Thesis for the Degree of Doctor of Philosophy

Effects of Light Emitting Diode (LED) Spectral Sensitivity on Molecular and Endocrine Regulation in Three Marine Teleosts

Hyun Suk Shin

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Advisor: Prof. Cheol Young Choi

by

Hyun Suk Shin

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Hyun Suk Shin

Approved as to style and content by:

Young Jin Chang, Ph.D.
Chairman

In-Seok Park, Ph.D.
Member

Sung Hwoan Cho, Ph.D.
Member

Byung Hwa Min, Ph.D.
Member

Cheol Young Choi, Ph.D.
Member

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List of Abbreviations

4-HNE	4-hydroxynonenal
aa	amino acid
AANAT	*arylalkylamine-N-acetyltransferase*
ANOVA	analysis of variance
BW	body weight
CAT	catalase
cDNA	complementary deoxyribonucleic acid
Cry	*Cryptochrome*
Ct	cycle threshold
CT	circadian time
Cys	cysteine
DD	constant dark
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
E_2	estradiol-17β
Exo-RH	extra-ocular rhodopsin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GSI	gonadosomatic index
GPX	glutathione peroxidase
GPCR	G protein-coupled receptor
HIOMT	hydroxyindole-O-methyltransferase
LED	light emitting diode
LD	12-h light:12-h dark
LL	constant light

LPO	lipid peroxidation
MDA	malondialdehyde
mRNA	mitochondrial ribonucleic acid
MT	melatonin receptor
MS-222	tricaine methanesulphonate
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
Per	*Period*
PMSF	phenylmethylsulfonyl fluoride
PNS	peri-nucleolus
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene diflouride
PYS	primary yolk stage
RACE	rapid amplification of cDNA ends
RH	rhodopsin
ROS	reactive oxygen species
RT	reverse transcription
SCN	suprachiasmatic nucleus
SD	standard deviation
SNP	simulated natural photoperiod
SOD	superoxide dismutase
SYS	secondary yolk stage
TBS	Tris-buffered saline
TYS	tertiary yolk stages
QPCR	quantitative polymerase chain reaction
VA	vertebrate ancient
VTG	vitellogenin
ZT	zeitgeber time

3종 해산 어류의 분자내분비 조절에 미치는 발광다이오드(LED) 파장의 영향

신 현 숙

한국해양대학교 대학원 해양생명환경학과

요 약

1. 넙치 *Paralichthys olivaceus*의 망막과 송과체에서 로돕신 및 엑소로돕신 유전자 발현

넙치의 광수용체의 일종인 로돕신 유전자 family의 분자적 메커니즘을 파악하기 위하여 로돕신과 엑소로돕신 mRNA의 발현량 변화를 관찰하였다. 넙치의 망막과 송과체에서 로돕신과 엑소로돕신 cDNA를 각각 분리하였으며, 28 시간 동안 명·암기(LD), 명기(LL) 및 암기(DD) 조건에서 넙치의 다양한 조직을 대상으로 이들 유전자의 발현량 변화를 분석하였다. 로돕신은 LD 광 조건의 ZT4와 28 시간대에서 높은 발현량이 관찰되었으나, ZT16 시간대에서는 가장 낮은 발현량이 관찰되었다. 엑소로돕신의 발현량은 로돕신과 반대의 경향을 보였으며, LD 광 조건에서는 밤 시간대인 ZT20에서 높은 발현량이 관찰되었다. 또한, 송과체 배양 실험(*in vitro*)에서도 비슷한 경향이 관찰되었다. LL과 DD 광 조건에서 로돕신과 엑소로돕신의 발현 경향은 LD 광 조건에서와 유사하게 나타났다. 이는 일반적(대조구)인 LD 광 조건에서 유지되고 있었던 생체리듬이 LL과 DD 광 조건에서도 어느 정도 지속되기 때문에 로돕신과 엑소로돕신의 발현 경향

이 유사한 것으로 보인다. 그러나 LD 광 조건의 경우와 비교해 본 결과, LL과 DD 광 조건에서는 엑소로돕신의 발현량 및 발현 폭이 약하게 관찰되었다. 더욱이 멜라토닌을 송과체에 처리한 *in vitro* 실험의 경우, 멜라토닌을 처리하지 않은 실험구에 비하여 LD 광 조건에서는 100 배, LL 광 조건에서는 10 배 그리고 DD 광 조건에서는 30,000 배 정도 발현량이 낮게 관찰되었다. 또한, 혈장 내 멜라토닌의 농도 변화를 관찰한 결과에서도 LD, LL 및 DD 광 조건 실험구 모두에서 엑소로돕신 유전자의 발현량 변화와 유사한 경향을 보였다. 따라서 넙치의 생체리듬은 외부 광주기 환경에 의해 광수용체의 일종인 로돕신과 엑소로돕신 유전자 그리고 혈액 내에서 멜라토닌의 분비를 통하여 빛의 정보를 전달함으로써 조절되고 있는 것으로 사료된다.

2. 멜라토닌 주입에 따른 넙치의 뇌와 망막에서 멜라토닌 수용체 유전자 발현

넙치의 생체리듬을 파악하기 위하여 3가지 타입 멜라토닌 수용체 mRNA의 조직별 발현 양상 및 멜라토닌 주입에 따른 멜라토닌 수용체 mRNA의 발현량 변화를 관찰하였다. 우선, 넙치의 다양한 조직별로 3가지 타입의 멜라토닌 수용체 발현량을 관찰한 결과, 모든 타입의 멜라토닌 수용체가 신경조직에서 높은 발현량을 나타내었다. 그러나 말초조직의 경우, 멜라토닌 수용체 1은 높은 발현량을 보였으나, 멜라토닌 수용체 2와 3은 낮은 발현량을 보이거나 발현이 거의 검출되지 않았다. 또한, 3가지 타입 멜라토닌 수용체의 발현량 변화를 낮과 밤(LD) 그리고 지속적으로 야간 환경으로만 설정한 실험구(DD)별로 관찰한 결과, 멜라토닌 수용체는 낮 시간대 보다는 밤 시간대에 주로 증가하는 경향을 보였다. DD 실험구에서도 LD 실험구에서의 멜라토닌 수용체 발현량의 경향과 유사하게 나타났으나, 멜라토닌 수용체의 발현량이 매우 낮은 점으로 보아, 낮과 밤에 따른 일주기 경향이 약화된 것을 알 수 있었다. 또한, 멜라토닌을 처리한 송과체 배양 실험(*in vitro*) 결과, 멜라토닌 수용체의 발현량이 매우 높게 증가한 것으로 보아, 멜라토닌이 멜라토닌 수용체와 결합함으로 인하여 멜

라토닌 수용체의 발현량이 높게 증가한 것으로 판단된다. 따라서 멜라토닌 수용체는 넙치의 뇌와 망막에서 광주기에 따른 생체리듬 조절에 중요한 역할을 수행하는 것으로 사료된다.

3. LED 파장별 흰동가리 *Amphiprion clarkii*의 생체리듬 관련 유전자 발현

적색, 녹색 및 청색 LED 파장이 흰동가리의 생체리듬에 미치는 영향을 파악하기 위하여, 시계 유전자(*Per2* 및 *Cry1*) mRNA의 발현량 변화를 관찰하였다. 다양한 LED 파장에 따른 흰동가리의 생체리듬 차이를 분석하기 위하여 멜라토닌 수용체 1, *Per2* 및 *Cry1*의 발현량 변화와 혈장 내 멜라토닌의 농도 변화를 분석하였다. 적색 LED 파장에서는 멜라토닌 수용체 1의 발현량이 다른 LED 파장 실험구에 비하여 유의적으로 높게 나타났으나, *Per2* 및 *Cry1*의 발현량은 다른 LED 파장 실험구에 비하여 유의하게 낮은 결과를 보였다. 또한, 혈장 내 glucose 농도는 적색 LED 파장 실험구에서 유의하게 높게 나타났는데, 이러한 결과는 적색 LED 파장이 물 분자에 빠르게 흡수되어 사라짐으로써 어류가 빛을 감지하기가 어렵게 되어, 결국 이는 어류에게 스트레스 요인으로 작용할 가능성이 있는 것으로 사료된다. 더욱이, 멜라토닌은 시계 유전자를 통해 생체리듬을 조절하며, 수중에서는 녹색 및 청색 LED 파장의 빛이 적색 LED 파장의 빛에 비하여 효과적으로 투과할 수 있는 것으로 보인다. 따라서 녹색 및 청색 LED 파장이 흰동가리의 생체리듬 조절에 적합한 파장인 것으로 사료된다.

4. LED 파장별 흰동가리 *Amphiprion clarkii*의 산화스트레스 유발 및 멜라토닌에 의한 스트레스 억제

청색, 녹색 및 적색 LED 파장이 흰동가리의 산화스트레스 유발에 미치는 영향 및 멜라토닌의 산화스트레스 억제 효과에 대하여 조사하였다.

각각의 LED 파장이 산화스트레스에 미치는 영향을 확인하기 위하여 멜라토닌의 합성을 좌우하는 시계 유전자인 *AANAT2* 및 항산화 효소(SOD 및 CAT) mRNA의 발현과 혈장 H_2O_2 및 멜라토닌의 농도를 측정

하였다. 적색 LED 파장에서 *AANAT2*, SOD 및 CAT mRNA는 다른 LED 파장에 비해 유의하게 높은 발현량이 관찰되었으며, SOD, CAT 활성, 혈장 H_2O_2 및 멜라토닌의 농도 또한 적색 LED 파장에서 유의적으로 높은 결과 값을 보였다. 이러한 연구 결과는 적색 LED 파장이 산화스트레스를 유도한다는 것을 시사한다. 또한, 본 연구에서는 산화스트레스에 대한 멜라토닌의 영향을 확인하기 위하여, 흰동가리에 멜라토닌을 주입(*in vivo*)하거나 송과체에 멜라토닌을 처리한 배양 실험(*in vitro*) 결과, *AANAT2*, SOD 및 CAT mRNA의 발현량, SOD, CAT 효소의 활성, 혈장 H_2O_2 그리고 지질과산화(LPO) 정도가 멜라토닌을 처리하지 않은 실험구에 비하여 유의하게 낮은 결과 값을 보였다. 이러한 연구 결과는 적색 LED 파장이 산화스트레스를 유도하고, 유도된 산화스트레스에 대하여 멜라토닌이 강력한 항산화제 역할을 수행한다는 것을 의미한다.

5. LED 파장별 흰동가리 *Amphiprion clarkii*의 성장호르몬 유전자 발현 및 성장 촉진 효과

성장호르몬은 척추동물의 성장과 발달에 필수적인 폴리펩타이드 호르몬이다. 흰동가리의 뇌하수체에서 전장의 성장호르몬 cDNA를 클로닝하였으며, 적색, 녹색 및 청색 LED 파장의 조명으로 흰동가리를 사육하면서 각각의 실험구별 흰동가리에서 성장호르몬 mRNA의 발현량 변화를 조사하였다. 성장호르몬의 발현량은 적색 LED 파장 실험구보다 녹색 및 청색 LED 파장 실험구에서 유의적으로 높은 값을 확인할 수 있었다. 이러한 결과는 장파장인 적색 LED 파장에 비하여 단파장인 녹색 및 청색 LED 파장이 흰동가리의 성장을 촉진시키는 것으로 보이며, 일반 형광등보다 LED 광원이 흰동가리의 성장을 촉진시키는데 효과적인 것으로 사료된다. 또한, 멜라토닌을 주입한 경우, 성장호르몬의 발현량이 대조구보다 유의하게 높은 값을 나타내었다. 이러한 연구 결과는 녹색 및 청색 LED 파장이 흰동가리 성장호르몬의 활성을 증진시키는 동시에, 멜라토닌 또한 흰동가리의 성장을 촉진시키는 요인인 것으로 사료된다.

6. LED 파장별 파랑돔 *Chrysiptera parasema*의 성숙 관련 호르몬 유전자 발현 및 성숙 촉진효과

형광등, 3가지의 LED 파장(적색, 630 nm; 녹색, 530 nm; 청색, 450 nm) 및 3가지의 세기(0.3, 0.6, 및 0.9 W/m^2)로 설정된 사육수조에서 파랑돔을 4개월 동안 사육하면서 각각의 실험구별 LED 파장 및 세기가 파랑돔의 성 성숙에 미치는 영향을 조사하였다. 각각의 조명에 의한 파랑돔의 성 성숙 여부 및 정도의 파악은 GSI, VTG, ER mRNA 및 단백질 발현량 그리고 혈장 E_2 농도를 측정하여 조사하였다. 그 결과, 녹색 및 청색 LED 실험구에서 VTG와 ER mRNA 발현량이 유의적으로 높게 나타났으며, GSI 수치 및 혈장 E_2 농도 또한 다른 LED 파장 실험구에 비하여 녹색 및 청색 LED 실험구에서 유의하게 높게 나타났다. 또한, 난소의 조직학적 관찰 결과에서도 단파장(녹색 및 청색 LED) 실험구에서 사육된 파랑돔에서 성숙한 난모세포가 다수 관찰되었다. 더욱이, 0.3과 0.6 W/m^2 세기의 LED 실험구에서 사육된 파랑돔에서 성숙한 난모세포를 다수 확인할 수 있었다. 이러한 연구 결과는 낮은 세기(0.3 W/m^2)의 녹색 LED 파장이 파랑돔의 성숙을 촉진시키는 데 효과적임을 확인할 수 있었으며, 또한 에너지 효율 측면에서 긍정적인 효과로 사료된다.

Chapter 1

General Introduction

Among the many factors that control circadian rhythms, light is the most important, modifying many of the physiological and behavioral changes that occur within a 24 h period (Pierce et al., 2008). Light emitting diode (LED), a new form of lighting technology that is still being developed, can be manufactured to output specific wavelengths (Migaud et al., 2007). Furthermore, LED have lower power requirements, electrical running costs, and a longer life span than the standard metal halide bulbs (Migaud et al., 2007). Narrow bandwidth light using such new technologies, and especially a high energy short wavelength, could thus provide much more efficient lighting systems than those currently used in the fish farming industry since they can be tuned to a species' environmental sensitivity by emitting narrow bandwidths (Villamizar et al., 2009). It is known that the spectral composition of incident light changes differentially in underwater environments and that there is a rapid attenuation with increasing depth (Lythgoe, 1979); the short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates in shallow waters (McFarland, 1991; Lythgoe et al., 1994; Myrberg and Fuiman, 2002). When farming the barfin flounder *Verasper moseri* using short wavelengths such as those in the blue spectrum, the flounders showed a high growth rate (Yamanome et al., 2009). In addition, it was shown that the blue spectrum prevented stress in the Nile tilapia *Oreochromis niloticus* (Volpato and Barreto, 2001), and that long wavelengths such as those in the red spectrum induced gonad development in the tropical damselfish *Chrysiptera cyanea* (Bapary et al., 2011).

First of all, light also controls the expression of the gene encoding rhodopsin (RH), a dim-light photoreceptor belonging to the G protein-coupled receptor

(GPCR) family (Khorana et al., 2002). RH is located in rod cells and transduces extracellular light signals into the cells (Yokoyama, 2000; Terakita, 2005). Most fish possess only one RH, with the maximum absorption corresponding to the photic environment (Minamoto and Shimizu, 2003). Recently, extra-ocular rhodopsin (Exo-RH), with a similar amino acid sequence to RH and belonging to RH group in the molecular phylogenetic analysis, was found in some teleosts (Mano et al., 1999; Philp et al., 2000). Exo-RH, a pineal opsin protein, is expressed solely in the pineal gland (Mano et al., 1999; Philp et al., 2000), such as the zebrafish *Danio rerio* (Pierce et al., 2008), the puffer fish *Takifugu niphobles* and the Atlantic salmon *Salmo salar* (Philp et al., 2000). Phylogenetic analysis indicates that this opsin belongs to the RH group. Exo-RH has been identified in some vertebrates, including the lamprey, teleosts, amphibians and mammals (Yokoyama and Zhang, 1997; Mano et al., 1999; Philp et al., 2000; Zhang et al., 2003). Exo-RH has been hypothesised to play an important role in the photoentrainment of the circadian clock (Zatz and Mullen, 1988; Cahill, 1996).

Also, the photoperiod, the most potent environmental stimulus, exerts an endogenous effect by causing a rhythmic change in melatonin levels (Bromage et al., 2001). Melatonin is mainly produced in the pineal gland and retina, and its plasma content is higher at night, between zeitgeber times (ZTs) 16 and 20, than during the day. It acts as a neuroendocrine messenger in the regulation of circadian and seasonal biological rhythms (Reiter, 1991; Falcón et al., 2007). These actions are mediated via melatonin receptors (MTs), which belong to the GPCR superfamily (Iigo et al., 1994a; Reppert et al., 1996). Vertebrates have three subtypes of MTs: MT1, MT2, and MT3 (Ebisawa et al., 1994). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Dubocovich, 1995; Reppert et al., 1996) and are believed to be expressed to mediate various physiological functions of melatonin in these tissues (Ikegami et al., 2009). The *arylalkylamine-N-acetyltransferase* (*AANAT*) enzyme is the precursor of melatonin;

which is the rate-limiting enzyme of melatonin synthesis (Iuvone et al., 2005; Klein, 2007). *AANAT* catalyzes the conversion of serotonin to *N-acetylserotonin*, which is then catalyzed by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin (Klein et al., 1997).

Period (Per), which was first characterized in *Drosophila* (Reddy et al., 1984), is a key protein in the circadian system of animals. In zebrafish, *Per2* is a circadian oscillator that is rapidly induced by light information from the suprachiasmatic nucleus (SCN) and appears to be involved in light-dependent clock resetting (Vallone et al., 2004). *Cryptochrome 1* (*Cry1*) regulates gluconeogenesis in the liver and the biological clock (Hirota et al., 2012).

Specifically, daily light conditions affect the survival and growth of fish (Barahona-Fernandes, 1979; Tandler and Helps, 1985). Under lighting regimes, the somatic growth of organisms including fish, is an integral biological process, and measures of growth may serve as indicators of organismal fitness since growth is related to the maintenance of homeostasis (MacKenzie, 1998). Melatonin may also modulate the central neural pathways involved in the regulation of growth hormone (GH) synthesis, as well as control the circadian rhythm of organisms (John et al., 1990). It has also been shown to affect the physiological processes involved in the growth and development of goldfish *Carassius auratus* (Bromage et al., 2001).

Additionally, certain LED spectra can have the negative effect of inducing stress in fish, including oxidative stress (Head and Malison, 2000; Van der Salm et al., 2004). Overproduction of reactive oxygen species (ROS) by environmental stress can increase lipid peroxidation (LPO), the oxidation of nucleic acid and proteins, and induce DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity and can thereby accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003). Complex antioxidant defense systems maintain homeostasis in changing environments and protect aerobic organisms against ROS and subsequent oxidative stress-induced damage

(Bagnyukova et al., 2007). Antioxidants can include enzymes such as superoxide dismutase (SOD), catalase (CAT), or glutathione peroxidase (GPX) and compounds such as melatonin, metallothionein, vitamin C or vitamin E (α-tocopherol) (McFarland et al., 1999).

Furthermore, it is well known that light and temperature are among the most important natural environmental factors that regulate reproduction of fish. Lighting characteristics including wavelength (quality), intensity (quantity), and periodicity (daily cycle) are the main factors that regulate seasonally dependent changes in reproductive and growth physiology of fish (Boeuf and Le Bail, 1999). In various reproductive hormones, estrogen activity is mediated by nuclear estrogen receptors (ERα and ERβ), and ERα is a member of a superfamily of transcription factors that induce target gene expression by binding *cis*-acting enhancer elements located in the promoter region of their responsive genes (Green et al., 1986). Furthermore, the induction of hepatic vitellogenin (VTG), which is a precursor yolk protein, in response to estrogens by an ER-mediated pathway has been well documented in several oviparous fish species (Ryffel, 1978; Nelson and Habibi, 2010). Thus, VTG and ER might serve as indicators of reproduction and maturation in fish. However, studies on the effect of LED light wavelengths on fish reproduction remain very limited (Bapary et al., 2009; Bapary et al., 2011).

In this study, endocrinological, physiological and biochemical markers were used to predict and assess the circadian rhythm, oxidative stress, growth and reproduction of fish illuminated by LED spectral sensitivity. Therefore, this study results might be applied to an artificial photic system aimed at effectively enhancing the growth and reproduction and regulating stress and circadian rhythm of fish reared under captive conditions.

Chapter 2

Expression of rhodopsin and Exo-rhodopsin genes in the retina and pineal gland of olive flounder *Paralichthys olivaceus*

1. Introduction

Fish live in aquatic environments with varying light intensity, and they often possess visual pigments with the absorption spectrum adapting to their habitats (Yokoyama et al., 1999). Fish that migrate from the pelagic zone to the benthic habitat, or vice versa, during ontogeny change their visual receptors (Helvik et al., 2001). The retina at the larval stages consists of only single cone cells; later during metamorphosis double cones and rods develop (Helvik et al., 2001). Because fish use environmental light signals for multiple physiological functions such as vision, photoentrainment of circadian rhythms, regulation of body colour and detection of seasonal changes in the photoperiod (Cole and Youson, 1982), they are useful model species to study the adaption of animals to the environmental light conditions.

Among the factors that influence circadian rhythms (physiological and behavioural changes within a period of 24 h), light is the most important one (Simonneaux and Ribelayga, 2003; Pierce et al., 2008). The biological circadian rhythm usually has a diurnal rhythm that is controlled by the external illumination time, often with seasonal periodicity (Bolliet et al., 2001; Pierce et al., 2008). The oscillation of the circadian rhythm is driven by an intra-cellular molecular clock and is self-sustaining, i.e. occurring even in the

absence of environmental cues (Deguchi, 1981; King and Takahashi, 2000; Pierce et al., 2008).

Light also controls the expression of the gene encoding RH, a dim-light photoreceptor belonging to the GPCRs family (Khorana et al., 2002). RH is located in rod cells and transduces extracellular light signals into the cells (Yokoyama, 2000; Terakita, 2005). It consists of a protein, opsin and a retinal chromophore and is the photoreceptor responding to external light signals in the retina (Deguchi, 1981; Khorana et al., 2002; Minamoto and Shimizu, 2003). RH has been identified in various animals including fish (Terakita, 2005). Most fish possess only one RH, with the maximum absorption corresponding to the photic environment (Minamoto and Shimizu, 2003). There are two ways to tune the absorption spectrum of visual pigments: one is to change the chromophore and the other is by changing the primary amino acid
structure of opsin proteins (Munz and McFarland, 1977). It is reported that only a few important amino acid substitutions can cause a large shift of absorption maximum in vertebrates (Nakayama and Khorana, 1991; Yokoyama and Radlwimmer, 1998).

Also, it has been generally considered that one fish has one type of RH, although some fish such as the coelacanth (Yokoyama et al., 1999), the ayu *Plecoglossus altivelis* (Hope et al., 1998), the pufferfish and the Atlantic salmon (Philp et al., 2000) have multiple RHs.

