan, 1833-1850]) showed the highest light sensitivity and food intake under red light. In addition, Choi et al. (2018) and Choe et al. (2018) reported that red wavelength light effectively reduces the stress of *L. amboinensis* exposed to rapid osmotic changes in the environment, enhancing immunity. However, compared with fish, knowledge about the molecular mechanism related to photoreaction in crustaceans is limited.

Therefore, in this study, I sought to find a new method to determine the correlation between sex 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, I investigated the effects of E_2 and red wavelength light, which are already reported to effectively control positive physiological responses to stress and enhance immunity (Choi et al., 2018), on maturation and vitellogenesis. Specifically, I measured the mRNA expression levels of the estrogen receptor (ER), CHH, VIH, and VTG receptor (VTG-R). In addition, the mRNA expression of VTG in hepatopancreas injected with E_2 at two concentrations or exposed to red wavelength (630 nm) irradiation was examined using in situ hybridization.

2. Materials and methods

2.1. Experimental species

For each experiment, cleaner shrimp *Lysmata amboinensis* (n = 480, length, 39.2 ± 3.6 mm; 1.78 ± 0.43 g) were purchased from the commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in 600-L circulation filter tanks [a tank consist of four mini tanks (reared 5 individual in each mini tanks) (size: 45 cm × 45 cm × 45 cm)]. A total of 160 individuals were used for 32 experiments (Control, E_2 0.5 μg/g injection, E_2 1.0 μg/g injection and red LED) × hours (3, 6, 12, 18, 24, 48, 96, 144 h); Use 5 individuals at once] and it was conducted triplication. 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 ℃, pH 8.0 and 35 psu.

2.2. Light condition and E_2 injection

The experiment was divided into two experiments (the E_2 injection groups and red LED group). E_2 were intra-peritoneally injected at two concentrations (0.5 and 1.0 μg/g body weight), respectively. Red LED group was exposed to 630 nm of 0.5 W/m^2 at bottom of aquarium and I used white fluorescent bulb as a control light source (Cont.). I sampled at 3, 6, 9, 12, 18, 24, 48, 96 and 144 hours after E_2 injections or red LED exposure. The irradiance level at the bottom layer of each tank with external light interception maintained at approximately 0.5 W/m^2 using a spectrometer (MR-16; Rainbow Light Technology, Taiwan) and a photo-radiometer (HD2102.1; DeltaOMHCO., 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 and hepatopancreas collection and stored at −80 ℃ until analysis.

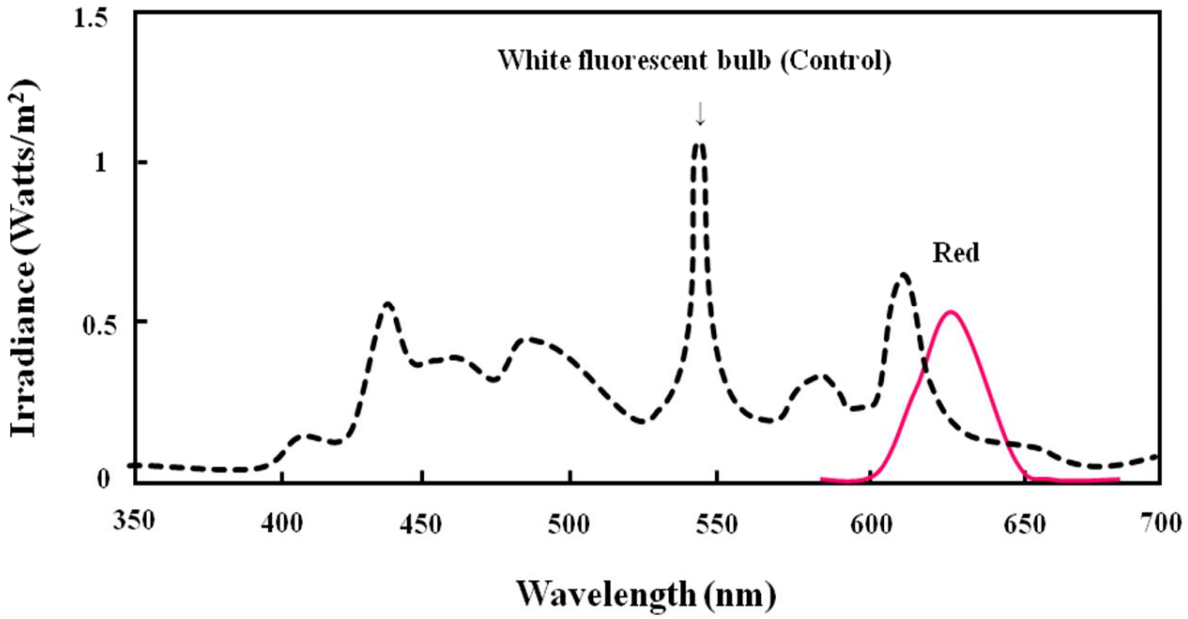
1.5
1
0.5
0
Irradiance (Watts/m²)
White fluorescent bulb (Control)
Red
350
400
450
500
550
600
650
700
Wavelength (nm)

Fig. 14. Spectral profiles of red light emitting diodes (LEDs) and white fluorescent bulb (Cont.) used in this study. The light intensities (0.5 W/m^2) were used for each type of LED.

2.3. Total RNA extraction and complementary DNA synthesis

Total RNA was extracted from eyestalk, hepatopancreas, and gonads (for analysis of hormones, the ER, and VTG-R, respectively) using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. Then, 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) 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.