Recently, Exo-RH, with a similar amino acid sequence to RH and belonging to RH group in the molecular phylogenetic analysis, was found in some teleosts (Mano et al., 1999; Philp et al., 2000). These new rhodopsins are expressed in fish pineal glands and considered to be related with non-visual photoreception as well as vertebrate ancient (VA) opsin (Soni and Foster, 1997; Kojima et al., 2000), although their exact physiological

functions remain unknown.

Exo-RH, a pineal opsin protein, is expressed solely in the pineal gland (Mano et al., 1999; Philp et al., 2000), such as the zebrafish *Danio rerio* (Pierce et al., 2008), the puffer fish and the Atlantic salmon (Philp et al., 2000). Phylogenetic analysis indicates that this opsin belongs to the RH group. Exo-RH has been identified in some vertebrates, including the lamprey, teleosts, amphibians and mammals (Yokoyama and Zhang, 1997; Mano et al., 1999; Philp et al., 2000; Zhang et al., 2003). Exo-RH has been hypothesised to play an important role in the photoentrainment of the circadian clock (Zatz and Mullen, 1988; Cahill, 1996).

A photoreceptive molecule in the pineal gland mediates photoentrainment of the endogenous circadian clock which drives rhythmic production of melatonin (Deguchi, 1979, 1981). The pineal gland also synthesises and secretes melatonin, a nocturnal hormone produced during the scotophase which regulates circadian and seasonal rhythms, including sleeping/waking cycles of vertebrates (Eskin, 1979).

The circadian rhythm has been studied in various organisms such as fruit flies (Stanewsky et al., 1998); they investigated that cryptochrome, period and timeless genes expression for circadian rhythm; and plants (Samach and Coupland, 2000); the genes related with circadian rhythm might regulate flowering by controlling their expression by the circadian clock; however, only limited studies on this topic have also been done in fish (e.g. golden rabbitfish and zebrafish) (Mano et al., 1999; Park et al., 2007a; Pierce et al., 2008), they investigated that changes of circadian rhythm genes, melatonin receptor subtypes and Exo-RH for searching the part of circadian rhythm in fish.

In this study, the mitochondrial ribonucleic acid (mRNA) expression of RH and Exo-RH were explored changes for examining the part of the photic

regulation molecular mechanism in the olive flounder, an economically important fish which migrates to benthic environments after inhabiting the pelagic zone during the fry stage. The full length complementary deoxyribonucleic acid (cDNAs) of RH and Exo-RH genes were cloned from the retina and pineal gland of the olive flounder, respectively, and determined the expression level of RH gene in the retina and Exo-RH gene in pineal glands (*in vivo* and *in vitro*) during a 28-h time period under the 12-h light:12-h dark (LD) photocycle, constant dark (DD), and constant light (LL) conditions. This study showed that the expression level of RH in retina was higher during the photophase, while the Exo-RH gene expression level in the pineal gland was higher during the scotophase.

2. Materials and Methods

2.1. Experimental fish and conditions

Olive flounders (body length, 15.0 ± 0.5 cm; body weight, 27.5 ± 2.5 g) were obtained from a commercial fish farm. Flounders were transferred into three 300-l circulation filter tanks and acclimated for 2 weeks under LD (lights on 07:00-19:00 h, 200 lux during the photophase in the water phase) condition. During the experiments, flounders were placed under LD, DD and LL conditions. They were fed commercial feed twice daily (09:00 and 17:00 h). Experimental fish were anesthetised with 200 mg/l tricaine methane-sulphonate (MS-222; Sigma, St. Louis, MO, USA) before blood was collected from the caudal vein using a 3-ml syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 × g, 5 min) and stored at 80°C until analysis. The fish were euthanised by spinal transection at 4 h intervals, and the retina and pineal gland were dissected and fixed immediately in Trizol solution (Gibco/BRL, Gaithersburg, MD, USA).

2.2. Total RNA extraction, cDNA synthesis and RH/Exo-RH cDNA isolation

Total RNA was extracted from the retina and pineal gland, respectively, using the TRIzol kit 167 (Gibco/BRL, Gaithersburg, MD, USA). The first-strand cDNA was synthesised using M-MLV 168 reverse transcriptase (Bioneer, Daejon, Korea) according to the manufacturer's instructions.

Gene-specific primers were designed for RH and Exo-RH (Table 1) using highly conserved regions from the Atlantic halibut *Hippoglossus hippoglossus* (GenBank accession no.: AAM17918), common sole *Solea solea* (CAA77254), fire clownfish *Amphiprion melanopus* (ADI59664) and pufferfish (AF201472). Polymerase chain reaction (PCR) amplification was performed using 2 × Taq Premix I (Solgent,

Daejeon, Korea) under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 20 s, 56°C for 40 s and 72°C for 1 min; and a final step of 7 min at 72°C.

The PCR products were purified and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* DH5α competent cells (RBC Life Sciences, Seoul, Korea). Plasmid DNA was extracted and sequenced using ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. 3′- and 5′-rapid amplification of cDNA ends (RACE) of RH and Exo-RH

For the RH and Exo-RH RACE reactions, 2.5 μg total RNA extracted from the retina and pineal gland, respectively, was used to synthesise the first-strand 5′- and 3′-RACE cDNAs using a CapFishing™ Full-length cDNA Premix Kit (Seegene, Seoul, Korea) following the manufacturer's protocol.

RH- and Exo-RH-specific primers were designed (Table 1) based on the partial sequences obtained above and paired with 5′-RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′) or 3′-RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′). The 5′-and 3′-RACE cDNAs were used as the template for the 5′- and 3′-RACE reactions, respectively. PCR was carried out as follows: 94°C for 5 min; 35 cycles of 94°C for 40 s, 62°C for 40 s and 72°C for 1 min; and a final step of 5 min at 72°C. PCR amplicons were cloned and sequenced as described earlier.

Table 1. Primers used in this experiment

Primer	DNA sequences
First round of RT-PCR	
RH-F	5′-CCC CTC TAA ACT ACA TCC TTC-3′
RH-R	5′-GCG ATA ACC ATG ATC ACA ACC-3′
Exo-RH-F	5′-AAC TAC GTG CTG CTC AAC C-3′
Exo-RH-R	5′-CAA GAA GGA GAT CAC CAT GAC-3′
5′ RACE	
RH	5′-TGC TCT GTG CTG TCA AGG AGG CTG C-3′
Exo-RH	5′-CTC ACC TCC CAG AGT GGC GAA GAA TC-3′
3′ RACE	
RH	5′-CAA CGA GTG ACC AGA GGG CAA TTT CAC C-3′
Exo-RH	5′-CAG CAG CAG GAG TCT GAA ACC ACC CAG-3′
Real-time PCR	
RH-F	5′-CAA GCC CAT CAG CAA CTT-3′
RH-R	5′-TAA CGA GAC CAG CCA ACA-3′
Exo-RH-F	5′-TGT TTG TCC TCC ATT TCT CTA TC-3′
Exo-RH-R	5′-GCG TGT CAC CTC TTT CTC-3′
β-actin-F	5′-GCA AGA GAG GTA TCC TGA CC-3′
β-actin-R	5′-CTC AGC TCG TTG TAG AAG G-3′
GAPDH-F	5′-GTC GGT ATC AAT GGA TTC GG-3′
GAPDH-R	5′-CAT GTA GAC CAT GTA CTC CAG-3′

2.4. Phylogenetic analysis

RH and Exo-RH phylogenetic analysis was conducted using RH and Exo-RH amino acid sequences from the following species that were aligned using BioEdit software (Hall, 1999): Atlantic halibut RH (GenBank accession no. AAM17918), common sole RH (GenBank accession no. CAA77254), flathead mullet *Mugil cephalus* RH (GenBank accession no. CAA77250), winter flounder *Pseudopleuronectes americanus* RH (GenBank accession no. AAT72123), fire clownfish Exo-RH (GenBank accession no. ADI59664), pufferfish Exo-RH (GenBank accession no. AF201472), ayu Exo-RH (GenBank accession no. BAC56700) and Atlantic salmon Exo-RH (GenBank accession no. AAF44619). The phylogenetic tree was constructed using the neighbour-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.5. Tissue distribution of RH and Exo-RH mRNA

To examine the tissue distribution of RH and Exo-RH mRNA, total RNA was extracted from the nervous and peripheral tissues. The brain, pineal gland, pituitary, retina, gill, liver and kidney were removed and immediately stored at 80°C. Total RNA was extracted from the tissues using a TRIzol kit (Gibco/BRL, Gaithersburg, MD, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Daejon, Korea) according to the manufacturer's instructions. PCR amplification was performed with the following primer sets: RH forward primer (5′-AGG AGT CCT TAT GAG TAC CC-3′), RH reverse primer (5′-CTT GAC AGC ACA GAG CAG-3′), Exo-RH forward primer (5′-TTG ATC ACC CGC AGT ACT AC-3′) and Exo-RH reverse primer (5′-AGG AGA TCA CCA TGA CGA TG-3′). PCR were performed with 2 × Taq Premix I (Solgent, Daejon, Korea) under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 20 s, 56°C for 40 s and 72°C for 1 min; followed by a final step of 7 min at 72°C. Amplification of

β-actin mRNA was also conducted to verify the quality of the RT products using a primer set for olive flounder β-actin cDNA: β-actin forward primer (5′-TCG AGC ACG GTA TTG TGA CC-3′) and β-actin reverse primer (5′-ACG GAA CCT CTC ATT GCC GA-3′). The amplified PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualised by illumination with UV light.

2.6. Determination of transcript levels of RH and Exo-RH using quantitative polymerase chain reaction (QPCR)

Duplicate retina and pineal organ samples were collected at 4 h intervals during a 28 h period. The first-strand cDNA was prepared as described earlier and used as the template for QPCR.

QPCR was performed with gene-specific primers designed based on the RH and Exo-RH cDNA sequences of the olive flounder (Table 1) using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, California, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions under the following condition: 95°C for 5 min followed by 40 cycles of 95°C for 20 s and 55°C for 20 s.

To confirm that the cDNAs for the diel samples were free from contaminating genomic DNAs, a negative control of RNA was prepared for use in QPCR in the same way as cDNAs, but omitting reverse transcriptase. To avoid the potential error of QPCR caused by poor RNA quality, β-actin (HQ386788) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AB029337) were used as the internal controls. The expression level of the genes in each cDNA sample was analysed based on the corresponding cycle threshold (Ct) values of gene in QPCR. The Ct level was defined as the PCR cycle at which the fluorescence signal crossed the threshold line in the exponential phase of the amplification curve. The expression level of RH and Exo-RH was normalized to the average of the Ct of β-actin and

GAPDH. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β -actin and GAPDH) were calculated [ΔΔCt = 2ˆ- (ΔCt_{sample} - $\Delta Ct_{internal\ control}$)]. The data were analysed using the delta - delta method (Livak and Schmittgen, 2001).

2.7. *In vitro* culture of the pineal gland and melatonin treatment

After the fish were anesthetised, the pineal gland was dissected and placed in ice-cold medium (pH 7.5) composed of 150 mM NaCl, 3.5 mM KCl, 1.0 mM $CaCl_2 \cdot H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 0.7 mM $NaH_2PO_4 \cdot 2H_2O$, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 1.0 mM $MgCl_2$, 10 mM HEPES and 0.88 g/l Eagle's MEM (Sigma) containing antibiotics (0.06 g/l penicillin and 0.1 g/l streptomycin; Penicillin-Streptomycin, Gibco, USA). The pineal gland was transferred to a 24-well culture plate containing 1-ml of medium and incubated at 20 ± 1°C in an incubator under LD (LD12:12, light switched on at 07:00 h), DD and LL conditions. The light intensity at the surface of the culture plate was approximately 700 lx during the photophase. The pineal gland and medium were sampled at 4 h intervals (n = 5～6) from ZT4 to ZT28, and each sample was centrifuged (20°C, 10,000 × g, 15 s) to separate the pineal gland and medium. Total RNA was then extracted from the pineal gland. The pineal gland culture medium (supernatant) was stored for measuring melatonin, and the tissue was stored at -80°C until RNA extraction.

Melatonin (Sigma) dissolved in ethanol was added to the culture medium in the ratio 1/1000e (v/v), and the indicated concentrations of melatonin (10 M) were obtained. Melatonin treatments were given at 07:00 h (ZT0). Cells were treated for 4 or 28 h without renewing the medium. Each sample was centrifuged (20°C, 10,000 x g, 15 s), and the supernatant was removed and stored at -80°C until RNA extraction.

2.8. Melatonin determination in plasma and the pineal gland culture medium by enzyme-linked immunosorbent assay (ELISA)

Immunoenzymoassay with a commercial ELISA kit (IBL, Hamburg, Germany) was used to determine the melatonin concentration in the plasma. Plasma and the pineal gland culture medium samples were purified through extraction columns immediately after defrosting in the centrifuge. Then, 50 µl of each sample was added to different wells of an ELISA plate pre-coated with the capture antibody, anti rabbit IgG (goat, polyclonal). The samples were incubated with melatonin-biotin and antiserum solutions for 15 h at 48°C. The wells were then washed with the assay buffer (phosphate buffer with Tween-20 and stabilizer), and the plate was incubated with the enzyme-labelled solution (anti-biotin-alkaline phosphatase in Tris buffer with stabilizers) for 2 h at room temperature with constant shaking. After the plate was washed, it was incubated with p-nitro-phenyl phosphate solution for 30 min, and 50 µl of stop solution (1 N NaOH with 0.25 M ethylene diamine tetraacetic acid) was then added to it. Absorbance was read at 405 nm. According to the manufacturer of the ELISA kit, the intra- and inter-assay coefficients of variation were 3.9 - 6.9% in the range of 28.8 - 266 pg/ml and 6.2 - 15.9% in the range of 3.5 - 281 pg/ml, respectively.

2.9. Statistical analysis

All data were analysed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, USA). A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to assess statistically significant differences among the different time points of diurnal and circadian variation. A value of $p<0.05$ was considered statistically significant. If necessary, values were transformed (logarithmic or square root transformations) to fulfil the conditions of the parametric analysis of variance ANOVA (normal distribution and homogeneity of variances). A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Full-length cDNA sequences of RH and Exo-RH

The expected amplicons for both RH and Exo-RH were obtained by RT-PCR with gene-specific forward and reverse primers and were cloned and sequenced. A 572- and 397 bp sequence were proved to be part of the RH and Exo-RH genes, respectively. On comparing the sequence of olive flounder, RH and Exo-RH with those deduced from the RH and Exo-RH cDNAs of other teleost species. Using the RACE method, the full-length cDNA sequences for both the RH (1,059) bp [GenBank accession no.: HQ413772]) and Exo-RH (1,059) bp [HM107825]) of the olive flounder were obtained, both of which were predicted to encode a protein of 352 amino acid (aa) residues. Olive flounder RH and Exo-RH showed high aa sequence similarity to other teleosts, Atlantic halibut, common sole, flathead mullet, winter flounder, fire clownfish, pufferfish, and Atlantic salmon (93 - 95% for RH and 87 - 96% for Exo-RH). Also, they shared common 70% aa sequence similarity with each other (Figs. 1 and 2), as a member of the RH family (a family including RH and Exo-RH) has a similar aa structure. The aa residues that are important for the function of RH were found to be conserved in both RH and Exo-RH. Examples are: the lysine residue (□) in the putative seventh transmembrane domain that attaches to the chromophore by Schiff base linkage (Wang et al., 1980); the counterion glutamic acid residue (Glu-113) in the predicted transmembrane domain III (Sakmar et al., 1989); the cysteine residues cysteine (Cys)-110 and Cys-187, which may form a disulphide bridge; and the palmitoylation sites Asn-302 and Pro-303 (Asn-303 and Pro-304 in Exo-RH), which are thought to be important in anchoring RH to the cell membrane by palmitic acid esterification (Ovchinnikov et al., 1988) (Figs. 1 and 2). For Exo-RH, the transmembrane embedded residues of the a-helices were predicted according to Baldwin's model (Baldwin et al., 1997).

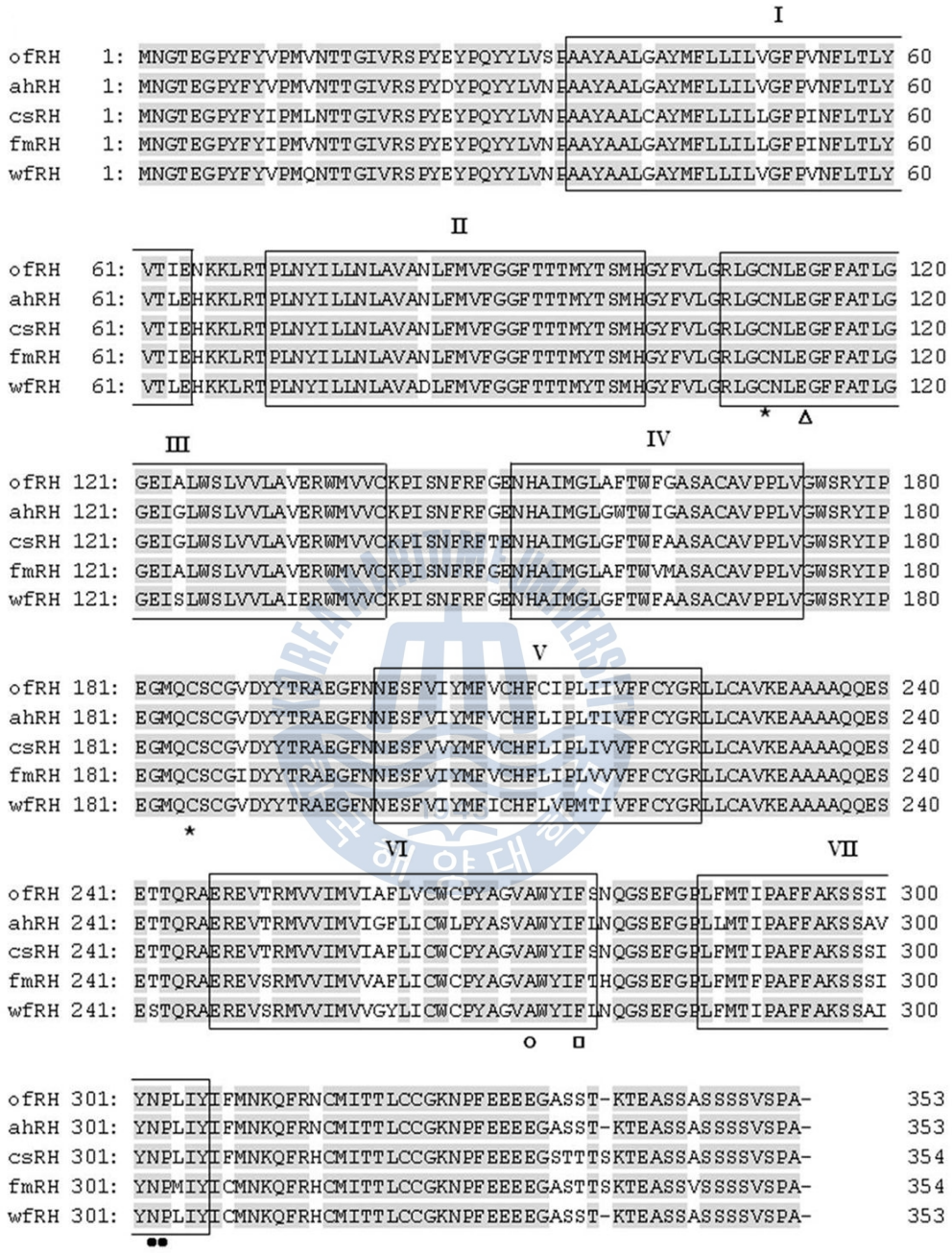

Fig. 1. Alignment of the amino acid sequences of olive flounder RH isolated in this study with those from the GenBank/EMBL/DDBJ databases. The GenBank accession numbers for the sequences used in the alignment are as follows: olive flounder (ofRH, HQ413772), Atlantic halibut (ahRH, AAM17918), common sole (csRH, CAA77254), flathead mullet (fmRH, CAA77250), and winter flounder (wfRH, AAT72123). The seven transmembrane domains are indicated by boxes. Amino acid residues involved in Schiff base formation (Phe, □) and their counterions (Thr, ■), those involved in disulphide bond formation (Cys, *), those involved in glycosylation (glutamate residue; Gly, Δ) and those involved in palmitoylation (Asn and Pro, ●) are indicated. Amino acids involved in the spectral tuning of rhodopsin are also indicated (Ala, ○).

Fig. 2. Alignment of the amino acid sequences from olive flounder Exo-RH isolated in this study with those from the GenBank/EMBL/DDBJ databases. The GenBank accession numbers for the sequences used in the alignment are as follows: olive flounder (ofExo-RH, ADI59669), fire clownfish (fcExo-RH, ADI59664), pufferfish (frExo-RH, AF201472), ayu (ayExo-RH, BAC56700), and Atlantic salmon (asExo-RH, AAF44619). Putative transmembrane domains I - VII are indicated by horizontal lines. The conserved functional opsin features include glycosylation sites (Asn, Δ), a disulphide bridge (Cys, *), a chromophore attachment site (Lys, □), a Schiff base counterion (Glu, ■); and a palmitoylation site (Asn and Pro, ●).

3.2. Phylogenetic analysis

The phylogenetic tree obtained by Clustal analysis of the sequences described in this section is shown in Fig. 3. Olive flounder RH and Exo-RH are grouped with other retinal RH and Exo-RH molecules, and separated into a retinal RH group and an Exo-RH group. In this tree, olive flounder RH was clustered in the retinal RH group, and olive flounder Exo-RH was clustered in the extra-ocular RH group.

3.3. Tissue distribution of RH and Exo-RH mRNA

The tissue distribution of RH and Exo-RH mRNA by RT-PCR were examined (Fig. 4). RH was predominantly expressed in the retina and slightly expressed in the peripheral tissues. Exo-RH was significantly expressed in the pineal gland and also expressed at a high level in the retina.

3.4. Diurnal and circadian variation in the expression of RH mRNA in the retina

The diurnal variation in the expression of RH mRNA in the retina by QPCR was examined (Fig. 5). RH mRNA was expressed at significantly higher levels during the photophase than the scotophase, and its expression peaked between ZT28 and ZT4. At the start of the scotophase, the RH expression levels were significantly low, and even lower levels were observed at midnight (ZT16) (Fig. 5A). Furthermore, RH mRNA expression at ZT16 was lower under DD than LD, but the amplitude was attenuated (Fig. 5B). Also, under LL condition, the expression was rhythmic as under LD and DD, but expression levels were significantly higher than LD and DD conditions about 1.2 to 1.5 times, and the amplitude was attenuated (Fig. 5C).