2.4 Quantitative PCR (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 eyestalk 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) 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. As an internal control, experiments were duplicated using β-actin. 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 $\Delta\Delta Ct = 2^{-(\Delta Ct_{sample}-\Delta Ct_{internalcontrol})}$.

2.5. Tissue preparation section

We used 1-day-old individuals from eggs produced in the shrimp. 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 then 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 then cut into 6 μm-thick sections using a freezing microtome (CM1510 S Cryostat, Leica Inc., Germany). All samples were processed quickly to avoid degradation of the RNA.

2.6. Analysis of in-situ hybridization

We modified the in-situ technique of Sumi et al., (2018). VTG mRNA sequence obtain by NGS technique. The template sequence for in-situ hybridization probe designed (MN119283) against the 3′-UTR and CDS (497 bp) of VTG identified by National Center for Biotechnology Information blast system (mRNA 73% homology with *Pandalopsis japonica* vitellogenin, Accesstion NO. KF731996), then was amplified by PCR amplification and ligated to TOPO-TA vector (Invitrogen). 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′) primer. As a result, anti-sense was confirmed by sequencing, and plasmid DNA was amplified by PCR amplification with antisense primer and T7 primer. Digoxigenin (DIG)-labeled probes were then created from DIG RNA Labeling Mix (Roche,

Germany) and the PCR product using anti-sense primer and T7 RNA polymerase for antisense labeling probes.

Fresh hepatopancreas tissue sections were hybridized with hybridization buffer (5 ml deionized formamide, 2.5 ml of 20 × SSC, 100 µl of 0.1% Tween-20, 92 µl of 1 M citric acid (pH 6.0), and DEPC-H_2O upto 20 ml total volume), yeast total RNA (50 µl), and with the RNA probe overnight at 65 °C. Subsequently hybridized sections were thoroughly washed with 75% hybridization buffer plus 25% 2× saline sodium citrate (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), 0.2× SSC (2 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, 22 °C), and PBST (5 min, 22 °C). For hybridization signal detection tissue sections were first incubated with a blocking solution (comprised 10% calf serum in PBST) for 1 h at 22 °C followed by overnight incubation at 4 °C with an alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted 1:2000 in blocking solution) (Roche). Subsequently a series of washing steps (6 × 15 min each wash 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 wash at 22 °C), color imagining was attained using labeling mix (1 ml alkaline tris buffer, 4.5 µl nitroblue tetrazolium, 3.5 µl 5-bromo-4-chloro-3- indoly phosphate disodium salt) and spraying the mix over the sections. Then the sections were kept in a dark and humid chamber for at 8 h to develop color. The slides were washed with PBST, fixed with 4% PFA for 1 h, mounted with Aquamount (Aqua Polymount, Polisciences Inc., Warrington, PA, USA), and cover-slipped. Stereo microscope (Nikon Eclipse Ci, Tokyo, Japan) was used to capture the images. For the quality check of the probe, gill tissue and non-treated hepatopancreas (negative control) were performed.

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., 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 means ± standard error (SE).

3. Results

3.1. Change 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 injection concentration of E_2. At a concentration of 1.0μg/g, a significant increase was observed between 3 and 6h, and continued to increase until 48 h and then decreased. At a concentration of 0.5 μg/g lower than 1.0 μg/g, it slightly increased from 3 to 18 h, but the significant increase was between 18 and 24 h, and then decreased. However, in the red LED group, there was no significant increase until 96 h, but a significant increase between 96 and 144 h (Fig. 15).

3.2. Change in CHH mRNA expression

To investigate the effects of E_2 injection and red LED on sex maturation in this species, I measured changes in CHH, known as ocular sex hormone promoting sexability. In the group injected with E_2, CHH mRNA expression tended to decrease with increasing E_2 injection concentration, the CHH mRNA expression no significant change. However, it increased from 3 h to 18 h in red light and remained high up to 144 h. (Fig. 16).

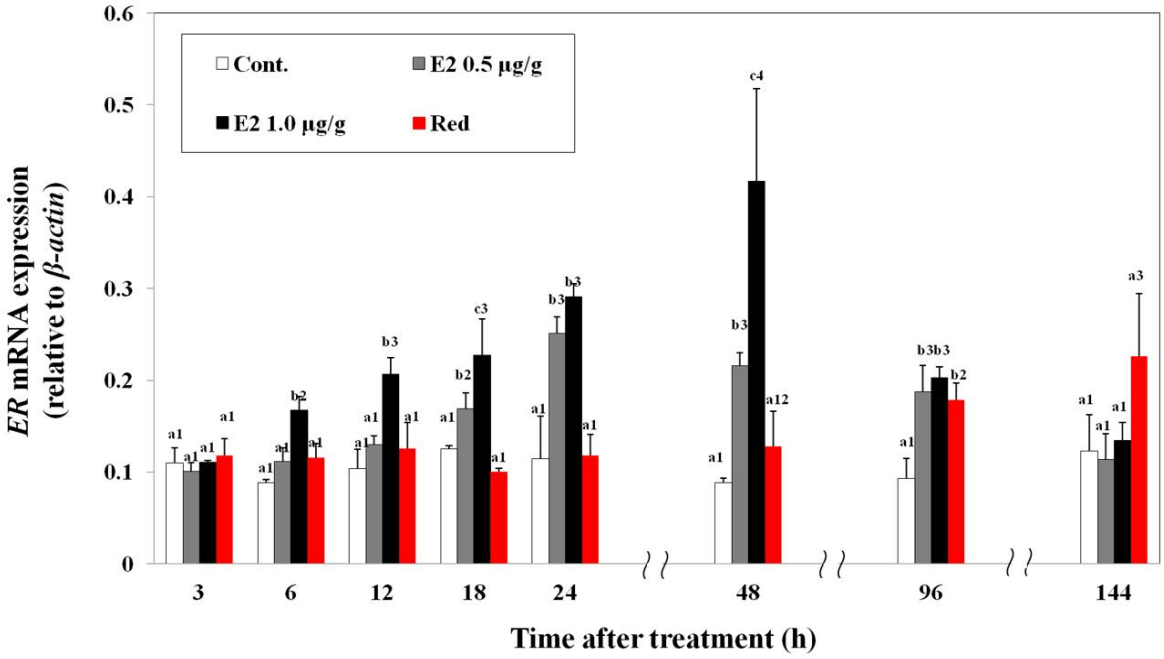
Cont.
E2 0.5 μg/g
E2 1.0 μg/g
Red
ER mRNA expression
(relative to β-actin)
0.6
0.5
0.4
0.3
0.2
0.1
0
3
6
12
18
24
48
96
144
Time after treatment (h)