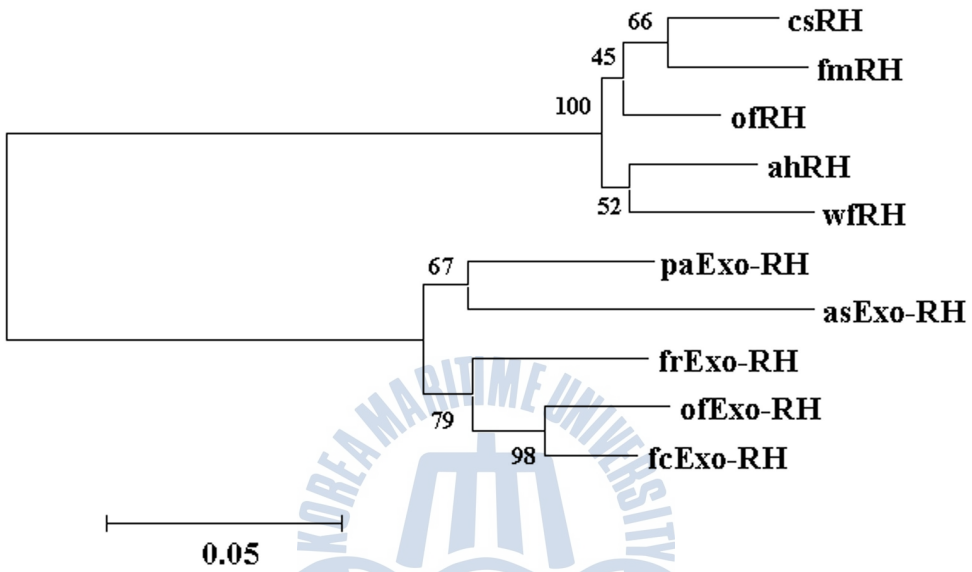

Fig. 3. Phylogenetic tree based on an amino acid alignment for RH and Exo-RH sequences in teleost fish. Bootstrap values (%) are indicated (1,000 replicates). The score between two protein sequences, which is a measure of their relative phylogenetic relationship, is represented by the horizontal distance in this tree, i.e. the shorter the distance, the more related they are. GenBank accession numbers for the sequences are: Atlantic halibut RH (ahRH, AAM17918); common sole RH (csRH, CAA77254); flathead mullet RH (fmRH, CAA77250); winter flounder RH (wfRH, AAT72123); fire clownfish Exo-RH (fcExo-RH, ADI59664); pufferfish Exo-RH (frExo-RH, AF201472); ayu Exo-RH (ayExo-RH, BAC56700); and Atlantic salmon Exo-RH (asExo-RH, AAF44619).

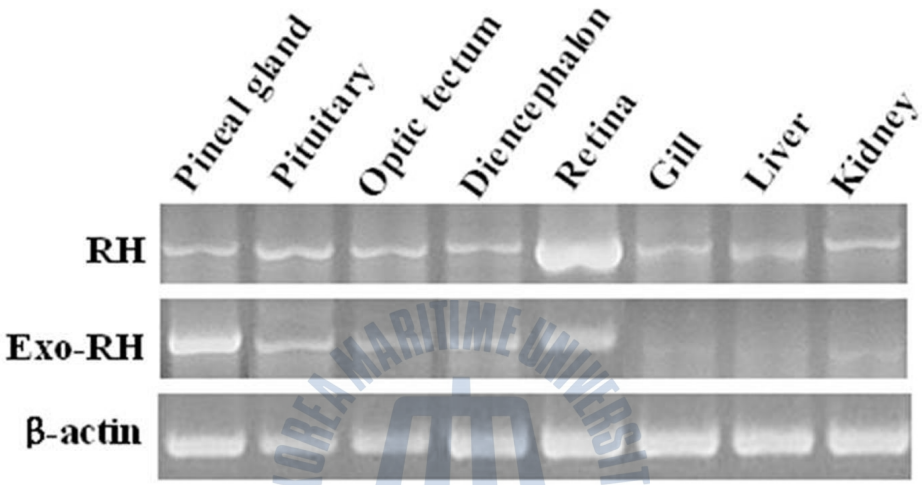

Fig. 4. Tissue distribution of RH and Exo-RH mRNA in olive flounder. β-actin mRNA was amplified to verify the integrity of each mRNA sample.

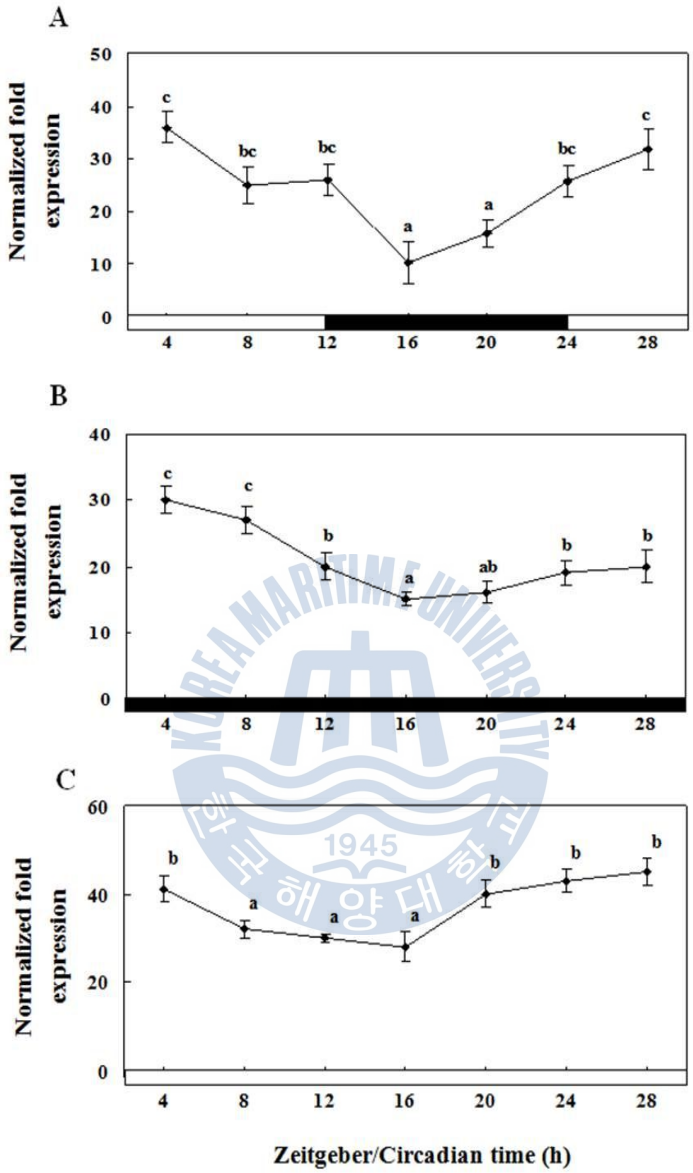

Fig. 5. Diurnal variations in the levels of RH mRNA in the retina of olive flounder as measured by QPCR. The fish were reared under a LD cycle (A), DD (B), and LL (C). Total retinal RNA (2.5 μg) was reverse transcribed and amplified. The results are expressed as normalised expression levels with respect to the β-actin and GAPDH levels in the same sample. The white bar

represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and Circadian time (CT) (P<0.05). All values represent means ± standard deviation (SD) (n=5).

3.5. Diurnal and circadian variation in the expression of Exo-RH mRNA in the pineal gland examined *in vivo*

The diurnal variation of Exo-RH mRNA expression in the pineal gland of olive flounder by QPCR were shown in Fig. 6. Exo-RH expression levels were found to be high at the start of the scotophase (ZT12), and significantly higher levels were observed during the scotophase than the photophase (Fig. 6A). Diurnal changes in Exo-RH expression were observed with a single peak at ZT20, and then the expression decreased at end of the scotophase (ZT24). Further, under DD condition, Exo-RH expression was rhythmic as under LD, with peaks at ZT20, but the amplitude was lower (Fig. 6B). Also, under LL condition, the expression was rhythmic as under LD and DD, but expression levels were significantly lower than LD and DD about 12,500 times, with peaks at ZT16, and the amplitude was lower (Fig. 6C).

3.6. Diurnal and circadian variation in the expression of Exo-RH mRNA in the pineal gland examined *in vitro*

In the cultured pineal gland, Exo-RH mRNA expression was found to be high at midnight (ZT16), and the levels were significantly higher during the scotophase than the photophase (Fig. 7A). The expression showed diurnal changes with one peak at ZT20 and then decreased at the end of the scotophase (ZT24). Further, under DD condition, Exo-RH expression in the cultured gland was as rhythmic as that under LD, with peaks at ZT20, but the amplitude was lower (Fig. 7B). Also, under LL condition, the expression was rhythmic as under LD and DD, but expression levels were significantly lower than LD and DD about 8,000 - 12,000 times, with peaks at ZT16, and the amplitude was lower (Fig. 7C).

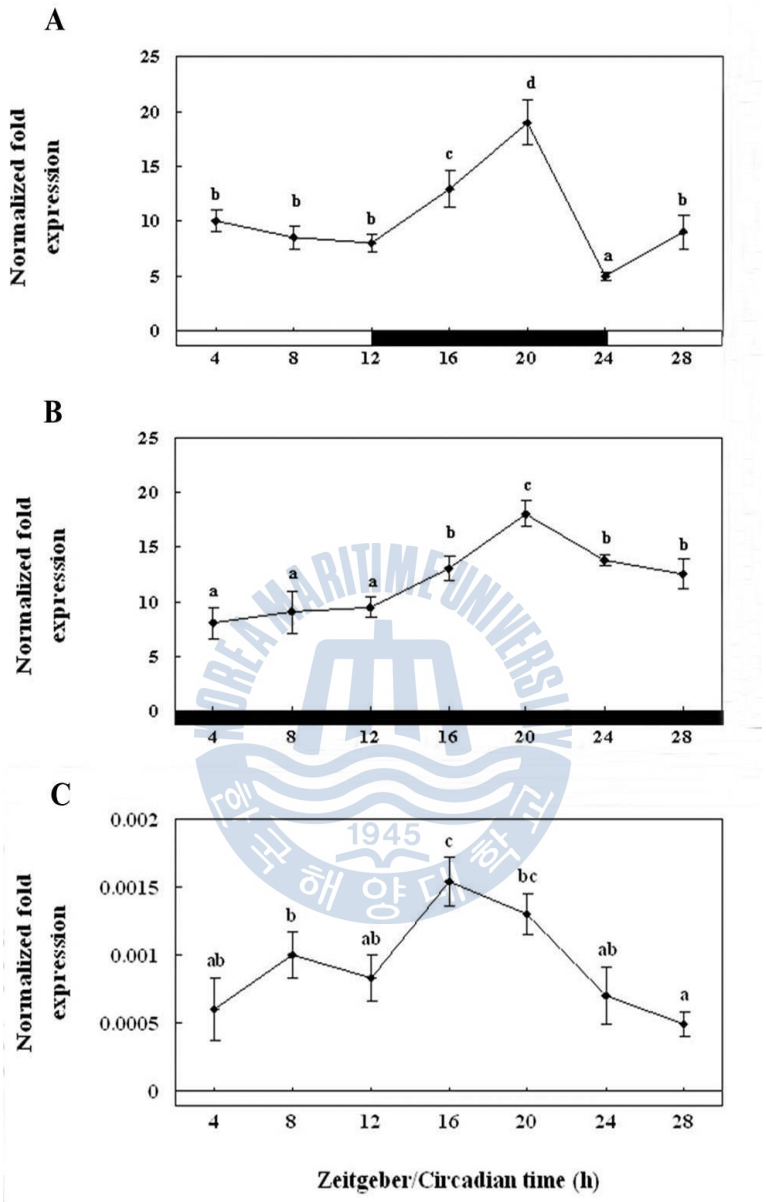

Fig. 6. Diurnal variations in the levels of Exo-RH mRNA in the pineal gland *in vivo* as measured by QPCR. The fish were reared under a LD cycle (A), DD (B), and LL (C). Each mean value and error bar indicates the pineal gland from 10 fish. Total pineal gland RNA (2.5 μg) was reverse transcribed and amplified. The results are

expressed as the normalised expression levels with respect to the levels of β-actin and GAPDH in the same sample. The white bar represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values represent means ± SD (n=5).

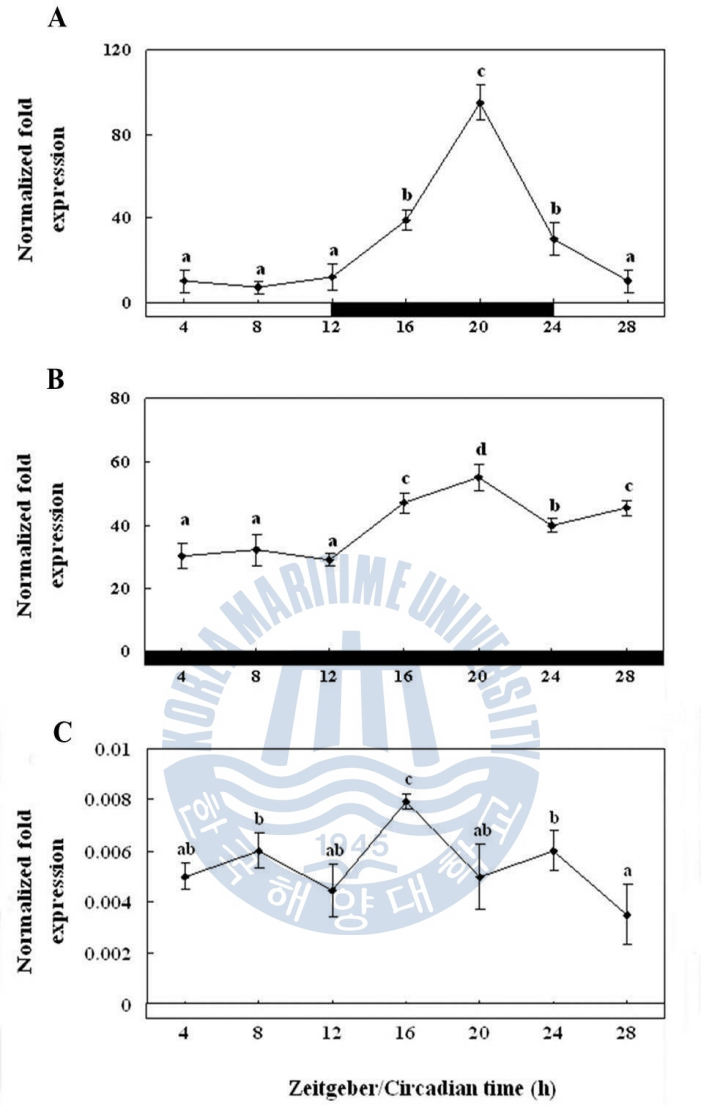

Fig. 7. Diurnal variations in the levels of Exo-RH mRNA in the cultured pineal gland *in vitro* as measured by QPCR. The pineal gland was maintained under a LD cycle (A), DD (B), and LL (C). Each mean value and error bar indicates the pineal gland from 10 fish. The white bar represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values represent means ± SD (n=5).

3.7. Diurnal and circadian variation in the expression of Exo-RH mRNA in the melatonin-treated pineal gland examined *in vitro*

Exo-RH mRNA expression was high at midnight (ZT20) in the cultured melatonin-treated pineal gland, and the levels were significantly higher during the scotophase than the photophase (Fig. 8A). The expression showed diurnal changes with one peak at ZT20 and then decreased at the end of the scotophase (ZT24). Similar results as Exo-RH mRNA expression in the non-treated pineal gland were observed with regard to the DD condition, and showed one peak at ZT16 (Fig. 8B). Further, under LD and DD, the Exo-RH expression levels were significantly lower in the melatonin-treated gland than in the non-treated one. Also, under LL condition, the expression was rhythmic as under LD and DD, but expression levels were significantly lower than LD and DD about 1.3 - 25.0 times, with peaks at ZT20, and the amplitude was lower (Fig. 8C).

3.8. Diurnal and circadian variation in the pineal gland culture medium melatonin levels

The melatonin levels in the pineal gland culture medium exposed to the normal 24 h LD cycle has been examined. The pineal gland culture medium melatonin concentrations were significantly higher during the scotophase (12.5 ± 0.6 pg/ml) than the photophase (3.5 ± 0.5 pg/ml) (Fig. 9A). Further, the pineal gland culture medium melatonin levels under the DD condition were similar to those under the 24 h LD cycle, although they were of a lower amplitude; they peaked significantly at ZT20 (12.0 ± 1.0 pg/ml) (Fig. 9B).

Also, under LL condition, the levels were rhythmic as under LD and DD, but levels were significantly lower than LD and DD about 1.33 times, with peaks at ZT16 (8.0 ± 0.7 pg/ml) and ZT20 (9.5 ± 0.9 pg/ml), and the amplitude was lower (Fig. 9C).

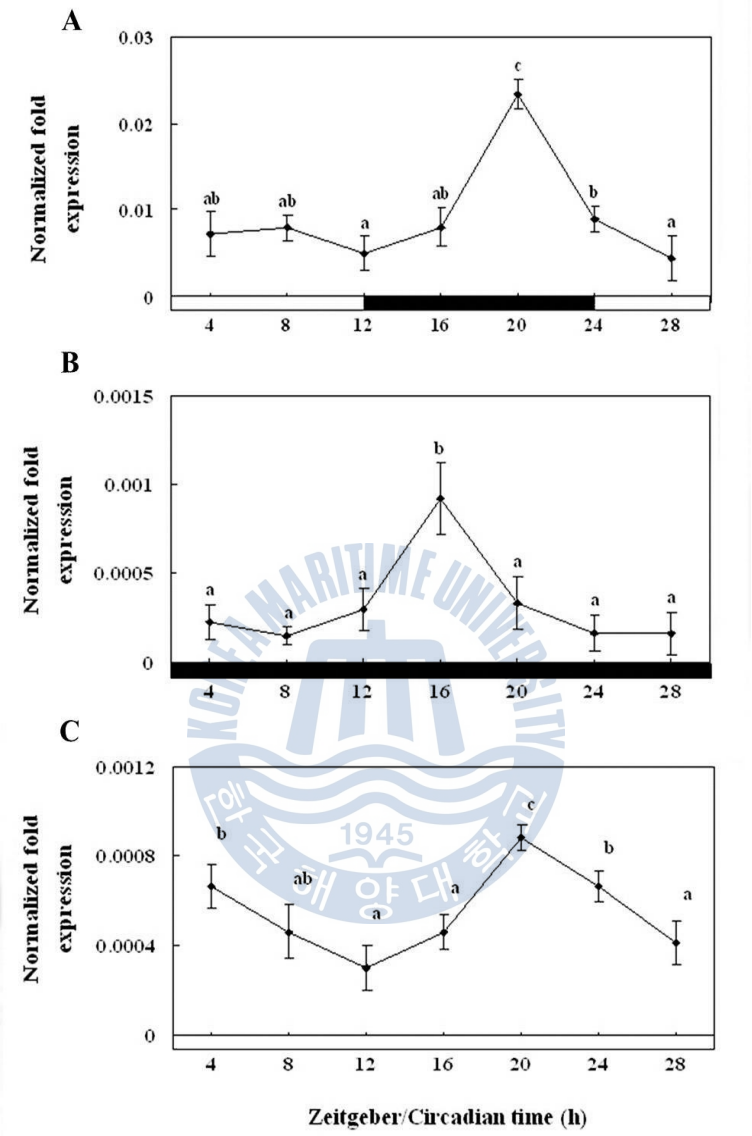

Fig. 8. Diurnal variations in the levels of Exo-RH mRNA in the cultured melatonin-treated *in vitro*, as measured by QPCR. The pineal gland was maintained under a LD cycle (A), DD (B), and LL (C). Each mean value and error bar indicates the pineal gland from 10 fish. The white bar represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and CT ($P<0.05$). All values represent means ± SD ($n=5$).

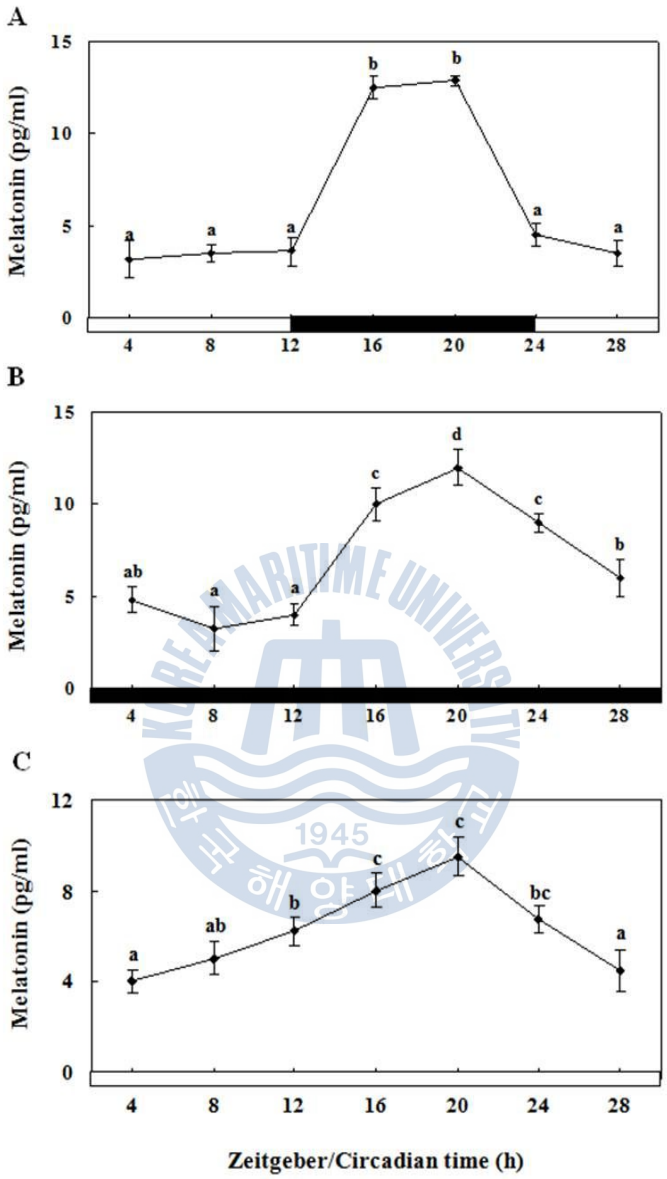

Fig. 9. ELISA of the melatonin levels of the pineal gland culture medium during the daily LD cycle (A), DD (B), and LL (C). The white bar represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values represent means ± SD (n=5).

3.9. Diurnal and circadian variation in plasma melatonin levels

The plasma melatonin levels in the olive flounders exposed to the normal 24 h LD cycle has been examined. Plasma melatonin concentrations were significantly higher during the scotophase (16.1 ± 1.9 pg/ml) than the photophase (5.2 ± 2.1 pg/ml) (Fig. 10A). Further, the plasma melatonin levels under the DD condition were similar to those under the 24 h LD cycle, although they were of a lower amplitude; they peaked significantly at ZT20 (10.0 ± 1.1 pg/ml) (Fig. 10B). Also, under LL condition, the levels were rhythmic as under LD and DD, but levels were significantly lower than LD and DD about 1.5 - 2.5 times, with peaks at ZT20 (6.5 ± 0.3 pg/ml) and ZT24 (6.7 ± 0.4 pg/ml), and the amplitude was lower (Fig. 10C).