Fig. 15. Changes of the ER mRNA expressions in the hepatopancreas in *L. amboinensis*. The tissues were taken at 3, 6, 12, 18, 24, 48, 96 and 144 hours after E_2 in jection or irradiation of red LED. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in same experimental methods and exposure time ($P < 0.05$). All values are means ± SE (n=5).

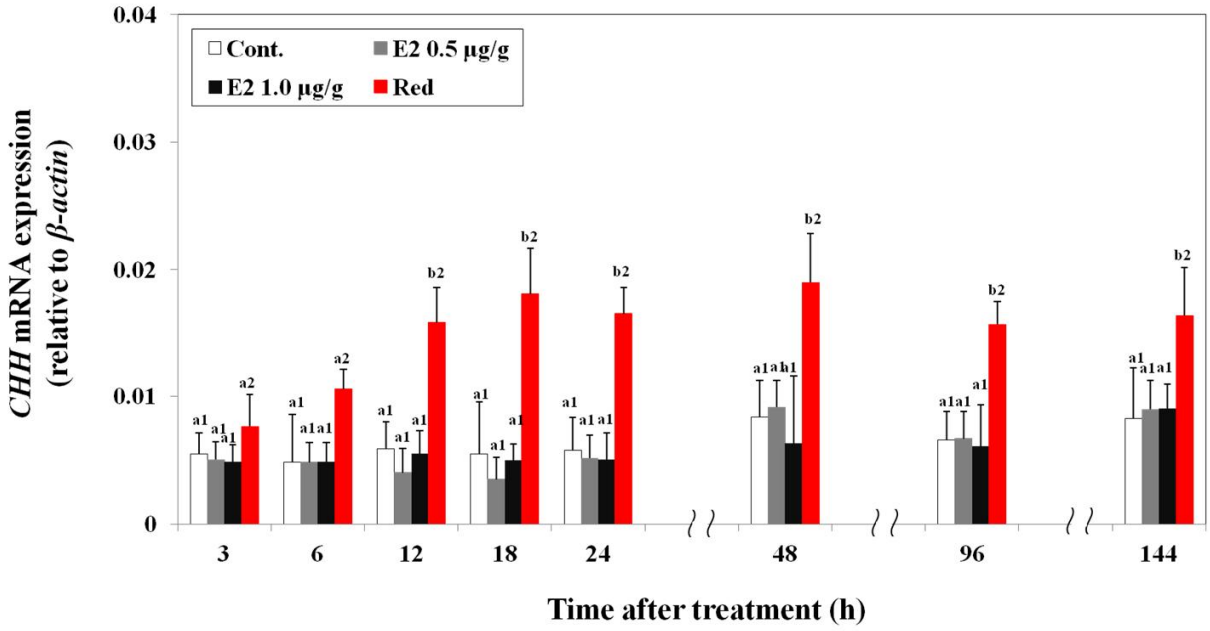
Cont.
E2 0.5 µg/g
E2 1.0 µg/g
Red
CHH mRNA expression
(relative to β-actin)
0.04
0.03
0.02
0.01
0
3
6
12
18
24
48
96
144
Time after treatment (h)

Fig. 16 Changes of CHH mRNA in the eyestalk in *L. amboinensis*. The tissues were taken at 3, 6, 12, 18, 24, 48, 96 and 144 hours after E_2 injection or irradiation of red LED. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in same experimental methods and exposure time ($P < 0.05$). All values are means ± SE (n=5).

3.3. Change in VIH mRNA expression

To investigate the effects of E_2 injection and red LED on sex maturation in this species, I measured changes in VIH, known as ocular sex hormone suppressing sexability. As a result, the results were in conflict with the results of CHH. In the group injected with E_2, the VIH mRNA expression no significant change. However, it remained to 24h in red LED and decreased from 48h to 144h (Fig. 17).

3.4. Change in VTG-R mRNA expression

To investigate the effects of E_2 injection and red LED on vitellogenesis in this species, we measured changes in VTG-R, known as indicator vitellogenesis. As a result, VTG-R mRNA expression was increased in both E_2 injrction and red irradiation group. However, the increasing time of VTG-R mRNA expression was different in both treatments (From 3 h in the E_2 treatment group; form 48 h in the Red irradiation group). In addition, E_2 injection groups showed peak value at 18h, and thereafter decreased (Fig. 5).