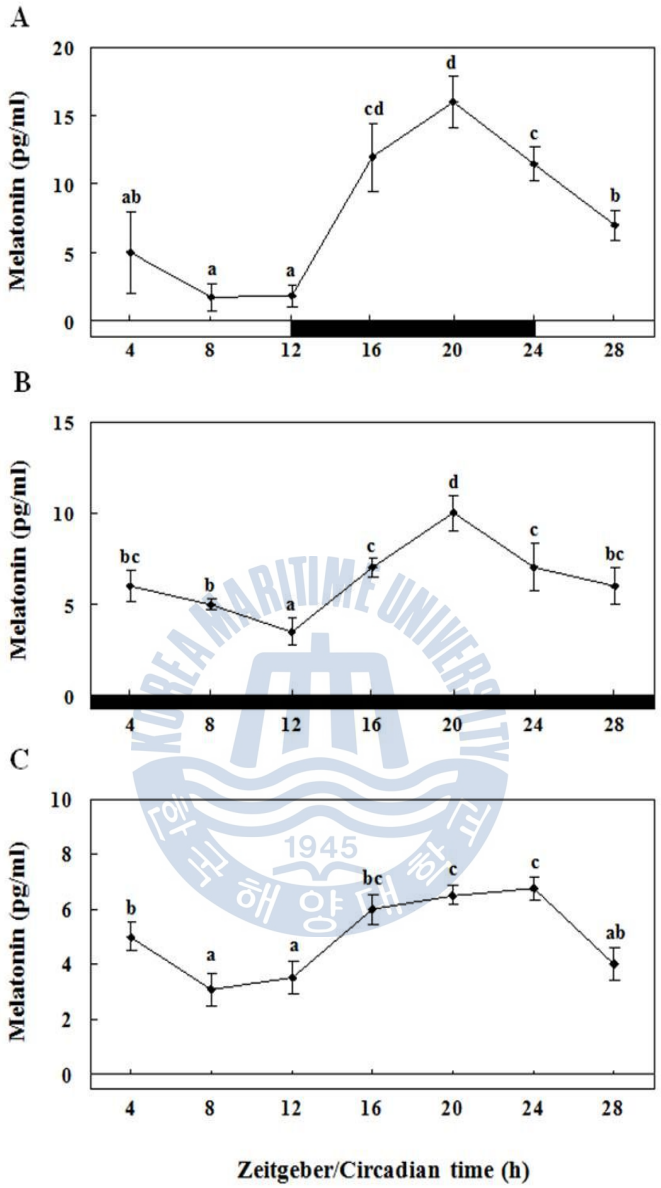

Fig. 10. ELISA of plasma melatonin levels of olive flounders during the daily LD cycle (A), DD (B), and LL (C). The white bar represents the photophase and the black bar, the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values represent means ± SD (n=5).

4. Discussion

The changes in the mRNA expression of RH and Exo-RH for examining the part of the photic regulation molecular mechanism in the olive flounder using the photoreceptors, RH and Exo-RH were investigated. Also, the relationship between the detection of external light and circadian rhythm by cloning full-length RH and Exo-RH cDNA and examining the changes in the expression of the RH family genes encoding these pigments. Importantly, the expression of Exo-RH mRNA was investigated in cultured pineal glands to determine whether it is specifically expressed in the pineal gland during the circadian cycle.

An extra-retinal opsin photoreceptor has been identified in the pineal gland of some vertebrates (Hope et al., 1998; Philp et al., 2000), but its physiological function has not been studied.

Molecular phylogenetic analysis indicated that olive flounder RH, which is expressed in the retina, and Exo-RH, which is expressed in the pineal gland, clustered with the vertebrate RH and Exo-RH groups, respectively (Fig. 3). However, Exo-RH, phylogenetically separated from the RH groups as amember of the RH family (a family including RH and Exo-RH), has a similar aa structure to RH. This tree agrees with the findings of Minamoto and Shimizu (2003), who showed that RH and Exo-RH, separated from the RH groups were phylogenetically very similar, according to phylogenetic analysis of ayu RH and Exo-RH. In addition, Zhang et al. (2003) reported that Exo-RH had evolved from RH, as determined by the divergence of functional regulatory elements that resulted in its specific expression in the pineal gland.

In this study, the differential expression of RH and Exo-RH mRNA were observed in the nervous and peripheral tissues of olive flounder by RT-PCR

analysis: RH is expressed predominantly in the retina, while Exo-RH is strongly expressed in the pineal gland. However, these genes were slightly expressed in the peripheral tissues, it indicated that RH and Exo-RH were specific to retina and pineal gland, respectively (Fig. 4). These results showed that whether RH accepts the photic information in the retina and RH is retinal visual pigments responsible for light capture and initiation of the photo-transduction cascade in rod cells, and if Exo-RH plays a central role in the processing of photic information transduced by RH to control the circadian rhythm in the olive flounder. In addition, Exo-RH is also significantly expressed in the retina, and given the structural homology of Exo-RH and RH, it may function as a photoreceptor (Mano et al., 1999; Philp et al., 2000; Zhang et al., 2003). Considering these results, RH and Exo-RH accept the photic information and control the circadian rhythm.

I hypothesise that both RH and Exo-RH receive photic information and control circadian rhythm. Thus, the relationship between the detection of external light and circadian rhythm was investigated by examining changes in the expression of RH family genes, e.g. RH and Exo-RH. Exo-RH can function as an extra-retinal photoreceptor, e.g. by resetting the circadian rhythm during the photoperiod, and RH controls circadian rhythm under LD condition (Schantz et al., 1999). When the olive flounder was exposed to LD in this study, RH was expressed at a significantly higher level during the photophase and was expressed at a lower level during the scotophase (Fig. 5A). Under the DD and LL conditions, RH mRNA expression exhibits circadian rhythmicity as under LD condition (Fig. 5B, C). These results suggest that the retina may contain a functional light-entrainable circadian oscillator that synchronises RH expression levels in a circadian rhythm. In addition, light down-regulates RH expression, attenuating transcription and amplitude, but not the period (Im et al., 2007). RH is known to be a dim-light photoreceptor

that is predominantly expressed during the night than during the day, when there is light (Kim et al., 2007). Unfortunately, previous studies in fish, like the cuttlefish *Sepia officinalis*, European eel *Anguilla anguilla* L. and skate *Raja erinacea* and *Raja ocellata*, were general studies that merely cloned RH cDNA (O'Brien et al., 1997; Bellingham et al., 1998; Hope et al., 1998), and few studies have investigated the expression levels of RH mRNA during diurnal rhythm (Yokoyama, 2000; Im et al., 2007).

To examine the roles played by Exo-RH as a putative photoreceptor of the pineal gland and as a controller of circadian rhythm, its expression levels were assessed in the pineal gland during diurnal rhythm. Exo-RH expression was significantly increased at the start of the scotophase, ZT12 and peaked during the night at ZT20 (Fig. 6A). These observations are in agreement with the findings of Pierce et al. (2008), who observed a peak of Exo-RH mRNA expression in the zebrafish pineal gland during the scotophase. These results are also supported by another study which indicated that Exo-RH is related to melatonin, a key hormone for the control of circadian rhythm (Zatz and Mullen, 1988). Collectively, these results indicate that Exo-RH plays a central role in the control of circadian rhythm at night.

This study showed that the expression of Exo-RH mRNA between the pineal gland and retina was compared and Exo-RH is strongly expressed in the former. This result is in agreement with the findings of Zatz and Mullen (1988) and Cahill (1996), who reported that Exo-RH and RH have a similar function, but Exo-RH controls the biological clock and melatonin secretion during the photoperiod, whereas retinal RH is expressed in dim light.

The expression of Exo-RH was compared in cultured pineal glands in relation to the diurnal rhythm. Exo-RH mRNA expression was high at the start of the scotophase, at ZT12, and then peaked during the night, at ZT20, similar to the *in vivo* results. Mano et al. (1999) examined that expression of

Exo-RH in the pineal gland, and Cahill (1996) reported that Exo-RH may play an important role for photoentrainment of the circadian clock and/or for acute regulation of melatonin production, whereas retinal RH serves for twilight vision. Therefore, I hypothesised that Exo-RH mRNA is expressed at night along with melatonin to control circadian rhythm. Cahill (1996) reported that the biological clock of the pineal gland responds to light and controls the secretion and suppression of melatonin depending on the presence or absence of light, whereby the expression of genes related to the control of circadian rhythm is controlled.

Exo-RH mRNA expression was examined in pineal glands (*in vivo* and *in vitro*) under DD condition and found that its expression is rhythmic, as under LD condition, with peaks at ZT20. The difference of expression levels between *in vivo* and *in vitro* (*In vitro* group, expression levels were significantly higher about 3-5 times than *in vivo* group) were observed, I hypothesised that Exo-RH mainly expresses in pineal gland and there are very close relationship between Exo-RH and pineal gland. Furthermore, under DD and LL conditions, amplitude was lower but it was rhythmic as under LD. It appears to be regulated in a circadian manner (Takemura et al., 2006), since all conditions showed similar variation in Exo-RH mRNA expression. It is likely that Exo-RH mRNA expression in all conditions is under circadian regulation (Park et al., 2007a). Also, Exo-RH expression levels under LL condition were significantly lower than LD and DD conditions, this result is in agreement with the findings of Iigo et al. (1995) and Park et al. (2007a), who reported that the rhythm of melatonin-binding sites in the brain diminished after exposure to LL condition in goldfish, and MT1 mRNA expression levels under LL condition were significantly lower than control in the golden rabbitfish *Siganus guttatus*, respectively. Therefore, I hypothesised that Exo-RH is related to melatonin, a key hormone for the control of

circadian rhythm in night (Zatz and Mullen, 1988), so Exo-RH mRNA expression levels were very low under LL condition.

Also, Exo-RH mRNA expression in the cultured melatonin-treated pineal gland under DD condition was rhythmic, as under LD condition, with a peak at ZT20, but the expression level was significantly lower than in the cultured pineal gland not treated with melatonin. In addition, Exo-RH mRNA expression under LL condition was rhythmic, as under LD and DD conditions, with a peak at ZT20 and expression level was similar with DD condition. Skene et al. (1996) reported that melatonin feeds back to regulate the primary circadian clock located in the SCN and thus serves as a strong entraining factor. Therefore, the pineal glands were treated with exogenous melatonin to examine the feedback function of decreasing Exo-RH expression levels through results of LL and DD conditions.

Plasma melatonin levels showed rhythmic changes under DD and LL conditions like they did under LD condition, with a peak at ZT20. Furthermore, the melatonin levels of the pineal gland culture medium showed the results similar with plasma melatonin levels. Therefore, the melatonin was examined which is directly secreted from pineal gland. This result is in agreement with the findings of Vera et al. (2010), who measured the levels of melatonin in the cultured pineal gland of the Atlantic cod and Atlantic salmon. They observed significantly higher melatonin levels in the night, indicating the control of circadian rhythm by the nocturnal production of melatonin. The teleost pineal gland contains an intrinsic light-sensitive circadian oscillator that regulates daily rhythms of genes, related with circadian rhythm, expression and melatonin production (Cahill, 1996; Begay et al., 1998; Gothilf et al., 1999; Ziv et al., 2005). Takemura et al. (2004) found that the exposure of the golden rabbitfish to the ‘brightness’ of midnight during the full and new moon periods resulted in a rapid decrease

in the melatonin concentration in the blood circulation. Similarly, the exposure of the cultured pineal gland of the golden rabbitfish to the full moon or new moon periods suppressed melatonin synthesis (Takemura et al., 2006). Therefore, it is likely that Exo-RH mRNA expression in the pineal gland is under circadian regulation both *in vivo* and *in vitro*. In addition, the level of Exo-RH mRNA expression under DD and LL conditions was significantly lower than under LD condition, indicating that light is an important factor affecting circadian rhythm, and melatonin actions by photoperiod in natural habitats as well as by endogenous clocks. Although there may be differences in circadian rhythm between fish and other vertebrates, these findings may be analogous to the mechanism in several other vertebrates, where attenuation and/or displacement under prolonged constant conditions are typical attributes of many clock-regulated rhythms in which light acts as the main environmental input to adjust rhythmic patterns on a daily basis (Karaganis et al., 2009). Further, these results are in agreement with the RH expression levels observed under DD and LL conditions in this study, on the basis of which these results suggest that while the circadian rhythm pattern in fish is maintained under LD, DD and LL conditions, thereby hampering the transcription of genes involved in circadian rhythm regulation, like Exo-RH. Therefore, light is an important factor that controls circadian rhythm. These results coincide with those of Ikegami et al. (2009) and Park et al. (2007a).

In conclusion, this result suggests that RH and Exo-RH potentially mediates the effects of environmental photocycle on pineal circadian rhythms and melatonin synthesis. Also, Exo-RH, as a member of the RH family, is expressed depending on the light dependent increase in RH expression. I hypothesise that Exo-RH controls circadian rhythm by increasing its levels in the pineal gland, an important organ for the generation and maintenance of

circadian rhythm. Furthermore, additional studies will be necessary to understand the molecular base of photic regulation in fish.

Chapter 3

Expression of three melatonin receptors in the brain and retina of olive flounder *Paralichthys olivaceus*: profiles following exogenous melatonin

1. Introduction

Organisms exhibit many activities that vary according to periodic changes in environmental factors. The photoperiod, the most potent environmental stimulus, exerts an endogenous effect by causing a rhythmic change in melatonin levels (Bromage et al., 2001). Melatonin is produced mainly in the pineal gland and retina, and its plasma content is higher at night, between ZTs 16 and 20, than during the day. It acts as a neuroendocrine messenger in the regulation of circadian and seasonal biological rhythms (Reiter, 1991; Falcón et al., 2007). These actions are mediated via MTs, which belong to the GPCR superfamily (Iigo et al., 1994a; Reppert et al., 1996).

Vertebrates have three subtypes of MTs: MT1, MT2, and MT3 (Ebisawa et al., 1994). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Dubocovich, 1995; Reppert et al., 1996) and are believed to be expressed to mediate various physiological functions of melatonin in these tissues (Ikegami et al., 2009). A variety of vertebrates reportedly express MT1 and MT2 (Reppert et al., 1995b; Roca et al., 1996). High MT1 mRNA expression has been detected in the SCN (Von Gall et al., 2002; Dubocovich et al., 2003), where the master circadian clock system is located in mammals (Gauer et al., 1993). Therefore, MTs are

thought to convey photoperiod information to control the circadian rhythm in the SCN (Masana et al., 2000). The pineal gland is also a candidate for the master circadian clock in teleost fish (Cahill, 1996) because this photoreceptive gland functions as both an endocrine organ and a circadian oscillator (Collin et al., 1989). The light signals captured by this gland play important roles in a variety of physiological functions (Simonneaux and Ribelayga, 2003).

The mammalian MT2 is expressed in the retina and may mediate the actions of melatonin in terms of retinal physiology (Reppert et al., 1995a). The MT1 and MT2 are expressed in the brain regions involved in processing light information, such as the thalamic region and optic tectum, in rainbow trout (Mazurais et al., 1999), and MT2 expression has been reported in the retina of sea bass (Sauzet et al., 2008). In contrast, MT3 expression has been identified in the brain and retinas of non mammalian species such as zebrafish *D. rerio*, golden rabbitfish *S. guttatus*, African clawed frog *Xenopus laevis*, and chicks, suggesting that MT3 plays functional roles in neural tissues, and that it corresponds to 'quinone reductase-2,' a cytosolic enzyme that might be involved in detoxification processes (Ebisawa et al., 1994; Reppert et al., 1995b; Wiechmann and Smith, 2001; Mailliet et al., 2005; Park et al., 2007b).

Melatonin activity exhibits a circadian rhythm in fish, and control of the circadian rhythm is reportedly associated with a high density of melatonin-binding sites in the brain (Yuan et al., 1990). The brain of goldfish *Carassius aurata* reportedly exhibits a regional distribution of melatonin-binding sites, with the highest number occurring in the optic tectum-thalamus region (Iigo et al., 1994b).

MTs were recently cloned in sea bass, and MT1 and MT2 expression levels were investigated by in situ hybridization in the retina (Sauzet et al.,

2008). In addition, Confente et al. (2010) investigated the expression of three types of MT genes, comparing day-night and seasonal variations. Ikegami et al. (2009) investigated the expression levels of four subtypes of MT genes in the diencephalons of pufferfish *Takifugu niphobles* with lunar-related spawning cycles. Previous studies have shown that MTs perform the important role of regulating melatonin activity according to the circadian rhythm, but knowledge of the relationship between the expression of MTs and the control of circadian rhythm is limited, i.e. no previous studies have examined the relationship between melatonin treatment and circadian rhythm. An analysis of the expression patterns of MTs would provide a better understanding of the molecular basis of melatonin activity.

The aim of this study was to establish the molecular basis of circadian rhythm control by MTs in the olive flounder. The changes in mRNA expression of three types of MTs were observed in the retina, pineal gland, and optic tectum over a 28 h time period under both LD and DD photocycles. In addition, to investigate the effect of melatonin on MT mRNA expression, the cultured pineal gland samples were treated with exogenous melatonin and examined the changes in MT mRNA expression.

2. Materials and Methods

2.1. Experimental fish and conditions

Olive flounder (body length 15.0 ± 0.5 cm; body weight 27.5 ± 2.5 g) were obtained from a commercial fish farm and allowed to acclimate in three 300-l circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20 ± 1°C and LD (lights on 07:00-19:00 h, 200 lux during photophase and 0 lux during the dark phase at the water phase) for 2 weeks.

For the DD experiment, the flounders were kept for 1 day and provided commercial feed twice daily (09:00 and 17:00 h). The fish were anesthetized with MS-222 (Sigma, St. Louis, MO, USA) before blood collection. Blood was collected from the caudal vein using a 1-ml syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 × g, 5 min) and stored at -80°C until the analysis, then killed at 4 h intervals to collect the retina, pineal gland, and optic tectum.

2.2. Tissue distribution of three subtypes of MT mRNAs

To examine tissue distribution of three subtypes of MT mRNAs, total RNA was extracted from the nervous and peripheral tissues. The brain, retina, olfactory bulb, pituitary, gill, liver, kidney, and skin were removed and immediately stored at -80°C. Total RNA was extracted from the whole brain using a TRIzol kit (Gibco-BRL, Grand Island, NY, USA). RT was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. PCR amplification was performed with specific primer sets (Table 2) and using a 2 × Taq Premix I (Solgent, Daejeon, Korea). PCR was carried out as follows: initial denaturation at 95°C for 2 min, 33 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, and

extension at 72°C for 1 min, followed by 7 min at 72°C for the final extension. Amplification of β-actin mRNA was also conducted to verify the quality of the RT products using a primer set for olive flounder β-actin cDNA (Table 2). The amplified PCR products were electrophoresed on 1% agarose gels, detected by staining with ethidium bromide, and visualized by illumination with UV light.

2.3. QPCR

Total RNA was extracted from the whole brain using a TRIzol kit (Gibco-BRL). RT was performed using M-MLV reverse transcriptase (Bioneer) according to the manufacturer's instructions. QPCR performed using cDNA from the protocol above, was conducted to determine the relative expression levels of MT1 (GenBank accession no. ADI59670), MT2 (HQ844737), and MT3 (HQ844738) mRNAs using total RNA extracted from the whole brain.

Primers for QPCR were designed with reference to the known sequences of the olive flounder (Table 2). PCR amplification was conducted using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 20 s, and annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin. The efficiencies were found to be as follows: β-actin=94.7%, MT1=95.1%, MT2=95.0%, and MT3=95.3%. As an internal control, experiments were duplicated with β-actin; all data were expressed as change with respect to the corresponding β-actin calculated ΔCt levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated [$\Delta\Delta Ct=2^{\wedge}-(\Delta Ct_{sample}-\Delta Ct_{internal\ control})$]. The data were analyzed using the delta-delta method (Livak and Schmittgen, 2001).

Table 2. Primers used in this experiment

Primer	DNA sequences
cDNA amplification from different tissues	
MT1-F	5′-CCT CAT CTT CAC CAT CG-3′
MT1-R	5′-GTG GTT TGA TTG CTA CAG CC-3′
MT2-F	5′-ACT GCT ACA TCT GTC ACT CG-3′
MT2-R	5′-AGT AGG CCA TGA AGT AGC TG-3′
MT3-F	5′-TGT ACA GTC TGA GGA ACA CC-3′
MT3-R	5′-TGA GGC AGC TGT TGA AGT AC-3′
β-actin-F	5′-TCG AGC ACG GTA TTG TGA CC-3′
β-actin-R	5′-ACG GAA CCT CTC ATT GCC GA-3′
QPCR	
MT1-F	5′-CCT CAC CTC CAT CTT CCA-3′
MT1-R	5′-ATG TAG CAG TAG CGG TTA ATG-3′
MT2-F	5′-CAG AAT GTC AGC ACT TCC T-3′
MT2-R	5′-CCT CCT CGG TCT TCA CTT-3′
MT3-F	5′-TGC TGG TGG TGT CTT ACT-3′
MT3-R	5′-GTC GCT CGG TTT CAG TTT-3′
β-actin-F	5′-GCA AGA GAG GTA TCC TGA CC-3′
β-actin-R	5′-CTC AGC TCG TTG TAG AAG G-3′

2.4. *In vitro* cultures of the pineal gland and melatonin treatments

Cultures of the pineal gland were measured using the modified methods of Park et al. (2007b). After anesthetizing the fish, the pineal gland was dissected (06:00 h) from the fish and placed in an ice-cold medium (pH 7.5), which was composed of 150 mM NaCl, 3.5 mM KCl, 1.0 mM $CaCl_2 \cdot H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 0.7 mM $NaH_2PO_4 \cdot 2H_2O$, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 1.0 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, and 0.88 g/l Eagle's minimum essential medium (Sigma) containing antibiotics (0.06 g/l penicillin and 0.1 g/l streptomycin and penicillin-streptomycin; Gibco-BRL). The pineal gland was transferred into a 24-well culture plate with 1 ml of the medium and placed in an incubator at 20 ± 1°C under LD and DD conditions (LD12:12, light on at 7:00 h). The light intensity at the surface of the culture plate was approximately 700 lux during the light phase. The pineal gland culturing was started at 07:00 h and sampling conducted at 4 h intervals (n=5～6) from ZT4 to ZT28. Each sample was centrifuged (20°C, 10,000×g, 15 s), and the supernatant was removed and stored at -80°C until RNA extraction.

Melatonin (Sigma) dissolved in ethanol was added to the culture medium in the ratio 1:1,000e (v/v), and the indicated concentrations of melatonin (10 μM) were added. Melatonin treatments were given at 07:00 h (ZT0). Cells were treated for 4 or 28 h without renewing the medium. Each sample was centrifuged (20°C, 10,000×g, 15 s), and the supernatant was removed and stored at -80°C until RNA extraction.

2.5. Melatonin determination by enzyme-linked immunosorbent assay

To determine melatonin concentration in the plasma, the immunoenzyme-assay method was used with a commercial ELISA kit (IBL, Hamburg, Germany). Plasma samples were purified in the centrifuge using extraction

columns immediately after defrosting. Next, 50-μl of each sample was added to different wells of an ELISA plate pre-coated with capture antibody. The samples were then incubated with the melatonin-biotin and antiserum solutions for 15 h at 4°C, after which the wells were washed with the assay buffer (phosphate buffer with Tween and stabilizer), and the plate was incubated with the enzyme labeled solution (antibiotin-alkaline phosphatase in TRIS buffer with stabilizers) for 2 h at room temperature and constant shaking. Following a second washing of the plate, it was incubated with the p-nitro-phenyl phosphate solution for 30 min before adding 50 μl of the stop solution (1N NaOH with 0.25M ethylenediamine tetracetic acid). The absorbance was read at 405 nm.