3.5. VTG mRNA expression by in-situ hybridization

Progress in the gill tissue for the quality check of the probe did not detect the expression of color, and the same results were obtained in the non-treated hepatopancreas (Figs. 6A and 6B). In-situ hybridization of VTG mRNA confirmed with 18h and 144h, the highest expression of VTG-R mRNA in each experiment, compared to 3h. As a result, VTG mRNA expression in hepatopancreas tissue was similar to that of VTG-R mRNA expression, and was highest at 18 h in E_2 injection group. In addition, the higher was observed in the group of red irradiation after exposure 144h, however, the E_2 injection group showed a tendency to decrease (Fig. 6C)

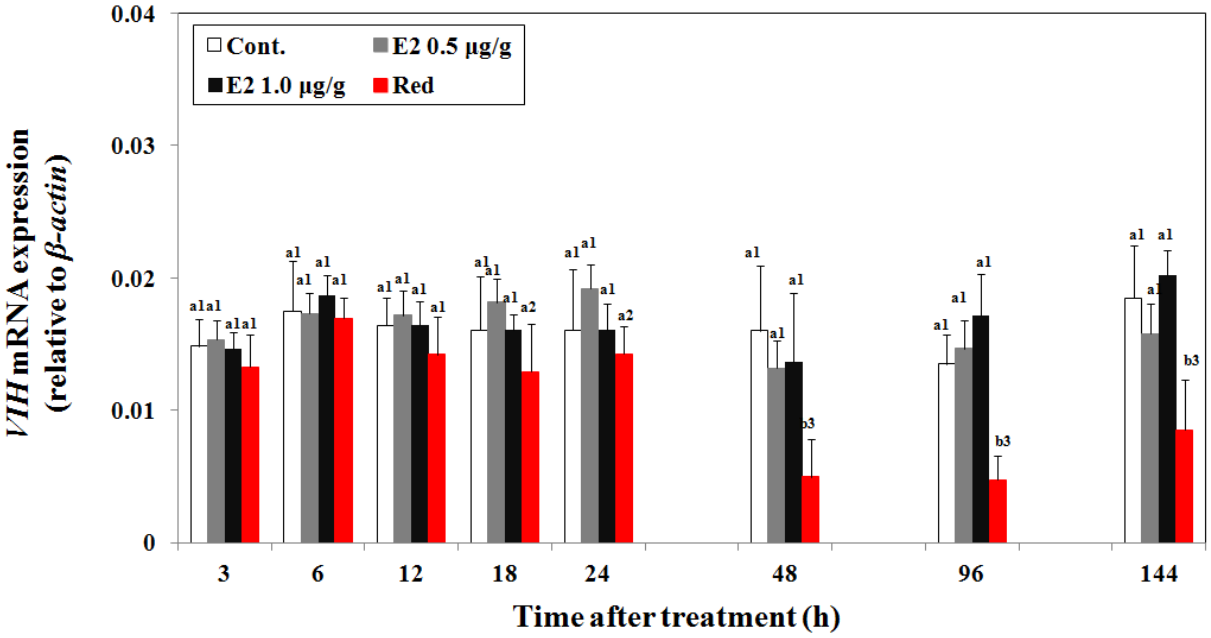
Cont.
E2 0.5 μg/g
E2 1.0 μg/g
Red
VIH mRNA expression
(relative to β-actin)
0.04
0.03
0.02
0.01
0
3
6
12
18
24
48
96
144
Time after treatment (h)

Fig. 17. Changes of VIH mRNA in the eyestalk in *L. amboinensis*. The tissues were taken at 3, 6, 12, 18, 24, 48, 96 and 144 hours after E_2 in jection irirradiation of red LED. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in same experimental methods and exposure time ($P < 0.05$). All values are means ± SE (n=5).

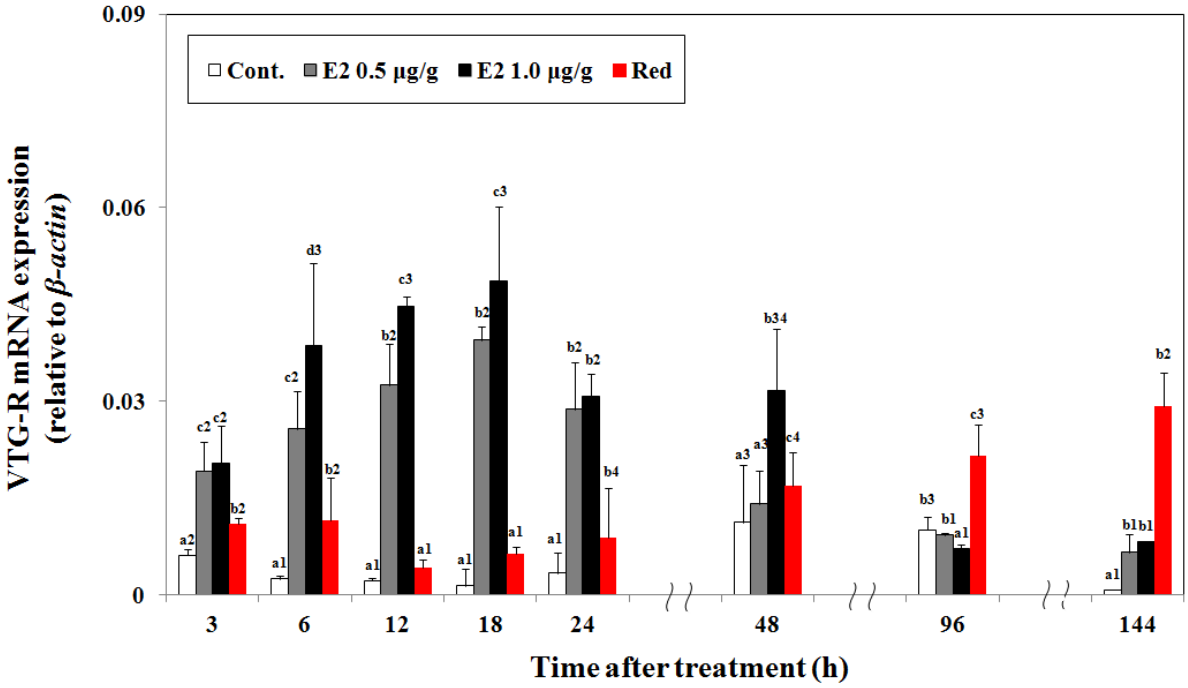
Cont.
E2 0.5 μg/g
E2 1.0 μg/g
Red
VTG-R mRNA expression
(relative to β-actin)
0.09
0.06
0.03
0
3
6
12
18
24
48
96
144
Time after treatment (h)

Fig. 18. Changes of VTG-R mRNA in the gonad in *L. amboinensis*. The tissues were taken at 3, 6, 12, 18, 24, 48, 96 and 144 hours after E_2 injection or irradiation of red LED. The different lower cases indicate significant difference of exposure time. The different number indicate significant difference between each experiment group in same experimental methods and exposure time ($P < 0.05$). All values are means ± SE (n=5).

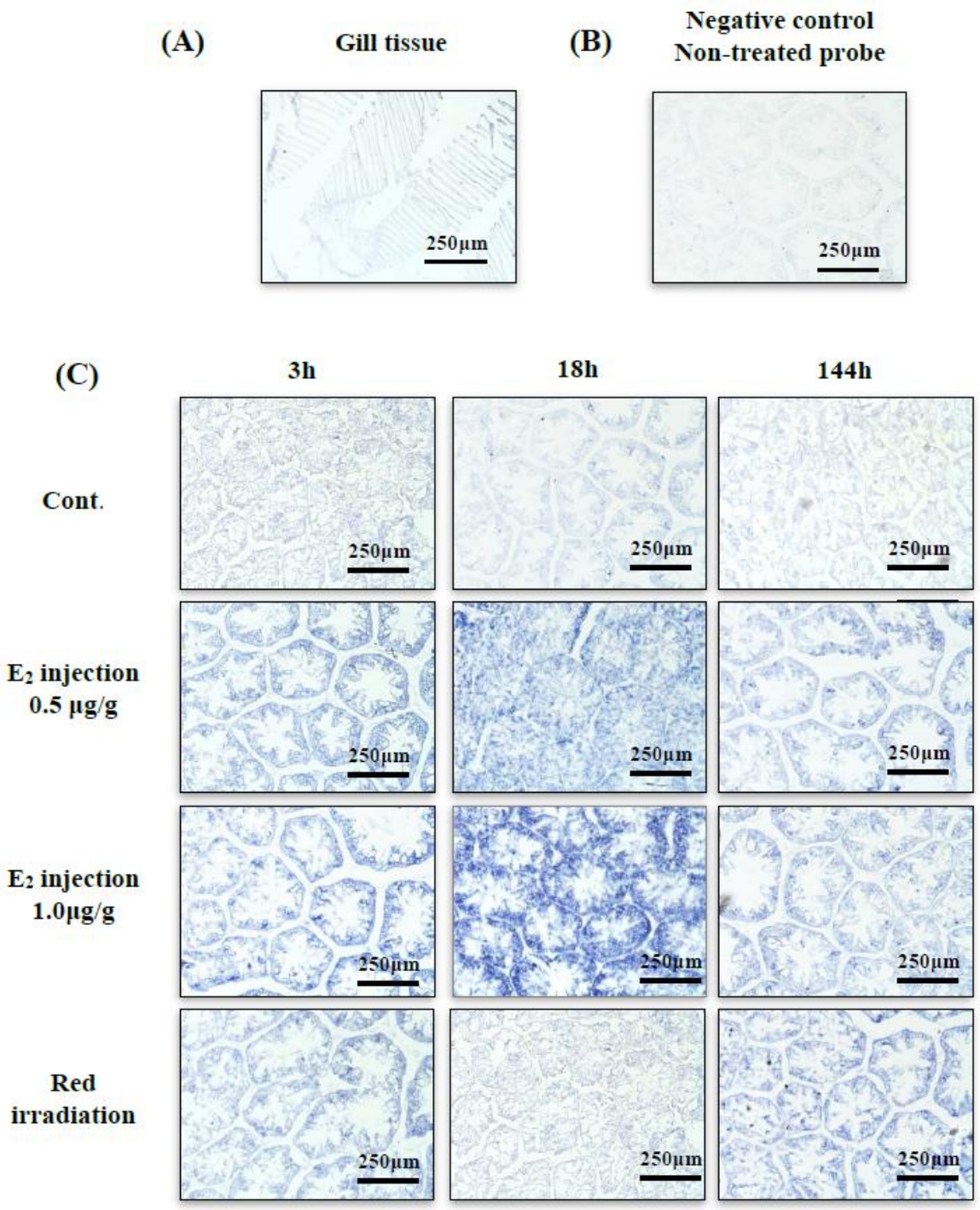
(A)
Gill tissue
(B)
Negative control
Non-treated probe
250μm
(C)
3h
18h
144h
Cont.
E2 injection
0.5 μg/g
E2 injection
1.0μg/g
Red
irradiation

Fig. 19. In-situ htbridization images of VTG mRNA in gill (A) and hepatopancreas (B, C). To probe quality (A) and Non-treated probe image (B). The tissues were taken at 3, 18 and 144 hours after E_2 injection or irradiation of red LED. As a result of the gill tissue for the quality check of the probe (A), non-probe treatment results were presented in hepatopancreas tissue (B). In-situ hybridization of VTG mRNA confirmed with 18h and 144h, the highest expression of VTG-R mRNA in each experiment, compared to 3h (C). Scale bars = 250 μm.