2.6. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). ANOVA followed by a Tukey's post hoc test was conducted to detect statistically significant differences among the different time points of diurnal variations. If necessary, values were transformed (logarithmic or square root) to fulfill the conditions of the parametric ANOVA (normal distribution and homogeneity of variances). A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Tissue distribution of MT mRNAs

Fig. 11 shows the tissue distribution of the MT mRNAs. MT1 and MT3 mRNAs were strongly expressed in the neural and peripheral tissues. The mRNAs of all MT subtypes were expressed in the pineal gland, pituitary, optic tectum, diencephalon, cerebellum, telencephalon, and retina. MT1 mRNA was expressed in almost all peripheral tissues except the kidney, whereas the other mRNAs were weakly expressed or undetected.

3.2. Diurnal and circadian variations in MT mRNA expression in the retina, pineal gland, and optic tectum

The diurnal variation in MT mRNA expression in the retina, pineal gland, and optic tectum of the olive flounder by QPCR were examined (Figs. 12-14). All tissues examined showed significant differences ($P<0.05$) in mRNA expression with time under the LD conditions. The mRNA expression levels of all three subtypes peaked at night and decreased at the start of daytime, but the time to peak and expression levels varied among the subtypes. Statistically significant results were as follows: the MT1 mRNA expression levels were approximately 2-fold lower than those of the other subtypes in the retina, whereas the MT1 and MT3 mRNA expression levels were approximately 3-fold higher in the pineal gland. In addition, the MT1 and MT2 mRNA expression levels were approximately 1.3-fold higher than those of MT3 in the optic tectum, which is in agreement with the tissue distribution results (Fig. 11). Finally, under DD conditions, the MT mRNA expression levels were as rhythmic, but significantly lower with lower amplitude, compared to those under LD conditions.

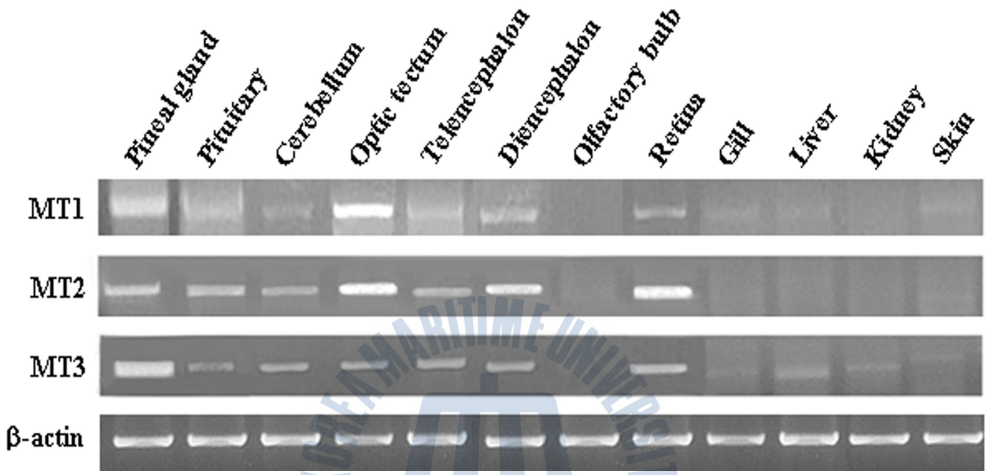

Fig. 11. MT mRNAs blots showing the tissue distribution of the three subtypes of MT mRNAs in olive flounder. β-actin mRNA was amplified to verify the integrity of each mRNA sample.

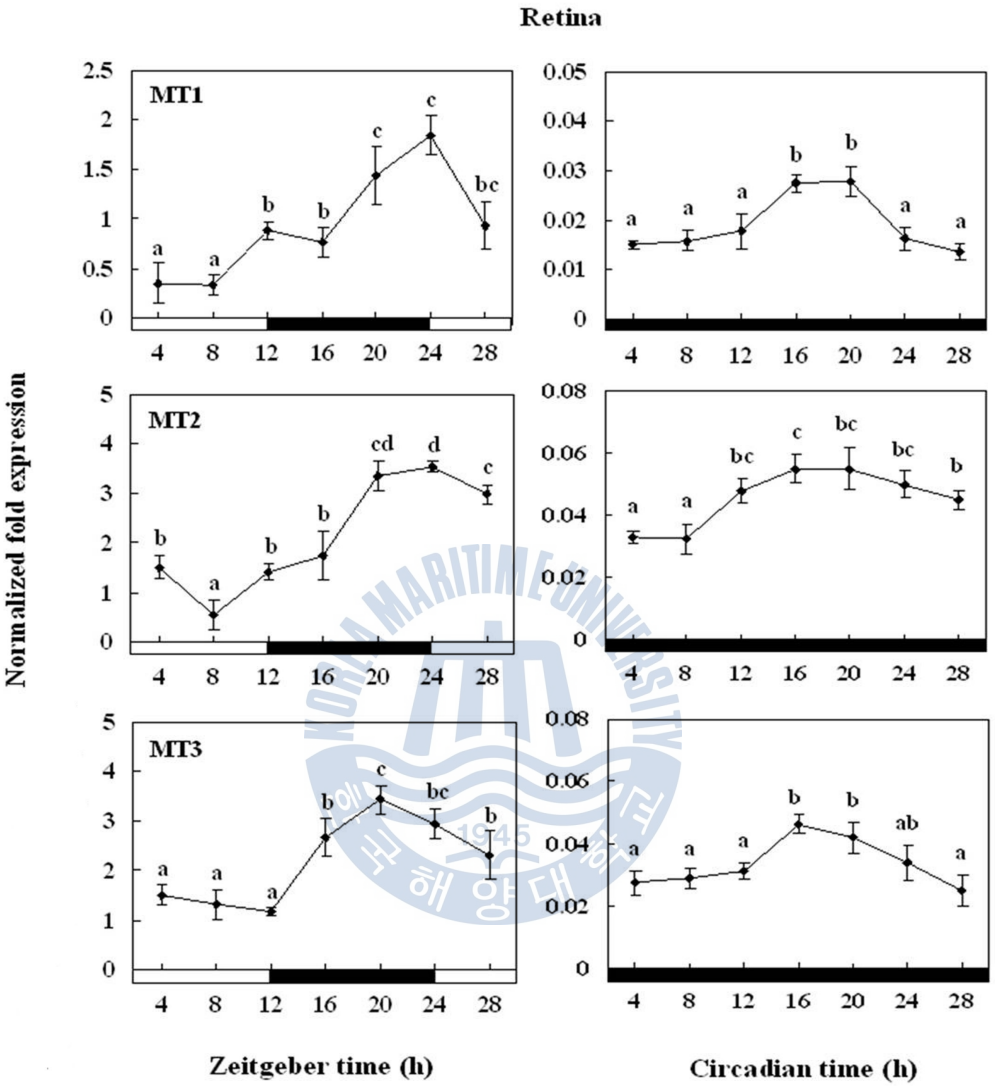

Fig. 12. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by QPCR analysis, in the retina of olive flounder. The fish were reared under 12:12 LD and DD conditions. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

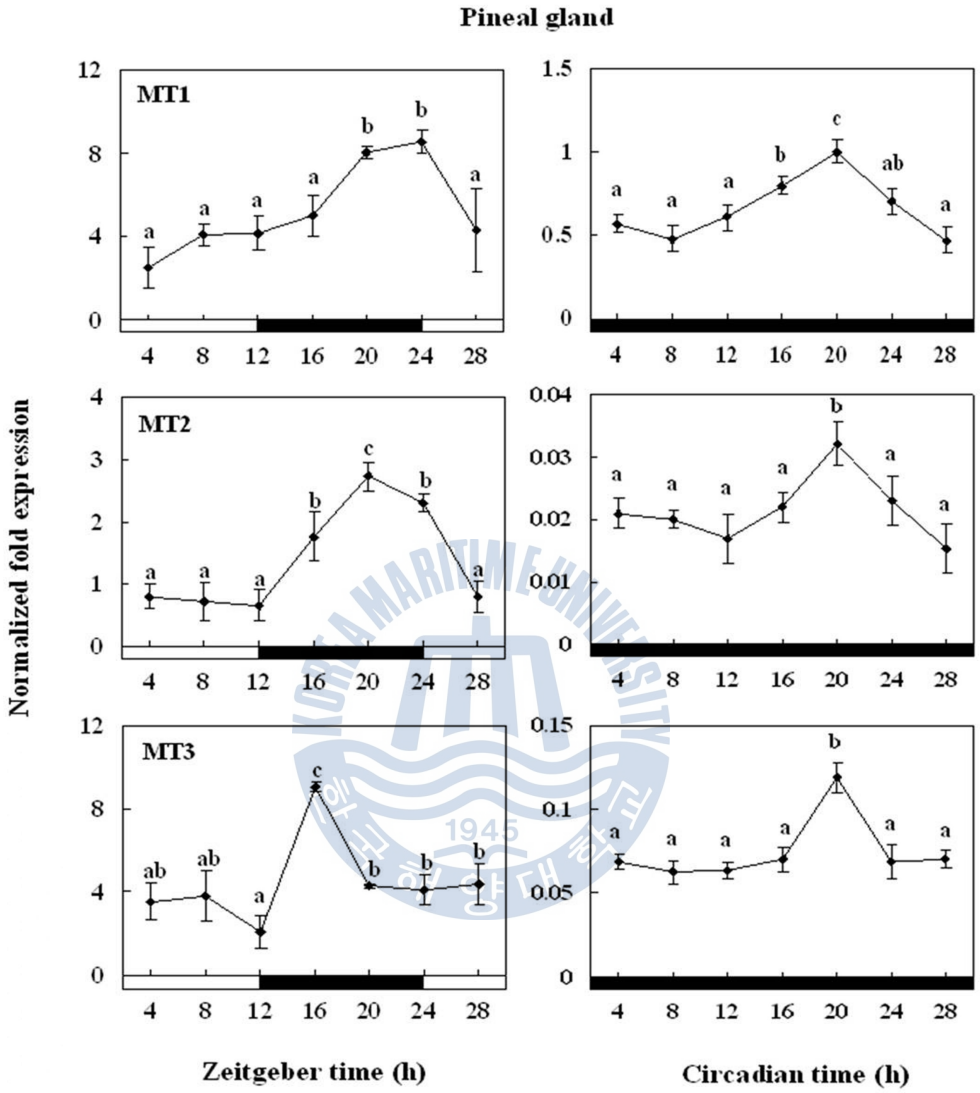

Fig. 13. Diurnal and circadian variation in the mRNA expression levels of the three MT subtypes, as measured by QPCR analysis, in the pineal gland of olive flounder. The fish were reared under 12:12 LD and DD conditions. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

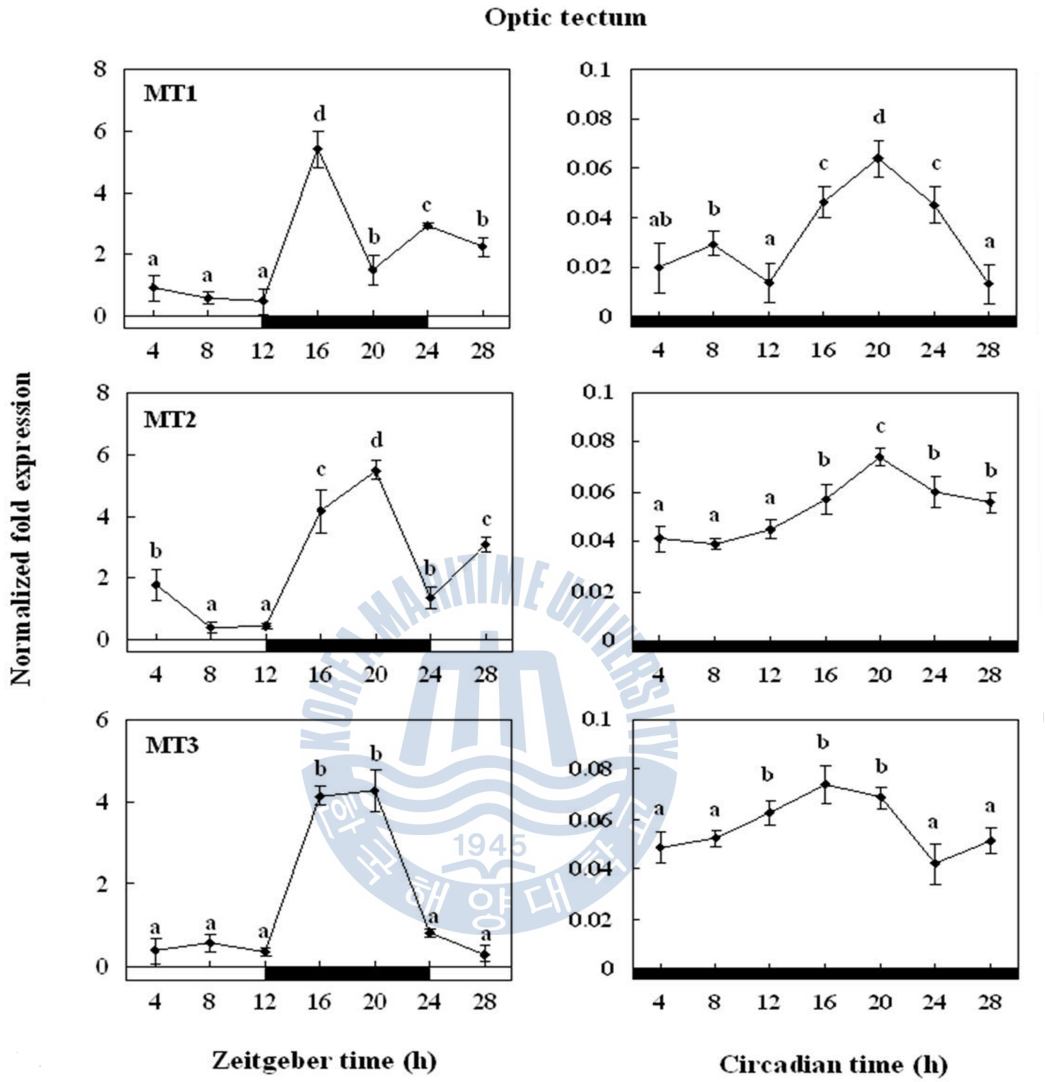

Fig. 14. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by QPCR analysis, in the optic tectum of olive flounder. The fish were reared under 12:12 LD and DD conditions. The white bars represent the photophase and black ones the scotophase. Different letters to indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

3.3. Diurnal and circadian variations in MT mRNA expression in untreated and melatonin-treated cultured pineal gland samples

As an *in vitro* experiment, the diurnal variations in the MT mRNA expression in untreated and melatonin-treated cultured pineal gland samples were examined (Figs. 15 and 16). In the untreated samples, the mRNA expression of all three subtypes peaked at nighttime and decreased at the start of daytime. Specifically, the peak amounts of MT1 and MT3 mRNAs were three to four times higher than that of MT2 mRNA at night. Similarly, in the melatonin-treated samples, the mRNA expression peaked at night and decreased at the start of daytime, but these samples showed approximately 5～40-fold higher peak amounts than the untreated samples. Under DD conditions, the MT mRNA expressions were as rhythmic as those under LD, but the expression levels and amplitude were significantly lower.

3.4. Plasma melatonin concentrations

Fig. 17 shows the plasma melatonin levels in olive flounder exposed to a normal 24 h LD cycle. The melatonin levels were low during the light phase, increased by approximately 3-fold during the dark phase, and then decreased to low daytime values before lights-on (ZT24). The melatonin concentrations were significantly higher during the dark phase than during the light phase (16.0 ± 1.9 pg/ml vs. 5.0 ± 2.0 pg/ml; $P<0.05$) (Fig. 17A).

The plasma melatonin levels under the DD condition were similar to those under the 24 h LD cycle, although they were of lower amplitude; they peaked significantly at ZT20 (10.0 ± 1.1 pg/ml) (Fig. 17B).

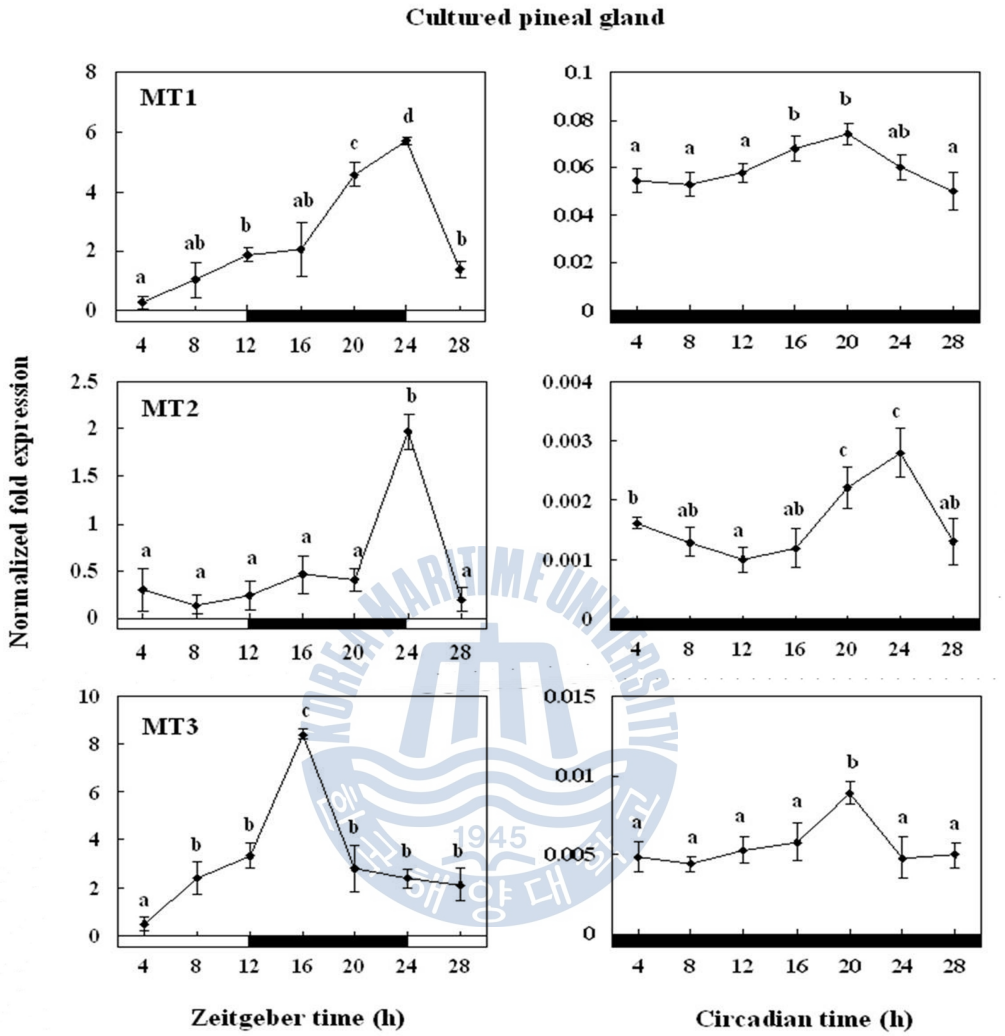

Fig. 15. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by QPCR analysis, in the untreated cultured pineal gland samples (*in vitro*) of olive flounder. The fish were reared under 12:12 LD and DD conditions. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

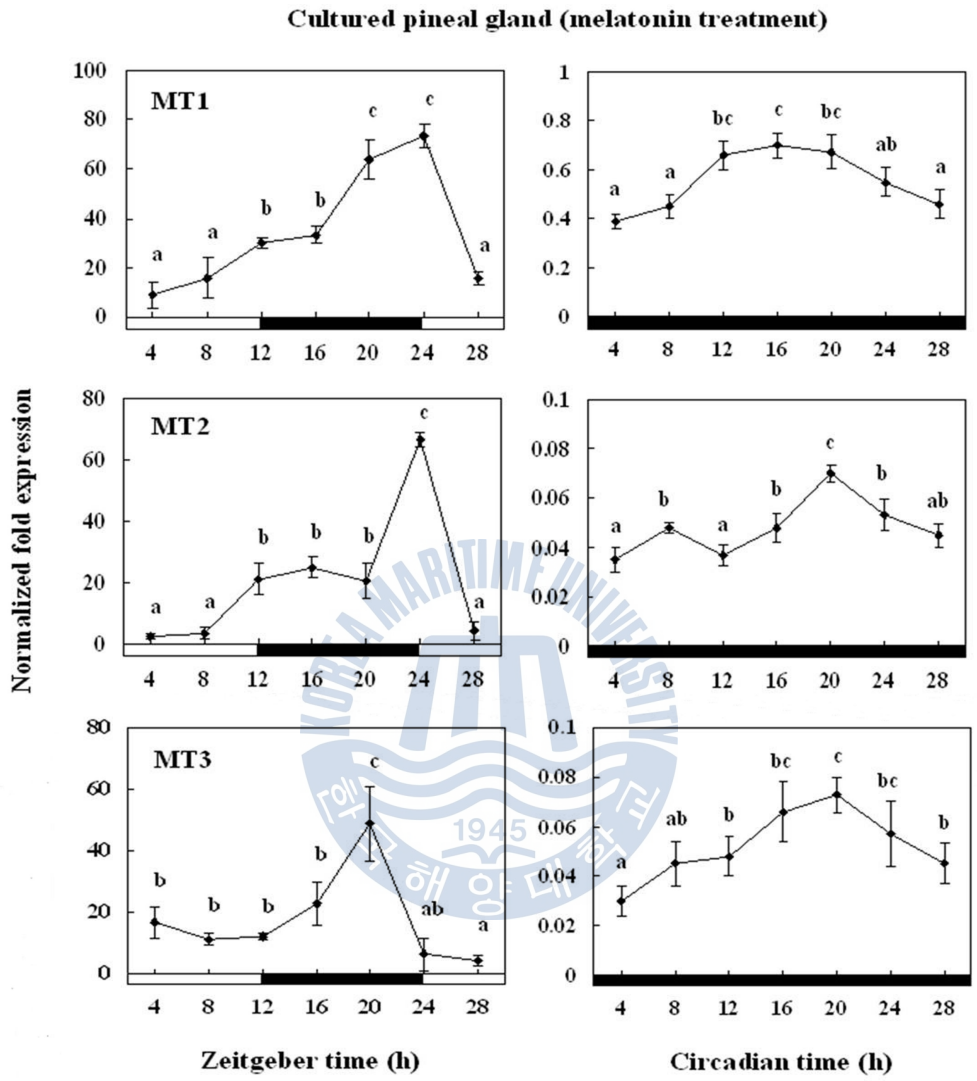

Fig. 16. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by QPCR analysis, in the melatonin-treated cultured pineal gland samples (*in vitro*) of olive flounder. The fish were reared under 12:12 LD and DD conditions. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

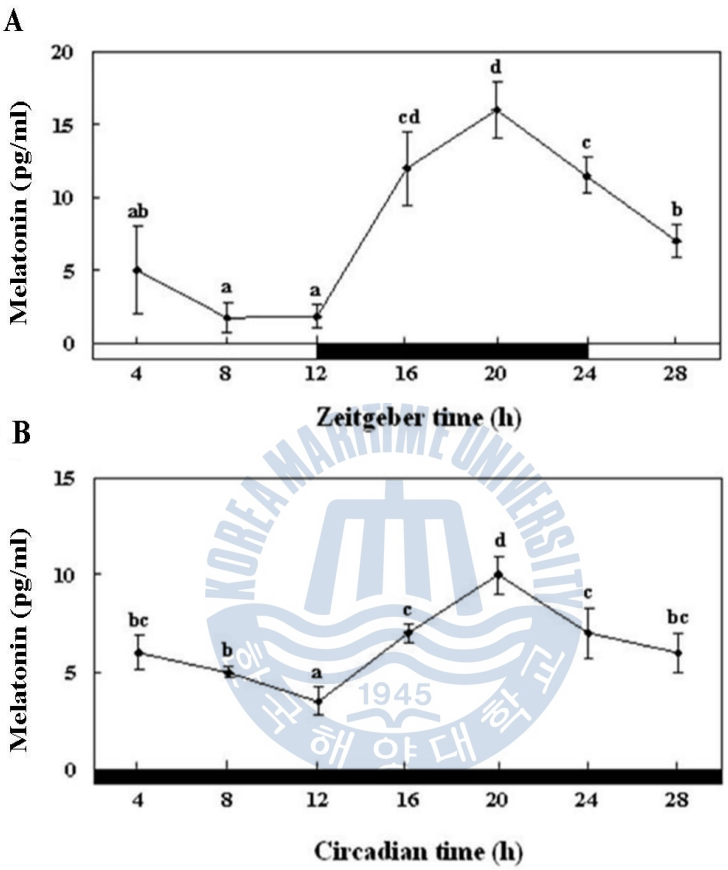

Fig. 17. Plasma melatonin measurement by ELISA in olive flounder during the daily LD (A) and DD (B) cycle. The white bars represent the photophase and the black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT (P<0.05). All values are the means ± SD (n=5).

4. Discussion

There were changes observed in MT mRNA expression in the retina, pineal gland, and optic tectum during diurnal and circadian variations to examine the role of MTs in circadian rhythm control in olive flounder. Specifically, the changes were investigated in MT mRNA expression during diurnal and circadian variations by treating cultured pineal gland samples with melatonin.

In terms of their tissue distribution, all three genes were strongly expressed in the pineal gland, pituitary, optic tectum, diencephalon, cerebellum, telencephalon, and retina. The expressions of these genes in parts of the visual system, such as the retina, confirm that they play a role in visual processing (Ikegami et al., 2009). Daily and circadian variations of melatonin-binding sites under LD conditions have been noted in the retina of goldfish (Iigo et al., 1997) and European sea bass (Bayarri et al., 2004) as well as in the whole brain of goldfish (Iigo et al., 1995) and gilthead seabream *Sparus aurata* (Falcón et al., 1996). MT2 and MT3 were either not expressed or were expressed at very low levels in peripheral tissues such as the gill and liver. Park et al. (2007a) showed that in fish, MT1 plays a role in circadian rhythm control in peripheral tissues as well as the brain, the central control organ of circadian rhythm. All three MTs were also expressed in the central nervous systems, such as the optic tectum and diencephalon. These results are in good agreement with the high number of melatonin-binding sites in the optic tectum-thalamus region. Iigo et al. (1994b) reported a high number of melatonin-binding sites in the optic tectum-thalamus region of goldfish. The pineal gland receives light information from the retina and acts as the central organ secreting melatonin. Therefore, hypothesize that melatonin targets are mainly present in central

areas integrating the visual/light information. These findings also suggest that the three types of MTs participate in mediating the melatonin activity involved in processing light information in the brain, and their distribution suggests that they may be involved in diverse functions of melatonin, such as circadian and annual control of behavioral and physiological rhythms, regulation of sleep, and neuronal apoptosis (Iigo et al., 1994a).

The expression of the three genes in the retina, pineal gland, and optic tectum varied diurnally. Specifically, they showed significantly high expression levels in the scotophase, with increasing expression at the start of night time (ZT12) and peak expression at ZT16 or ZT20 or ZT24 of approximately 3～18-fold. Under the DD conditions, the mRNA expression for the three genes exhibited similar circadian rhythmicity as under LD conditions. Zawilska et al. (2006) reported that melatonin synthesis occurs in a light-dependent rhythmic manner controlled by an endogenous circadian clock, with high levels during the dark phase and low levels during the light phase. In retina, the expression levels of the three genes significantly decreased, suggesting that they are regulated by light. Melatonin is an autocrine (neural retina) and paracrine (retinal pigment epithelium) regulator of retinal functions such as cyclic biosynthesis of melatonin and circadian activity of photoreceptor cells (Sauzet et al., 2008). The diurnal expression of the three genes may be important for the circadian regulation of the retinal functions (Ikegami et al., 2009). Despite the low amounts of mRNAs, circadian variations were still visible under DD conditions. Both light and the circadian clock may therefore be responsible for the diurnal expression of the three genes.

Previous studies have shown that melatonin produced by the pineal gland is released into circulation and exerts various biological actions, such as the control of reproduction and prolactin levels in seasonal breeders as well as the regulation of several circadian rhythms when reaching MT-rich target

tissues (Zawilska and Nowak, 1999). The optic tectum, which is related to the circadian rhythm, also exhibits a high density of melatonin-binding sites, and MTs show significant expression changes following secretion of melatonin (Iigo et al., 1994b; Ikegami et al., 2009). Thus, the major functions of melatonin in the optic tectum are considered to be visual signal transduction. In agreement with previous results (Ebisawa et al., 1994; Reppert et al., 1995b, 1996; Wiechmann and Smith, 2001), this study showed that MT1, MT2, and MT3 mRNA expression levels were slightly higher in the optic tectum than those of the MT genes in the retina. These findings indicate that the MT genes control the circadian rhythm in the brain at the SCN.

Retinal melatonin acts primarily in the eye, where it is involved in the control of circadian rhythm, as well as in the brain and pineal gland (Zawilska and Nowak, 1992; Cahill and Besharse, 1995). In the retina of golden rabbitfish, the expression of three types of MTs peaked at scotophase, ZT15, and ZT18 during a diurnal rhythm (Park et al., 2006, 2007a, 2007b). These previously reported results are similar to this study result in terms of the changes in MT mRNA expression in the retina (ZT20 and ZT24). Specifically, the MT2 and MT3 mRNA expression levels were approximately 2-fold higher than those of MT1 in the retina. MT2 and MT3 therefore control the physiological effects of melatonin in the retina.

The expression of the MTs peaked at scotophase during the diurnal rhythm in the cultured pineal gland samples which was similar to the *in vivo* results. Park et al. (2007a) investigated changes in the melatonin concentration and MT1 mRNA expression in cultured pineal gland samples of golden rabbitfish and found that MT1 mRNA was highly expressed in scotophase compared to the photophase. These results indicate that MTs genes are co-expressed in the pineal gland and share regulatory mechanisms of oscillation in the circadian clock. Light may therefore have different

influences on the expression of the three genes depending on tissues and regulation by melatonin (Iigo et al., 1995).

In summary, similar to the results reported by Park et al. (2007a), the expressions of the MTs were found that peaked at scotophase were rhythmic under both DD and LD conditions, but that they were of lower amplitude under the former. Specifically, the expression levels were approximately 10～100-fold higher than in the cultured pineal gland samples not treated with melatonin. Therefore, MT expression varies according to melatonin secretion levels, and melatonin controls the MT expression levels. These results were also supported by another result that the plasma melatonin concentration peaked at scotophase (ZT20) during the diurnal rhythm. Zawilska et al. (2006) reported that the plasma melatonin concentration in turkey greatly increases in scotophase, indicating that melatonin is a timekeeping hormone that plays a central role in circadian rhythm control. Therefore, melatonin may control the circadian rhythm directly by binding with MTs, and melatonin influences the molecular clock by phasing its circadian activity. Although the SCN of fish is a circadian oscillator is not known, the master clocks could be located in the hypothalamus in addition to the eyes and pineal organ (Falcón et al., 2007).

In conclusion, MT1, MT2, and MT3 show high expression levels in the brain regions receiving light information, particularly the optic tectum-thalamus region, as well as the retina, the organ that primarily handles light information. These results suggest that MTs play a central role in controlling the diurnal and circadian rhythms in the brain and retina of olive flounder.

Chapter 4

Effects of LED spectral sensitivity on circadian rhythm-related genes in the yellowtail clownfish *Amphiprion clarkii*

1. Introduction

Among the many factors that control circadian rhythms, light is the most important, because it affects many of the physiological and behavioral changes that occur within 24 h periods (Pierce et al., 2008). Because of the central importance of the day-night light cycle (the photoperiod) for the survival of organisms, light-sensitive circadian clocks have evolved in most animals, including fish. The photoperiod exerts an endogenous effect by causing rhythmic synthesis and release of the "time-keeping" hormone melatonin, which affects rhythmic physiological functions in fish (Bromage et al., 2001). Many activities in organisms vary according to periodic changes in environmental factors. Melatonin is mainly produced in the pineal organ and retina, and its concentration in the plasma is higher during the night than the day. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of the circadian rhythm but also affects seasonal biological rhythms (Reiter, 1991).

The effects of melatonin are mediated by MTs, which belong to the G protein coupled receptor superfamily (Reppert et al., 1996). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Reppert et al., 1996) and mediate various physiological functions of

melatonin in these tissues. Reppert et al. (1996) used recent molecular techniques to examine amino acid structures and revealed the existence of three different subtypes of melatonin receptors, MT1-MT3. In particular, MT1 has been widely identified in vertebrates, and high levels of MT1 expression have been detected in the SCN (Reppert et al., 1996), where the master circadian clock system is located in mammals (Masana et al., 2000). Therefore, photoperiod information conveyed via MTs can control the circadian rhythm in the SCN and regulate the circadian rhythm by feedback mechanisms involving the clock genes (Okamura et al., 2002; Park et al., 2007a).

However, there are few studies of circadian rhythm regulation systems in the fish brain (Shi et al., 2004; Park et al., 2007a). A recent report showed that the master circadian clock in teleost fishes may be the brain and pineal gland and that illumination during the night affects melatonin synthesis in a chromatic- and intensity-dependent manner in the pineal glands of all examined teleost fish species (Ekström and Meissl, 1997).

Per, which was first characterized in *Drosophila* (Reddy et al., 1984), is a key protein in the circadian system of animals. *Per* genes that code for a cytoplasmic heterodimer have been identified in vertebrates, and the role of these cytoplasmic heterodimers as circadian oscillators has been studied extensively. Four types of *Per* (*Per1-Per4*) have been reported in zebrafish (*D. rerio*; Delaunay et al., 2000). In zebrafish, *Per2* is a circadian oscillator that is rapidly induced by light information from the SCN and appears to be involved in light-dependent clock resetting (Vallone et al., 2004).

Cry1 regulates gluconeogenesis in the liver and the biological clock (Hirota et al., 2012). Hirota et al. (2012) reported that the small molecule, KL001, regulates *Cry* activity. These authors found that *Cry* gene inhibits gluconeogenesis by blocking enzymes and activates the gluconeogenesis

pathway, as indicated by an increase in the *Cry* expression when KL001 was injected into mouse hepatocytes.

Recently, studies of light effects have been performed by using LED instead of natural light or metal halide lights (Shin et al., 2011). LED are a new form of lighting technology that is still being developed and can be manufactured to output specific wavelengths (Migaud et al., 2007). Furthermore, LED have lower power requirements, lower electrical running costs, and longer life spans than standard metal halide bulbs (Migaud et al., 2007).

However, LED studies have only recently examined the effects of photoperiod and light intensity in fish (Pierce et al., 2008). Although studies have documented the responses of fishes to various spectra (Villamizar et al., 2009; Shin et al., 2011, 2012), investigations into changes in the control of fish circadian rhythms due to exposure to various light spectra remain very limited.

In the current study, the effects of specific spectra on the circadian rhythm of the yellowtail clownfish (a high-value ornamental fish) were investigated by measuring changes in the expression of the MT1 gene, expression of the clock genes *Per2* and *Cry1*, and plasma glucose in relation to daily rhythms after exposure to red, green, or blue LED spectra.

2. Materials and methods

2.1. Experimental fish and conditions

Yellowtail clownfish (body length, 5.5 ± 0.3 cm; body weight, 2.4 ± 0.5 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and were allowed to acclimate for two weeks in twelve 300-l circulation filter tanks in the laboratory. The fish were exposed to a simulated natural photoperiod (SNP) (Migaud et al., 2007). A white fluorescent bulb (27 W) was used for the control group, and light intensity near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 27 ± 1°C and a LD period (light on 07:00 h and light off 19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h). For the experimental groups, the fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LED (Daesin LED Co. Kyunggi, Korea) for 28 h. The LED were set 50 cm above the surface of water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, Colorado, USA) (Fig. 18).

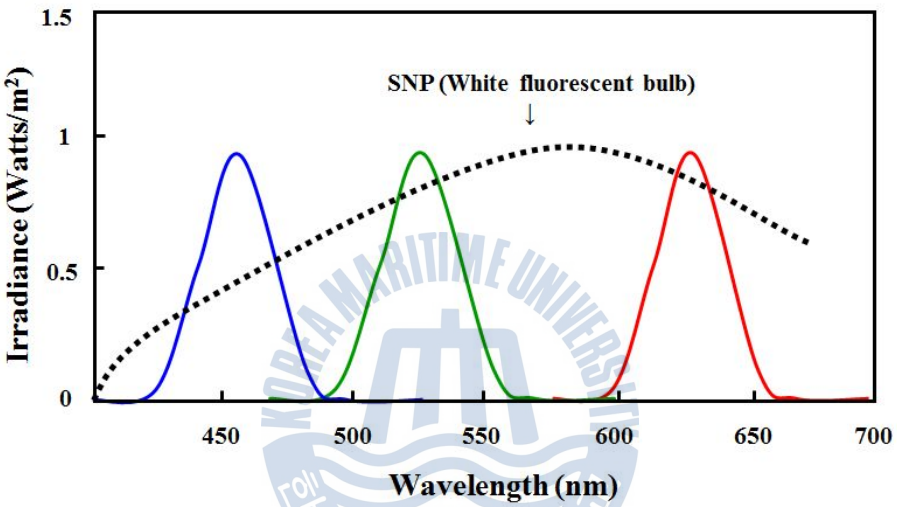

Fig. 18. Spectral profiles of blue (B), green (G), and red (R) LED used in this experiment. SNP; simulated natural photoperiod, B; blue, G; green, R; red.

2.2. QPCR

Total RNA was extracted from the livers using a TRIzol kit (Gibco/BRL, USA). RT was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. QPCR was performed using cDNA. QPCR was conducted to determine the relative expression levels of MT1 (GenBank accession no. JN418212), *Per2* (JQ809468), *Cry1* (JQ809469), and β-actin (JN039369) mRNA using total RNA extracted from the brain. The following QPCR primers were designed with reference to the known sequences of the yellowtail clownfish: MT1 forward (5′-GTC ATC GGC TCC ATC TTC-3′) and reverse (5′-GTT TAT CGT ATT TGA GGC TGT G-3′) primers; *Per2* forward (5′-CAG GAG GAA GAG AAA GTG AC-3′) and reverse (5′-TTT GTT GTT TGG GTT GGG-3′) primers; *Cry1* forward (5′-CAC TAA CAA CCC CTG CTT-3′) and reverse (5′-CAT GAT GGC GTC GAT CCA-3′) primers; and β-actin forward (5′-CCA ACA GGG AGA AGA TGA C-3′) and reverse (5′-TAC GAC CAG AGG CAT ACA-3′) primers. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. The QPCR was performed as follows: 95°C for 5 min, followed by 35 cycles each of 95°C for 20 s and 55°C for 20 s. As internal controls, experiments were duplicated with β-actin and GAPDH, and all data were expressed relative to the corresponding β-actin and GAPDH calculated Ct levels.

2.3. Melatonin determination by ELISA

The melatonin concentration in the plasma was determined using the ELISA kit (IBL, Hamburg, Germany) according to the manufacturer's instructions. The absorbance was read at 405 nm.

2.4. Plasma glucose analysis

Plasma samples were separated by centrifugation (4°C, 10,000×g, 5 min), and then the plasma glucose level was measured using a dry multiplayer analytic slide method in a biochemistry autoanalyzer (Fuji Dri-Chem 4000; Fujifilm, Tokyo, Japan).

2.5. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey's post hoc test was used to assess statistically significant differences among the different time points of the circadian variation and the different light spectra. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Expression of MT1, *Per2*, and *Cry1* genes in the brain

The effect of different LED light spectra on the expression of MT1, *Per2*, and *Cry1* genes in the brain by using QPCR were examined (Fig. 19). The expression of MT1 was significantly higher during the scotophase than during the photophase for all light spectra. In contrast, expressions of *Per2* and *Cry1* were significantly higher during the photophase than during the scotophase for all light spectra. MT1 expression in the group exposed to red LED was significantly higher than the expression in the groups exposed to other LED, and *Per2* and *Cry1* expressions in the groups exposed to green and blue LED were significantly higher than the expressions in the groups exposed to red LED.

3.2. Plasma melatonin levels

The effects of the different light spectra on plasma melatonin levels by using a microplate reader were observed (Fig. 20). Plasma melatonin levels were significantly higher during the scotophase than during the photophase for all light spectra. Plasma melatonin levels in the group exposed to red LED were significantly higher than the levels in the groups exposed to other LED.

3.3. Plasma glucose levels

The effects of the different light spectra on plasma glucose level by using a biochemistry autoanalyzer were observed (Fig. 21). Plasma glucose levels were significantly higher during the scotophase than during the photophase for all light spectra. However, glucose levels were significantly higher in the group exposed to red LED than in the other LED groups and the control group.

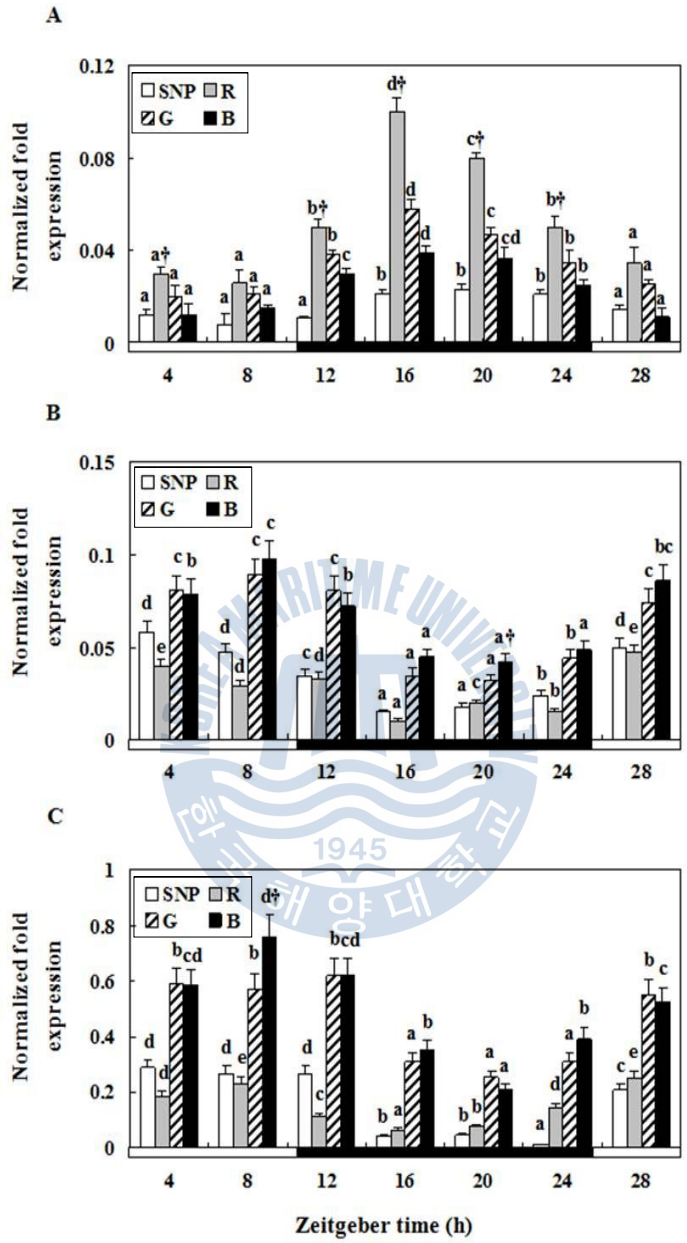

Fig. 19. Changes in the expression levels of MT1 (A), *Per2* (B), or *Cry1* (C) genes in the brain of yellowtail clownfish under lighting conditions with red (R), green (G), blue (B) LED, and SNP, as measured by QPCR. The white bar represents the photophase and black bar represents the scotophase. Values

with different characters are significantly different between the ZTs within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05). All values are means ± SD (n=5).

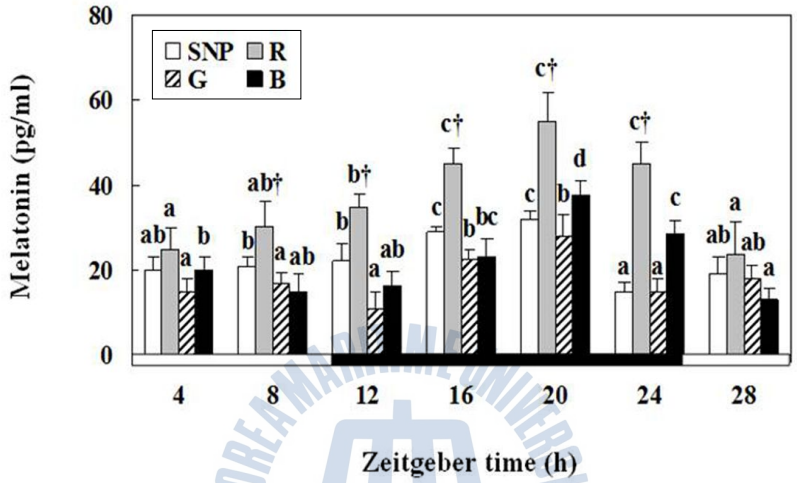

Fig. 20. Plasma melatonin levels obtained in yellowtail clownfish under lighting conditions with red (R), green (G), blue (B) LED, and SNP, as measured using a microplate reader. The fish were reared under a LD cycle (12:12). The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different between the ZTs within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05). All values are means ± SD (n=5).

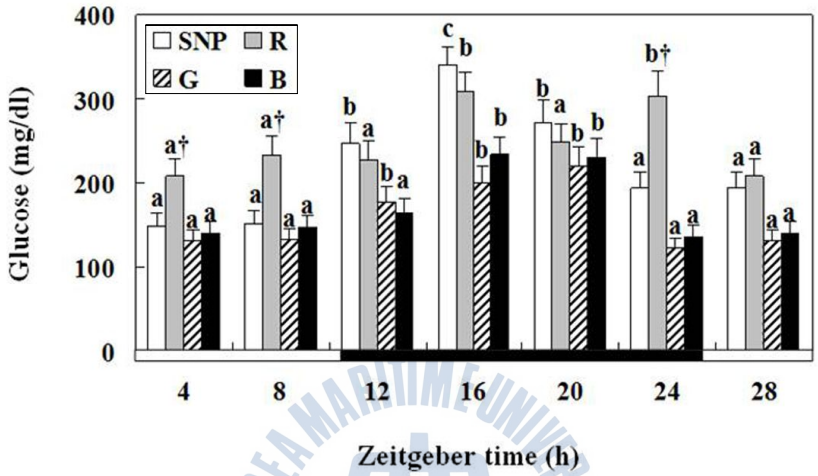

Fig. 21. Plasma glucose levels obtained in yellowtail clownfish under lighting conditions with red (R), green (G), blue (B) LED, and SNP, as measured using a microplate reader. The fish were reared under a LD cycle (12:12). The white bar represents the photophase and black bar represents the scotophase. Values with different characters are significantly different between the ZTs within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05). All values are means ± SD (n=5).