4. Discussion

Unlike fish, crustaceans are sexually mature through various hormones secreted from a special tissue called x-organ / sinus gland complex in eyestalk, and many studies have been conducted (Nagaraju, 2011). Recently, the discovery of vertebrate-type sex steroid hormones in hepatopancreas and ovary (Subramoniam, 2000; Guan et al., 2013) suggests that various sexual maturation mechanisms exist. These findings have led to the need for further studies on the interaction with sex hormones associated with sex-related X-organ / sinus gland complex.. Therefore, in this study, I investigated whether the vertebrate-type sex steroid E_2 and red light wavelength were effective in the control of eyestalk hormone in sexual maturation.

As a result of investigating the effect of irradiation of E_2 injection and red wavelength on the expression of ER mRNA, the expression of ER mRNA was significantly different between injected E_2 concentrations of 0.5 μg/g at 24 h and 1.0 μg/g at 6 h in the E_2 injection group respectively. Sexual maturation regulated by E_2 in the vertebrate ssuch as fish already known (Pakdel et al., 1991). However, it has been reported that sexual maturity occurs by hormones secreted from the eyestalk in crustaceans (Yano et al., 2000), but the role of E_2 in crustaceans has not yet been reported. Quinitio et al. (1991) reported that E_2 is closely related to changes in VTG during the ovarian cycle in *Pandalus kessleri* (Czerniavsky, 1878) and *Penaeus monodon* (Fabricius, 1798). In addition, the injection of E_2 in *P. monodon* stimulated the synthesis of VTG in the ovary and hepatopancreas, and the amount of ER as an E_2 receptor was increased. This phenomenon has been reported as evidence of maturation (Merlin et al., 2015). Therefore, in this study, the increase of expression level of ER mRNA in E_2 injection group is considered to be the result of sexual maturation by stimulating E_2 maturation promotion. In contrast, the injection of E_2 increased the ER expression level, but did not affect the expression level of the hormone secreted by the X-O/SG complex. In other words, the sex steroid hormone E_2 seems to induce sexual maturation of the hepatopancreas

and ovary. However, Subramoniam (2011) reported that the pathways involved in sex maturation by sex steroids including E_2 and hormone secreted by the X-organ/sinus gland complexes of eyestalk are different pathways for sexual maturation in crustaceans. Therefore, from the results of Subramoniam (2011), it is considered that E_2 in *L. amboinensis* used in this study did not affect the expression of VIH and CHH mRNA, and E_2 directly influenced egg maturation.

On the other hand, in general, the organs that receive the light signal are the retina, which recognizes the color and intensity by the photoreceptor in the retina. However, crustaceans accept 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 X-organ / sinus gland complex. As in Choi et al. (2018) report that the red wavelength light affected the stress reduction and immunity enhancement of *L. amboinensis*, the red wavelength was suggested to be a physiologically variable wavelength for *L. amboinensis*. To date, the red wavelength light has been used as a wavelength to minimize stress during the dissection process of crustacean samples (Calado, 2009), but Pudadera and Primavera (1981) found that the red wavelength light lead to incomplete maturation, and also reported that the red wavelength would not be sensitive to aquatic organisms because it was rapidly absorbed in water and could not transmitte into the deep water. However, when *L. amboinensis* was irradiated with red light, changes in the expression level of the hormone secreted from the X-O/SG complex were observed. CHH mRNA tended to increase significantly from 3 h after irradiation, and the expression of VIH mRNA which inhibits maturation tended to decrease significantly from 3 h. There are many studies on the effects of CHH on maturation in shrimp. De Kleijn et al. (1995) found that CHH plays a trigger in the development of yolk protein and oocyte, whereas Khayat et al. (1998) have reported conflicting results that CHH inhibits ovarian protein synthesis and mRNA expression. However, in the present study, it is suggested that CHH, which triggers sex maturation, is also a hormone induced by red wavelength, considering that red light has a wavelength that promotes sexual maturation. In addition, in the group irradiated with the red wavelength, the

expression level of ER did not increase more rapidly than that of the group injected with E_2, but the expression level of ER increased significantly from 96 h (day 4) after the irradiation of the red wavelength to 144 h (6th day). The increase of ER mRNA expression related to sexual maturation in red wavelength light is considered to be related not only to the secretion of the eyestalk hormone but also to the secretion and action pathway of E_2 as a steroid hormone. In addition, the results of VTG-R mRNA expression and in situ hybridization to VTG mRNA can support the E_2 injection method and red irradiation as a method of promoting egg maturation and vitellogenesis.

Conclusionally, E_2 injection seems to affect sexual maturation of *L. amboinensis* but does not affect eyestalk hormone. On the other hand, red light induces not only the increase of ER mRNA but also the significant expression of CHH mRNA and a decrease in the expression of VIH mRNA, It was confirmed that the red wavelength light is effective for sex maturation. In particular, we suggest that the red irradiation method is a method of promoting vitellogenesis by regulating mRNA expression of VTG and VTG-R by regulating the secretion of eyestalk hormones and E_2 during maturation (based on ER mRNA expression induction). Therefore, the red wavelength irradiation method can be used as an efficient new method to replace the maturation method by eyestalk ablation.