4. Discussion

In this study, the expression of the MT1, *Per2*, and *Cry1* genes were examined in yellowtail clownfish that were exposed to different LED spectral groups (red, green, and blue). Also, the effects of specific LED spectra on the circadian rhythm of the fish were investigated by measuring plasma melatonin levels.

First, this study showed that MT1 gene expression was significantly higher in the group exposed to red LED (Fig. 19A) than in groups exposed to either green or blue LED. Migaud et al. (2006) reported that most light energy is wasted in the form of unsuitable wavelengths (longer wavelengths, red light) that are rapidly absorbed by water molecules and therefore cannot be detected by fishes. Meanwhile, Villamizar et al. (2009) reported that blue wavelengths stimulate the visual system of fishes to a degree that is sufficient to facilitate feeding. In addition, Falcon and Meissl (1981) and Villamizar et al. (2009) reported that the fish pineal gland has double spectral sensitivity to light in the blue-green region. In the present study, the fish could not detect red wavelengths because red light is rapidly absorbed by water molecules and does not reach the depths where the fish reside. I hypothesized that MT1 expression would be significantly higher in the group of fish exposed to red LED than in the groups exposed to other LED. This result showed that, at night, the levels of MT1 mRNA were significantly higher in these fish than in those in the SNP group and the groups exposed to blue-green LED. I hypothesized that fish cannot detect red light and thus perceive it as night; therefore, MT1 mRNA expression levels would be significantly higher in the group exposed to red LED than to other light spectra.

To date, the use of molecular approaches to examine the circadian system

has been limited to only few model fish species. However, the importance of clock genes in the circadian rhythms of reproduction and feeding in several aquaculture fishes has been recently proposed. Furthermore, studies of the circadian system in fish using various spectra are very limited. Therefore, measured expression of clock genes in fish exposed to various LED spectra. Expressions of the clock genes, *Per2* and *Cry1*, were significantly higher in groups of fish exposed to blue-green LED (Figs. 19B and 19C) than in those exposed to red LED. While MT1 expression increased significantly during periods of darkness (Fig. 19A), clock gene expression increased significantly during periods of light. *Per2* and *Cry1* are induced by light exposure (Okamura et al., 2002). Hur et al. (2012) reported that the clock genes in the brain of the threespot wrasse *Halichoeres trimaculatus*, have different daily and circadian patterns. The opposite results for clock gene expression and MT1 expression suggest that a feedback loop exists between melatonin and clock genes. Cahill and Besharse (1995) reported that the circadian rhythm-controlling hormones, melatonin and dopamine, play opposing roles in the regulation of circadian rhythms. Dopamine, an amacrine and interplexiform cell neurotransmitter, functions as a chemical signal for light, producing light-adaptive physiology (Dowling and Ehinger, 1978). Melatonin, on the other hand, has dark-adaptive effects. Melatonin inhibits the release of dopamine by acting on the melatonin receptors, and dopamine inhibits the synthesis and release of melatonin from photoreceptor cells by acting on dopamine like receptors. Dopamine can also induce *Per2* and entrain the circadian clock (Cahill and Besharse, 1991). Thus, the melatonin-secreting photoreceptors and dopamine-secreting amacrine/ interplexiform cells form a cellular feedback loop that functions to regulate circadian physiology (Iuvone et al., 2005). These results support the hypothesis that the clock genes *Per2* and *Cry1* are inhibited by melatonin.

Moreover, melatonin influences the molecular clock to phase its circadian activity. Although it is not known whether the SCN of fish is a circadian oscillator, the master clocks are possibly located in the hypothalamus in addition to the eyes and pineal organ (Falcón et al., 2007).

The expressions of *Per2* and *Cry1* were induced by environmental factors and light. These observations are consistent with the involvement of clock genes and the light switch pattern of the circadian clock (Pando et al., 2001; Kim et al., 2012). In vertebrates, light responsive clock genes have been reported in goldfish *C. auratus* (Iigo et al., 2003) and zebrafish (Vallone et al., 2004), but detailed studies of clock genes in fish are limited.

Zhang et al. (2010) suggested that the clock component, *Cry1*, is a circadian regulator of hepatic gluconeogenesis as well as circadian rhythm. Through interaction with the Gs a subunit of G proteins, *Cry1* appears to modulate GPCR signaling. This may lead to temporal regulation of glucagon signaling, which is involved in the activation of hepatic gluconeogenesis. In the present study, plasma glucose levels were significantly higher throughout the entire photoperiod in fish exposed to a red LED spectrum than in those exposed to other light spectra, and that *Cry1* expression levels showed the opposite trend (Fig. 21). Hirota et al. (2012) reported that *Cry1* inhibits gluconeogenesis, and this study showed that *Cry1* mRNA expression was significantly lower under red LED than under blue or green LED. On the other hand, plasma glucose was significantly higher under red LED than under blue or green LED. These results are in agreement with a study by Karakatsouli et al. (2007) in which the plasma glucose of rainbow trout *Oncorhynchus mykiss*, was significantly higher in fish raised for 11 weeks under red light than in those raised under blue light. These authors concluded that red light induced stress and increased energy demands. Shin et al. (2012) measured the plasma H_2O_2 and lipid peroxidation levels in yellowtail

clownfish and examined oxidative stress induced by red-spectrum lighting. Among the fish studied by Shin et al. (2012), plasma glucose levels were highest in the red LED group. In addition, new lighting technology, such as LED units, may be beneficial to the aquaculture industry, because the operating cost is relatively low and the bandwidth of the light can be specified. It is common knowledge that the blue-green end of the visible spectrum penetrates seawater more efficiently than longer wavelengths (Wagner, 1990), and a number of reports suggest that fish are more sensitive to blue-green light than to red light (Max and Menaker, 1992; Ekström and Meissl, 1997; Bayarri et al., 2002).

In this study, plasma melatonin levels were significantly higher during the scotophase than during the photophase for all LED spectra. Moreover, melatonin levels were significantly higher throughout the entire photoperiod when fish were exposed to a red LED spectrum than to other spectra (Fig. 20). I hypothesized that fish cannot detect red light; therefore, they perceive illumination by red light as darker than illumination by other spectra, and melatonin levels remain high. This result is in agreement with those of various teleost studies, such as those on goldfish (Iigo et al., 2003). In these studies, teleost melatonin levels were significantly higher during the scotophase than during the photophase.

In conclusion, because red light could not be detected by the visual system of the yellowtail clownfish, due to the lack of photons, the fish perceived areas lit with red light as being darker than areas illuminated with blue-green wavelengths. In addition, red light induces stress and affects gluconeogenesis because of *Cry1* expression. MT1 expression was significantly higher in fish exposed to red LED than in those exposed to blue-green LED, further supporting the hypothesis that yellowtail clownfish cannot detect red light because of the lack of photons. The expression of the clock genes *Per2* and *Cry1* was significantly higher in fish exposed to blue-green LED than in those exposed to red LED, suggesting that red light inhibits the circadian rhythms and is a stressor.

Chapter 5

Effects of LED light spectra on oxidative stress and the protective role of melatonin in yellowtail clownfish *Amphiprion clarkii*

1. Introduction

Among the many factors that control circadian rhythms, light is the most important, modifying many of the physiological and behavioral changes that occur within a 24 h period (Pierce et al., 2008). Because of the central importance of the day-night light cycle (the photoperiod) in the survival of organisms, light-sensitive circadian clocks have evolved in most animals, including fish. The photoperiod exerts an endogenous effect by causing a rhythmic synthesis and release of the "time-keeping" hormone melatonin, which affects rhythmic physiological functions in fish (Bromage et al., 2001). Also, the oscillation of the circadian rhythm is driven by an intracellular molecular clock and is self-sustaining, i.e. occurring even in the absence of environmental cues (Pierce et al., 2008). Melatonin is produced mainly in the pineal organ and retina, and its plasma content is higher at nighttime than in the daytime. Moreover, the hormone not only acts as a neuroendocrine messenger in the regulation of circadian rhythm but also in seasonal biological rhythms (Reiter, 1991; Falcón et al., 2007).

In addition to its role in circadian regulation, melatonin has a number of other physiological functions, including clearing free radicals, improving immunity, and generally inhibiting the oxidation of biomolecules (Wu and

Swaab, 2005). Melatonin plays a role in antioxidant action by converting upon oxidation to a number of antioxidant compounds, including cyclic 3-hydroxymelatonin, N_1-acetyl-N_2-formyl-5-methoxykynuramine, and N_1-acetyl-5-methoxykynuramine (Reiter et al., 1997). Because of this array of compounds, melatonin is considered to be a broad spectrum antioxidant that is more powerful than glutathione in neutralizing free radicals and more effective than other antioxidants in protecting cell membranes (Reiter et al., 1997).

The *AANAT* enzyme is the precursor of melatonin; which is the rate-limiting enzyme of melatonin synthesis (Iuvone et al., 2005; Klein, 2007). *AANAT* catalyzes the conversion of serotonin to N-acetylserotonin, which is then catalyzed by HIOMT to form melatonin (Klein et al., 1997). In addition, the nocturnal rise in melatonin production is due to an increase in the activity of *AANAT* (Klein, 2007).

LED, a new form of lighting technology that is still being developed, can be manufactured to output specific wavelengths (Migaud et al., 2007). Furthermore, LED have lower power requirements, electrical running costs, and a longer life span than the standard metal halide bulbs (Migaud et al., 2007). Narrow bandwidth light using such new technologies, and especially a high energy short wavelength, could thus provide much more efficient lighting systems than those currently used in the fish farming industry since they can be tuned to a species environmental sensitivity by emitting narrow bandwidths (Villamizar et al., 2009). It is known that the spectral composition of incident light changes differentially in underwater environments and that there is a rapid attenuation with increasing depth (Lythgoe, 1979); the short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates in shallow waters (McFarland, 1991; Lythgoe et al., 1994; Myrberg and Fuiman, 2002). When farming the barfin

flounder *Verasper moseri*, using short wavelengths such as those in the blue spectrum, the flounders showed a high growth rate (Yamanome et al., 2009). In addition, it was shown that the blue spectrum prevented stress in the Nile tilapia *O. niloticus* (Volpato and Barreto, 2001), and that long wavelengths such as those in the red spectrum induced gonad development in the tropical damselfish *Chrysiptera cyanea* (Bapary et al., 2011).

However, certain LED spectra can have the negative effect of inducing stress in fish, including oxidative stress (Head and Malison, 2000; Van der Salm et al., 2004). Overproduction of ROS by environmental stress can increase LPO, the oxidation of nucleic acid and proteins, and induce DNA damage. It can also affect cell viability by causing membrane damage and enzyme inactivity and can thereby accelerate cell senescence and apoptosis (Kim and Phyllis, 1998; Pandey et al., 2003). Complex antioxidant defense systems maintain homeostasis in changing environments and protect aerobic organisms against ROS and subsequent oxidative stress-induced damage (Bagnyukova et al., 2007). Antioxidants can include enzymes such as SOD, CAT, or GPX and compounds such as melatonin, metallothionein, vitamin C, or vitamin E (α-tocopherol) (McFarland et al., 1999). Among these antioxidant compounds, melatonin plays a role in clearing free radicals and is known as a strong antioxidant within the antioxidant defense system (Wu and Swaab, 2005). Moreover, antioxidant enzymes found in the liver and kidneys of marine organisms and antioxidant compounds scavenge free radicals (Basha Siraj and Rani Usha, 2003). In fish, SOD and CAT are typical antioxidant enzymes that directly scavenge ROS.

Head and Malison (2000) and Van der Salm et al. (2004) reported that light spectra can induce stress in fish, but there have only recently been LED studies dealing with the effects of photoperiod and light intensity in fish (Boeuf and Le Bail, 1999; Bayarri et al., 2002; Pierce et al., 2008). Although

there are studies that show the response of fish to various spectra (Neumeyer, 1992; Villamizar et al., 2009; Bapary et al., 2011), investigations on the response of fish to various light spectra is still very limited (Head and Malison, 2000; Van der Salm et al., 2004).

Therefore, the expression changes of *AANAT2* mRNA were investigated in relation to the daily rhythm in experimental fish after exposure to red, green, and blue LED spectra, using the yellowtail clownfish, which is a high-value ornamental fish. In addition, the oxidative stress levels and the antioxidant response mechanism for specific spectrums were determined by measuring SOD and CAT expression and activities and plasma H_2O_2, LPO and melatonin levels in the yellowtail clownfish.

2. Materials and Methods

2.1. Experimental fish and conditions

Yellowtail clownfish (body length, 5.2 ± 0.5 cm; body weight, 2.1 ± 0.5 g) were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea) and were allowed to acclimate for 2 weeks in twelve 300-l circulation filter tanks in the laboratory. The fish were exposed to a SNP. A white fluorescent bulb (27 W) was used for the control group, and light intensity near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 27 ± 1°C and a LD period (lights on 07:00 h and light off 19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h). For the experimental groups, the fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LED (Daesin LED Co. Kyunggi, Korea) (Fig. 18). The LED were set 50 cm above the surface of water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2. The fish were anesthetized with 200 mg/l MS-222 (Sigma, St. Louis, MO, USA) prior to blood collection. Blood was collected from the caudal vein using a 3-ml syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 × g, 5 min) and stored at -80°C. The fish were euthanized by spinal transection at 4 h intervals, 07:00 h (ZT0), 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT24), to collect the pineal organ and liver under dim light.

2.2. QPCR

Total RNA was extracted from the livers using a TRIzol kit (Gibco/BRL, USA). RT was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. QPCR was performed using cDNA.

QPCR was conducted to determine the relative expression levels of *AANAT2* (GenBank accession no. JN032590), SOD (JN032591), CAT (JN032592), and β-actin (JN039369) mRNA using total RNA extracted from the pineal organ and liver. The following QPCR primers were designed with reference to the known sequences of the yellowtail clownfish: *AANAT2* forward (5′-CAT TCG TCT CTG TGT CTG G-3′) and reverse (5′-AAA GCC TCT CCT TGT CCC-3′) primers; SOD forward (5′-CAC GAG AAG GCT GAT GAC-3′) and reverse (5′-GAT ACC AAT GAC TCC ACA GG-3′) primers; CAT forward (5′-GGG CAA ATT GGT CCT CAA-3′) and reverse (5′-CGA TGT GTG TCT GGG TAG-3′) primers; and β-actin forward (5′-CCA ACA GGG AGA AGA TGA C-3′) and reverse (5′-TAC GAC CAG AGG CAT ACA-3′) primers. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. The QPCR was performed as follows: 95°C for 5 min, followed by 35 cycles each of 95°C for 20 s and 55°C for 20 s. As an internal control, experiments were duplicated with β-actin and GAPDH, and all data were expressed relative to the corresponding β-actin and GAPDH calculated Ct levels.

2.3. *In vitro* culture of the pineal organ and melatonin treatment

After the fish was anesthetized, the pineal organ was dissected out and placed in an ice-cold medium (pH 7.5) containing 150 mM NaCl, 3.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.7 mM NaH_2PO_4, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 10 mM HEPES, and 0.88 g/l Eagle's MEM (Sigma) containing antibiotics (0.06 g/l penicillin and 0.1 g/l streptomycin; Penicillin-Streptomycin, Gibco, USA). The pineal organs were transferred to a well of a 24-well microplate containing 1 ml of the medium and incubated at 20 ± 1°C

in an incubator under LD conditions (12L:12D, light switched on at 07:00 h). The lights were set at 40 cm above the surface of the pineal-organ culture plate, and the irradiance at the surface was maintained at approximately 0.96 W/m^2 (fluorescent bulb). The LED were approximately 0.9 W/m^2 during the light phase. The pineal organ was sampled at 4 h intervals from ZT4 to ZT28. One milliliter of each sample was centrifuged (20°C, 10,000×g, 15 s), and the supernatant was removed and remained tissues, in the bottom after centrifugation, stored at -80°C until RNA extraction.

Melatonin (Sigma, USA), dissolved in 0.9% physiological saline at the appropriate doses, was added to the culture medium in the following ratio: 1/1,000e (v/v) and the indicted concentrations of melatonin (10 μM) were obtained. Cultured pineal organs were treated for 4 to 28 h without renewing the medium. One milliliter of each sample was centrifuged (20°C, 10,000 × g, 15 s), and the supernatant was removed and removed and remained tissues, in the bottom after centrifugation, were stored at -80°C until RNA extraction.

2.4. Melatonin injection

Melatonin (Sigma) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the yellowtail clownfish (5.2 ± 0.5 g) were intraperitoneally injected at a dose of 200 μg melatonin/g body weight (BW) at a volume of 10 μl/g BW. The fish were injected at 07:00, and 5 fish were then sacrificed by decapitation at 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT24), and 11:00 h (ZT28).

After injection, the pineal organ and liver were sampled from 5 fish at 4 h intervals from ZT4 to ZT28. All of the fish survived the experimental period.

2.5. SOD and CAT activity analysis

Liver tissues were homogenized in ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was removed and the remaining pellet was used for analyses. SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities were determined using commercial kits supplied by Cayman Chemical (USA).

Each assay was performed in duplicate, and the enzyme units were recorded as U/ml, and the CAT activity was expressed in nmol/min/ml.

2.6. H_2O_2 assay

H_2O_2 concentrations were measured using the modified methods of Nouroozzadeh et al. (1994) and a Peroxidetect kit (Sigma, USA). Twenty microliters of olive flounder serum were added per well to flat-bottom 96-well microtitre plates. Concentrations are expressed as nM/ml.

2.7. LPO assay

LPO was quantified by measuring the amounts of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are the degradation products of polyunsaturated fatty acid (PUFA) hydroperoxides (Esterbauer et al., 1991), with a Lipid Hydroperoxide Assay Kit (Cayman Chemical) according to the manufacturer's instructions. LPO was expressed in terms of nmoles of MDA and 4-HNE per gram protein.

2.8. Melatonin determination by ELISA

The melatonin concentration in the plasma was determined using the ELISA kit (IBL, Hamburg, Germany). The absorbance was read at 405 nm.

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by Tukey's post hoc test was used to assess statistically significant differences among the different time points of the daily and circadian variation and the different light spectra. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Expression of *AANAT2* mRNA in the pineal organs

The effect of the different LED light spectra on the expression of *AANAT2* mRNA in the pineal organ by using QPCR were examined (Fig. 22). *AANAT2* mRNA was expressed at significantly higher levels during the scotophase than during the photophase for all light spectra. Moreover, for the pineal organ (*in vivo*) and cultured pineal organ (*in vitro*), *AANAT2* mRNA was expressed at significantly higher levels for the red LED group relative to the other LED ones (Fig. 22A and C).

Following melatonin injection into living fish or melatonin treatment of cultured pineal organs, the *AANAT2* expression patterns of the different LED groups during the daily rhythm were similar to the untreated LED ones. However, the expression levels of the melatonin-treated LED groups were significantly lower than the untreated LED ones (Fig. 22B and D).

3.2. Expression of SOD and CAT mRNA in the liver

The QPCR was used to examine the effects of the different LED regimes on the expression of SOD and CAT mRNA in the liver (Fig. 23). Both genes were expressed at significantly higher levels during the photophase than during the scotophase for all light spectra. Meanwhile, SOD and CAT mRNA levels were significantly higher in the red LED group than in the other LED groups (Fig. 23A and C). Moreover, after the injection of melatonin, the expression patterns of both genes during the daily rhythm were similar between treated and untreated LED groups, but the expression levels were significantly higher for the untreated LED groups (Fig. 23B and D).