Chapter 5.

General Discussion

Cleaner shrimp *Lysmata amboinensis* is an important species for which comprehensive research is urgently needed by the ecological aspect due to the unusual symbiotic relationship with the host fishes, the industrial aspect due to the world's second largest demand as an invertebrate aquarium species, and the physiological aspects due to the unique sexual maturation system. Especially, because they are unable to cultivate due to lack of aquaculture technology and depend on natural collection, they are classified as very expensive species and further research is needed.

In addition, due to the specificity mentioned above, it is considered to be a suitable species to represent crustaceans with high light sensitivity and unusual sexual maturation system of *L. amboinenesis*. So, the aim of this study was to evaluate the overall physiological response and the effects of specific light wavelengths on circadian rhythm, osmotic stress, sex maturation and vitellogenesis in cleaner shrimp *L. amboinensis*.

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).

In this research results, I confirmed 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. I 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. On the other hand, melatonin, which decreased the antioxidant stress and increased immunity, was higher in the DD group than in the red light group. This result suggests that red light irradiation may be positive in terms of stress and immunity, even though green light irradiation has a distinct expression of clock gene. Thus, there is doubt as to whether the change in the daytime clock gene due to inevitable activity due to the symbiotic relationship with host fish in basically a nocturnal crustacean *L. amboinensis* is positive effect for this species. Therefore, based on the results of only this study, it is difficult to confirm that the light of the red wavelength and the light of the green wavelength are effectively effective wavelengths in terms of physiological aspects of the present species. Nevertheless, the results of this study confirm the light response patterns of photoperiod and specific wavelength light in coral crustaceans, which are inevitably exposed to unspecific light, and this result can be a basis for future research.

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. Especially, in this study, I measured stress due to salinity and experimented with light of specific wavelength to reduce the stress. Most aquatic organisms are stressed when they are exposed to a salinity changing environment. I analyzed the stress in two aspects: the response to maintain homeostasis and the antioxidant response. As a result, I confirmed that the salinity change promoted the NKA activity, Glucose level and HSP 70 concentration of *L. amboinensis*, and decreased with time due to adapt to the salinity. In addition, green and red light showed that *L. amboinensis* decreased oxidative stress due to salinity change and present low antioxidant enzyme secretion. DNA damage of liver pancreatic cells was decreased in both green and red wavelengths in accordance with the two previous results.

However, the mechanism for the stress-relieving effect of light is still unknown. So, I would like to present two possibilities for the results of this study. First, the red wavelength light is likely to reduce salt stress due to increased melatonin, which enhances immunity and antioxidant function based on the results of previous biorhythm. Petrovsky (2001) and Mayo et al. (2002) reported that melatonin play up-regulation of immune function and antioxidant function. On the other hand, in general, most organism prefers the wavelength of light in inhabit where there is a lot of green light, and physiologically stable (Stevens et al., 2017). Therefore, unlike the red wavelength, the green wavelength is considered to be the most irradiated wavelength in the habitat of this species.

Although this study did not reveal the mechanism of photo-reaction to stress, it has been found that green and red light is an effective light to reduce the salt stress of *L. amboinensis*, and it is suitable as a light of the wavelength band preventing sudden environmental change light.

In general, for aquatic organisms, red wavelength light is known as light that causes stress or inhibit growth and sexual maturity. It has been reported that this is due to the low penetration into the water. On the other hand, red wavelength of light cannot penetrate into the habitat of aquatic organisms, which has been used as lighting light to minimize stress in research targets due to their low sensitivity to red wavelength light.

However, in relation to the results of the previous two studies, red wavelength light was found to be a factor causing changes in circadian rhythm, and especially effective wavelengths that reduce stress. In this regard, as a result of the effect on eyestalk hormone, which regulates sexual maturity and vitellogenesis, I confirmed that red wavelength light is effective for maturity by inducing not only an increase in ER, CHH, VTG-R mRNA but also a significant decrease in VIH mRNA expression. In addition, I also observed an increase in VTG mRNA in the hepatopancreas through in-situ hybridization. Considering that CHH and VIH have been released from X-organ/sinus glands complex present within the light-accepting organ eyestalk, the light signals in red wavelength light are judged to affect the nerves in X-organ/sinus glands complex and regulate the secretion of sex-maturation

hormones and induction of vitellogenesis. There is no aquaculture technology of this species to date, but in most crustaceans, eyestalk ablation and the treatment of sex steroid hormones such as E_2 were used for promoted sex maturation as a one of aquacultural technology. However, given the inefficiency and economic losses associated with the treatment of high E_2 hormones, it is judged that the use of red wavelengths light will be an effective method of inducing sexual maturity and vitellgenesis.

In conclusion, as mentioned above, the study found the overall physiological changes, the photoreaction effects of specific wavelengths irradiation, and the new method of controlling sexual maturation and vitellogenesis of *L. amboinensis*. I presented that schematic diagram showing the physiological response of *L. amboinensis* following specific light wavelengths identified in this study (Fig. 20). Although this study did not provide further information of detailed photoreaction mechanisms for changes in circadian rhythm, stress-reducing effects, sex maturation and vitellogenesis, the results of this study were based on the various hypotheses presented above. In addition, given that the *L. amboinensis* is a ornamental species, the method that red wavelength light irradiation alone can promote sexual maturation is deemed to be very effective in comparison to the eyestalk ablation or the treatment of high-priced sex steroid hormones such as E_2. Therefore, I expect that this result to be a useful for the aquaculture technology industry in the future.

Finally, although *L. amboinensis* is known as the species of ecological and physiological importance and is currently the second invertebrate trade species in the world (Wabnitz et al., 2003), as a result of poor development of aquaculture technology for various reasons to date, this species is expected to run out of its biological resources due to its dependence on natural collection. The results of this study may be used as basic data for the development of modelling aquaculture techniques.

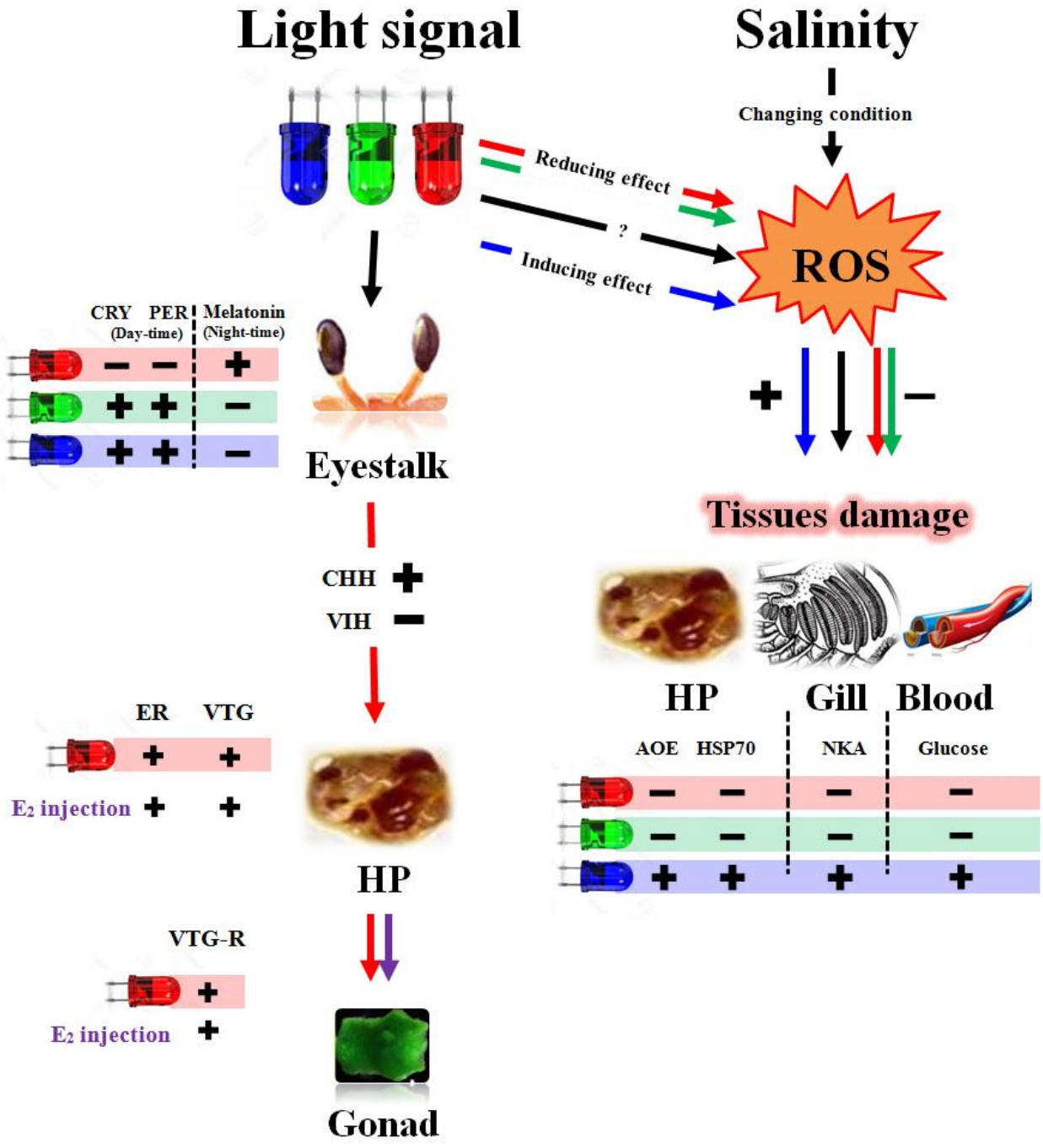
Light signal
Salinity
Changing condition
Reducing effect
?
Inducing effect
ROS
CRY PER
(Day-time)
Melatonin
(Night-time)
Eyestalk
Tissues damage
CHH
VIH
ER VTG
E2 injection
HP
Gill Blood
AOE HSP70
NKA
Glucose
VTG-R
E2 injection
Gonad

Fig. 20. Schematic diagram showing the physiological response of *L. amboinensis* following specific wavelength lights identified present study. Present the changes of physiological regulators in tissues (eyestalk, hepatopancreas, and gill) by irradiation of each wavelength lights. A line with black arrows (→) presents the expected physiological mechanism. A line with colored arrows presents the pathway of red (→), green (→), and blue (→) wavelength light to target tissue. The plus/minus sign was expressed as the result of higher (✚) or lower (▬) compared with the value measured in the non-irradiated shrimp. A question mark symbol (**?**) means a mechanism pathway that has not been revealed or expected. Each abbreviation is as follows: AOE, antioxidative enzyme; CHH, crustacean hyperglycemic hormone; CRY, cryptochrome; E_2, 17β -estradiol; ER, estrogen receptor; HSP70, heat shock protein; NKA, Na^+/K^--ATPase; PER, period; ROS, reactive oxigen species; VIH, vitellogenesis inhibiting hormone; VTG, vitellogenin; VTG-R, vitellgenin receptor.

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