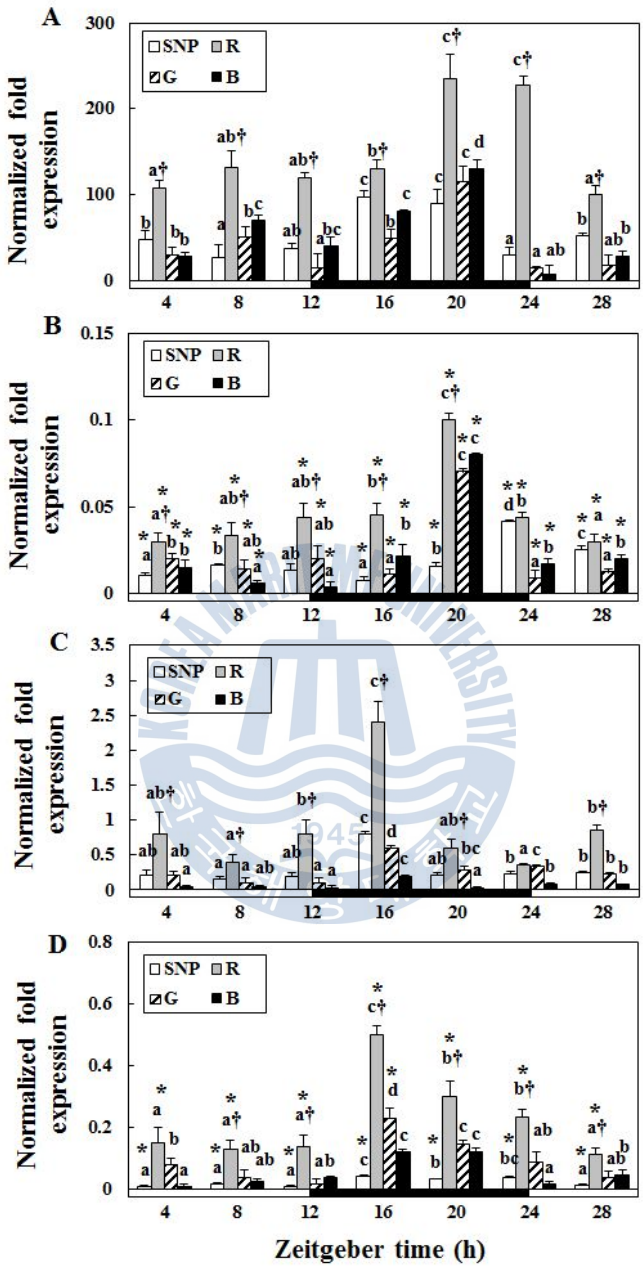

Fig. 22. Changes in the expression levels of *AANAT2* mRNA in the pineal organ (*in vivo*) (A), pineal organ injected with melatonin (*in vivo*) (B), cultured pineal organ (*in vitro*) (C), and cultured pineal organ treated with

melatonin (*in vitro*) (D) under lighting conditions using red (R), green (G), blue (B) LED, and SNP, as measured by QPCR. The fish were reared under a LD cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

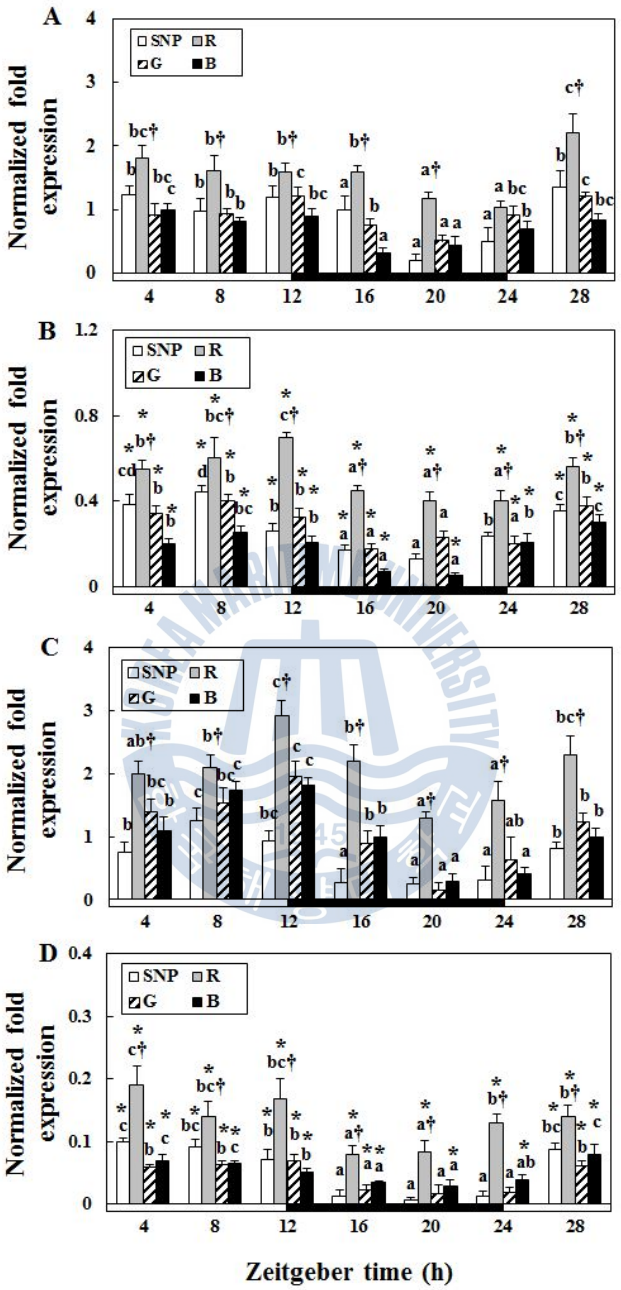

Fig. 23. Changes in the expression levels of SOD and CAT mRNA in the liver (A) and (C), and the melatonin-injected liver (B) and (D) of yellowtail clownfish under red (R), green (G), blue (B) LED, and SNP, as measured by

QPCR. The fish were reared under a LD cycle (12:12). Total liver RNA (2.5 μg) was reverse-transcribed and amplified. Results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

3.3. SOD and CAT activities in the liver

The effect of the different LED spectra on SOD and CAT activities in the liver by using a microplate reader were examined (Fig. 24). SOD and CAT activities were at significantly higher levels during the photophase than during the scotophase for all light spectra. In addition, the activities of both enzymes was significantly higher in the red LED groups than in the other LED ones and the control (Fig. 24A and C). In addition, the activity level patterns of both enzymes during the daily rhythm were similar between the melatonin-injected LED groups and the uninjected ones, but the activity levels were significantly lower in the injected LED groups (Fig. 24B and D).

3.4. Plasma H_2O_2 levels

The effects of the different LED spectra on the plasma H_2O_2 levels by using a microplate reader were observed (Fig. 25). The plasma H_2O_2 levels were at significantly higher levels during the photophase than during the scotophase for all light spectra, whereas the H_2O_2 levels were significantly higher for the red LED group than for the other LED groups and the control (Fig. 25A). After the injection of melatonin, the pattern of H_2O_2 plasma levels during the daily rhythm were similar between the injected LED groups and the uninjected groups, but the plasma levels were significantly lower for the injected groups (Fig. 25B).

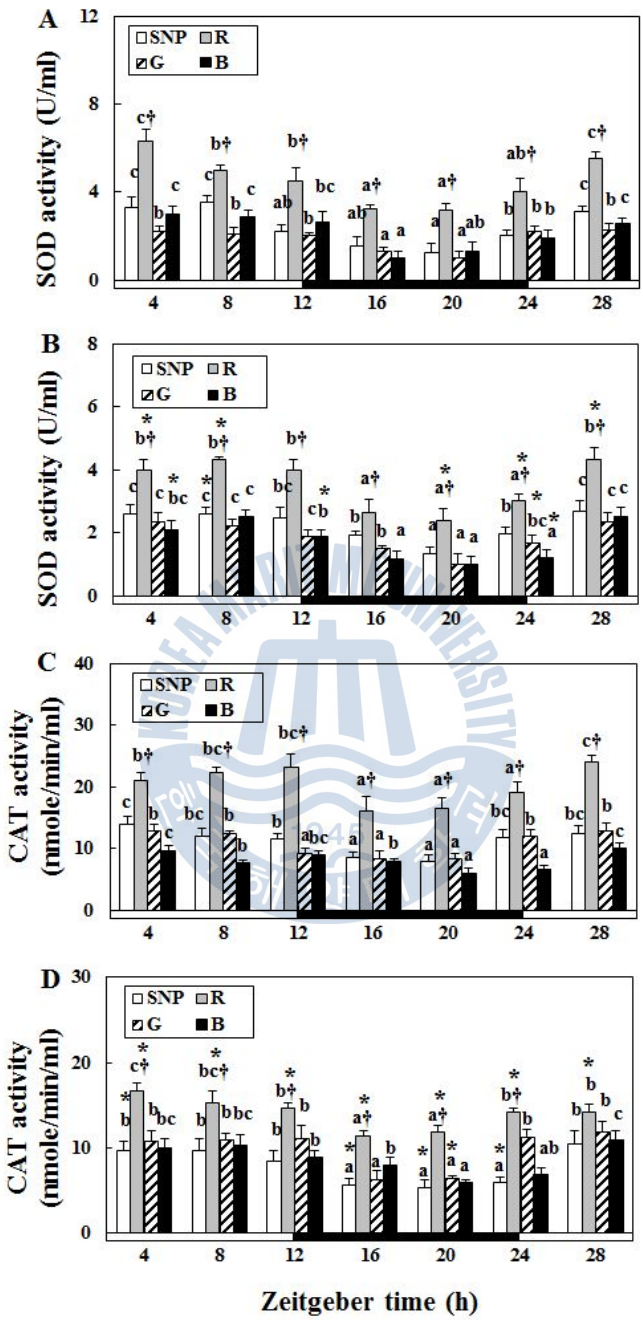

Fig. 24. Changes in the activity levels of SOD and CAT in the liver (A) and (C), and the melatonin-injected liver (B) and (D) of yellowtail clownfish

under red (R), green (G), blue (B) LED, and SNP, as measured by a microplate reader. The fish were reared under a LD cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

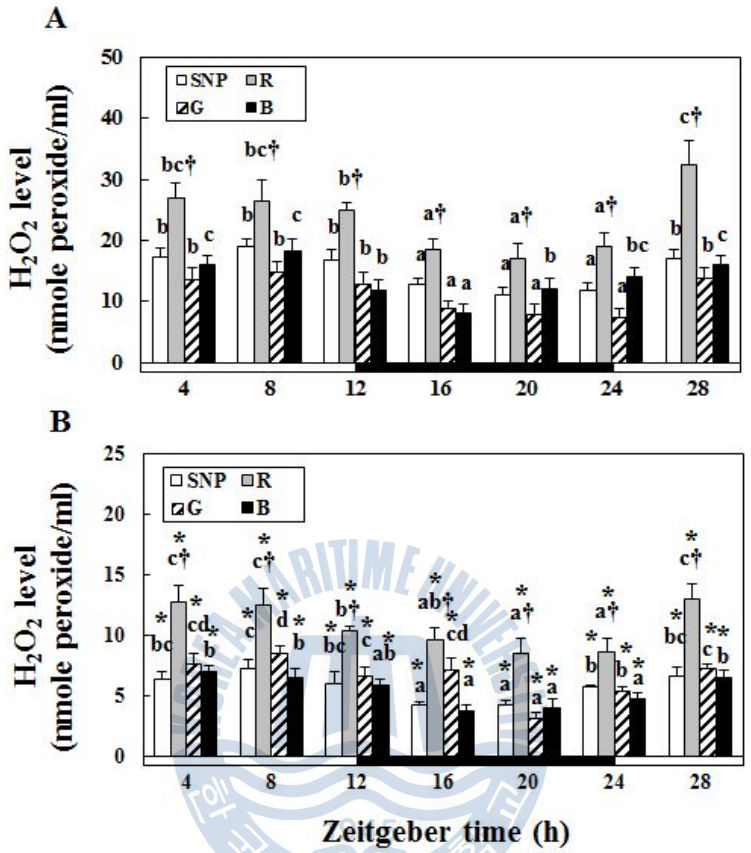

Fig. 25. Plasma H_2O_2 concentrations before (A) and after melatonin injection (B) in yellowtail clownfish under red (R), green (G), blue (B) LED, and SNP, as measured by microplate reader. The fish were reared under a LD cycle (12:12). The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

3.5. LPO levels

The effects of the different LED spectra on the LPO levels were observed (expressed in terms of the amounts of MDA and 4-HNE) by using a microplate reader (Fig. 26). The LPO levels were at significantly higher levels during the photophase than during the scotophase for all light spectra, whereas the LPO levels were significantly higher for the red LED group than for the other LED groups and the control (Fig. 26A). After the injection of melatonin, the pattern of LPO levels during the daily rhythm were similar between the injected LED groups and the uninjected groups, but the plasma levels were significantly lower for the injected groups (Fig. 26B).

3.6. Plasma melatonin levels

The effects of the different light spectra on plasma melatonin levels by using a microplate reader were observed (Fig. 27). Plasma melatonin levels were significantly higher during the scotophase than during the photophase for all light spectra. However, the melatonin levels were at significantly higher levels for the red LED group than for the other LED groups and the control (Fig. 27). Following the injection of melatonin, the pattern of plasma melatonin levels during the daily rhythm was similar between the injected and uninjected groups, whereas the plasma levels were significantly higher in the injected groups (Fig. 27).

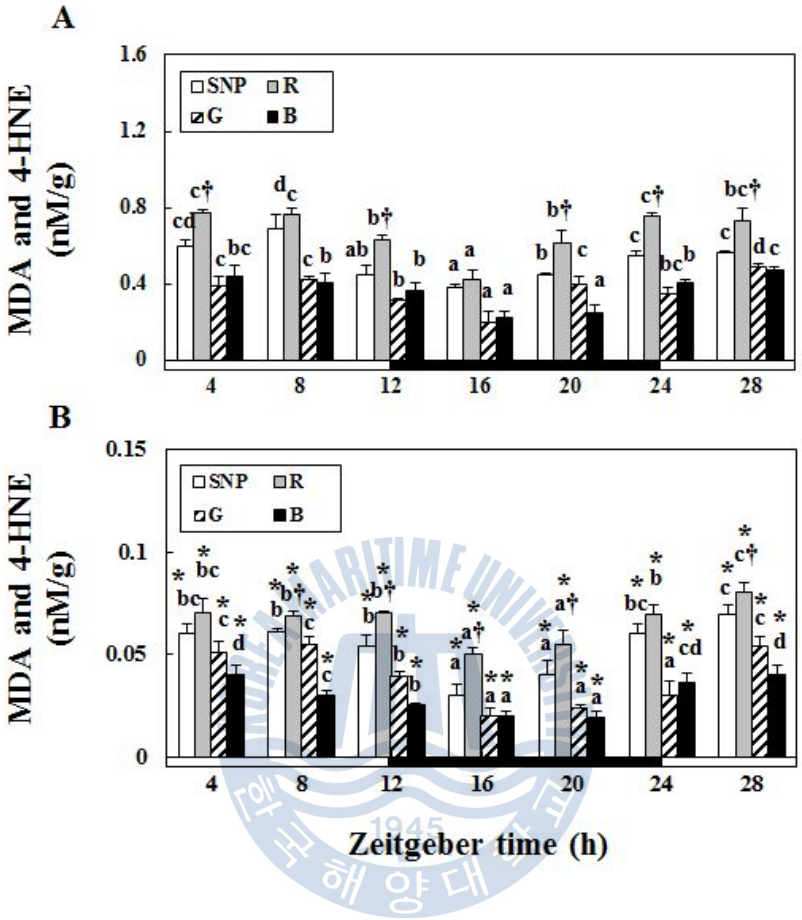

Fig. 26. MDA and 4-HNE levels before (A) and after melatonin injection (B) in yellowtail clownfish under red (R), green (G), blue (B) LED, and SNP, as measured by microplate reader. The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicates significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

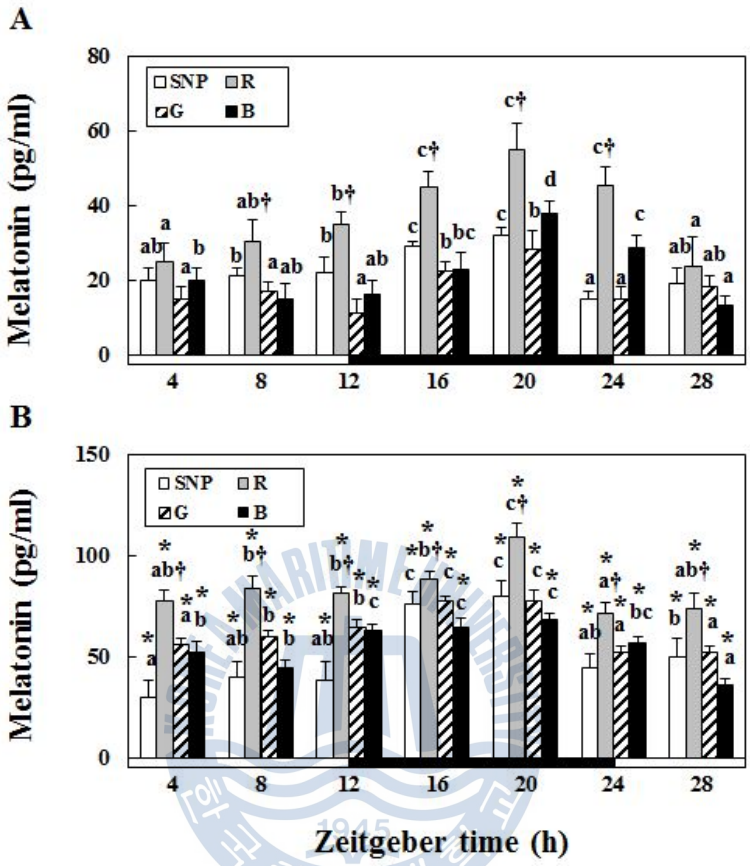

Fig. 27. ELISA melatonin plasma levels (A) and levels of melatonin injection (B) in yellowtail clownfish under lighting conditions using red (R), green (G), blue (B) LED, and SNP, as measured by microplate reader. The white bar represents the photophase, and the black bar represents the scotophase. Values indicated by different characters are significantly different between the ZT within the same light spectrum (P<0.05). The cross (†) indicate significant differences between different light spectra within the same ZT (P<0.05), and asterisks above letters indicate significant differences between melatonin uninjected or untreated group and injected or treated group (P<0.05). All values are means ± SD (n=5).

4. Discussion

In this study, the mRNA levels of the *AANAT2* enzymes, which synthesizes the precursor of melatonin, the mRNA expression and activities of the antioxidant enzymes SOD and CAT, and plasma H_2O_2 and melatonin levels were examined to determine the effects of different LED spectra (red, green, and blue) on oxidative stress in order to investigate the antioxidant response mechanisms. In addition, the *AANAT2* mRNA expression levels were observed in the pineal organ and SOD/CAT mRNA expression and activity changes in the liver of the yellowtail clownfish during the daily rhythm to examine the antioxidant capacity of melatonin.

First, this study showed that the *AANAT2* mRNA expression were significantly higher for the red LED spectrum than other spectra for both the pineal organ (*in vivo*) and cultured pineal organ (*in vitro*) (Fig. 22). Furthermore, SOD and CAT mRNA expression and SOD and CAT activities were found that they were significantly higher for the red LED spectrum than for the other spectra (Fig. 23, 24). Hence, these results suggest that red LED spectra produce oxidative stress in the yellowtail clownfish. Villamizar et al. (2009) have reported that the larvae of the European sea bass *Dicentrarchus labrax* when exposed to red LED spectra, showed a significantly lower growth and food intake rate relative to other spectra. With regard to these results, Villamizar et al. (2009) discussed the effect of the lack of appropriate photons for stimulating the visual system. In this study, the high expression levels of *AANAT2* mRNA in the red LED group suggest that melatonin was being synthesized as an antioxidant to protect against oxidative stress. Since melatonin synthesis in the pineal organ, the pattern of melatonin synthesis usually parallels *AANAT2* activity (Iuvone et al., 2005). *AANAT2* is the precursor of melatonin; which is the rate-limiting enzyme of melatonin

synthesis (Iuvone et al., 2005; Klein, 2007). *AANAT* catalyzes the conversion of serotonin to N-acetylserotonin, which is then catalyzed by HIOMT to form melatonin (Klein et al., 1997). Furthermore, melatonin is a strong antioxidant that can protect cell membranes from oxidative damage more effectively than other antioxidants (Reiter et al., 1997). Therefore, *AANAT2* mRNA expression, SOD, CAT expression and activity were analyzed in yellowtail clownfish injected (*in vivo*) or treated (*in vitro*) with melatonin to examine whether melatonin plays a role as an antioxidant against oxidative stress induced by specific LED spectra. Blanco-Vives et al. (2010) study reported that larvae exposed to red light exhibited a delay in growth and thus the yolk sac was visible, while in the blue light the yolk sac was completely absorbed. Larvae responded best to blue light, which appeared to be the most efficient light spectrum on the development on Senegal sole larvae (Blanco-Vives et al., 2010). These observations agree with these results that *AANAT2*, SOD and CAT expression and activity were of low levels in short wavelength such as blue and green LED lights, which appeared to be the most efficient light spectrums on the oxidative stress inhibition. Thus, the potential benefits of short wavelength such as blue light to adult fish as well as larvae. Also, the expression levels for *AANAT2*, SOD, and CAT were observed that they were significantly lower for the melatonin treated or -injected groups than for the uninjected or untreated ones (Figs. 22 and 23). These results indicated that melatonin reduces oxidative stress and are in agreement with those of Reiter et al.'s (2007) study, which reported that melatonin reduces oxidative stress. Therefore, I hypothesized that red LED light induced oxidative stress in yellowtail clownfish, and melatonin injection or treatment removed the ROS, as a strong antioxidant, that had occurred in fish. So, *AANAT2* gene expression, as a precursor of melatonin, indicated indirect antioxidant parameters against red LED light spectrum in this study. Also, Rosa Nogués

et al. (2006) reported that there is sufficient melatonin to scavenge free radicals, thus antioxidant enzymes (SOD and CAT) have no need to increase and may even decrease. Melatonin is thought to directly detoxify free radicals, such as highly toxic hydroxyl radicals, via electron donation, with an electron-rich aromatic indole ring that functions as an electron donor, thereby reducing electrophilic radicals (Reiter et al., 1997; Martinez et al., 2005; Wu and Swaab, 2005).

Consistent with this model for melatonin function, the plasma H_2O_2 levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~2 fold in melatonin-injected fish than the uninjected ones (Fig. 25). These results are in agreement with the studies of Allegra et al. (2003) and Martinez et al. (2005), which reported that melatonin scavenges ROS induced by red spectrum. In addition, the plasma H_2O_2 levels in the rat *Rattus norvegicus* treated with the environmental toxicants, polychlorinated biphenyls (PCB), were significantly higher than those in rats that intraperitoneally received melatonin (Venkataraman et al., 2008). Venkataraman et al. (2008) concluded that melatonin plays a strong antioxidant role by scavenging ROS.

Induced ROS from red LED spectrum can oxidatively damage the cellular elements in its target organ such as muscle. In this study, LPO levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~100 fold in melatonin-injected fish than the uninjected ones (Fig. 26). Red LED spectrum induced oxidative stress and then caused oxidative damage in tissue of yellowtail clownfish. These results are in agreement with Baydas et al. (2002) and Venkataraman et al. (2008), which reported that melatonin prevents oxidative stress induced by PCB, and then LPO levels were lower than the unsupplemented melatonin group in adult rats. It seems to be

melatonin prevents oxidative stress induced by red LED spectrum.

In this study, plasma melatonin levels were measured and they were at a significantly higher level during the scotophase than during the photophase for all LED spectra. Moreover, the melatonin levels were significantly higher for the red LED spectrum than for the other spectra over the course of the entire photoperiod (Fig. 27). These results indicated that the red spectrum induced oxidative stress in yellowtail clownfish and that more melatonin was produced under the red LED to scavenge for increased ROS. In addition, plasma melatonin levels rapidly increased after melatonin injection. I can conclude that melatonin levels were temporarily increased by the injection of the highest dose of melatonin. This result is in agreement with those of various teleost studies such as those on bloch (Renuka and Joshi, 2010), gilthead sea bream (Falcón et al., 1996), and goldfish (Iigo et al., 2003). In these studies, the teleost melatonin levels were significantly higher during the scotophase than during the photophase, similar to the results from this study. Increasing plasma melatonin levels have been shown to increase following induction of *AANAT* mRNA synthesis (Namboodiri et al., 1987; Ganguly et al., 2002).

In conclusion, the red spectrum is a factor in inducing oxidative stress in yellowtail clownfish. This study showed that melatonin likely plays a strong antioxidant role to reduce oxidative stress in fish. Light is known as a factor that affects various physiological changes in fish. Hence, additional studies will be necessary to understand the oxidative stress mechanism in fish in response to different light spectra.

Chapter 6

Effects of LED light spectral sensitivity on the growth of the yellowtail clownfish *Amphiprion clarkii*

1. Introduction

The influence of environmental factors on the growth and reproduction of fish has been studied extensively for more than 10 years. Salinity, pH, and oxygen availability are known to play a major role on the capacity to develop and grow, but light is not only essential to almost all life, both plants and animals, on earth, it also modulates a number of physiological and behavioral changes that occur within a 24 h period as part of the circadian rhythm (Pierce et al., 2008). Specifically, daily light conditions affect the survival and growth of fish (Barahona-Fernandes, 1979; Tandler and Helps, 1985). Under lighting regimes, the somatic growth of organisms, including fish, is an integral biological process, and measures of growth may serve as indicators of organismal fitness, since growth is related to the maintenance of homeostasis (MacKenzie et al., 1998).

Growth, food intake, and digestion are related to specific behavioral rhythms, including reproduction, with pineal hormones (such as melatonin) being the assumed regulators of these mechanisms (Underwood, 1989; Zachmann et al., 1992). For example, the administration of melatonin to goldfish maintained under a short (but not long) photoperiod for several days caused an accelerated weight gain and growth (de Vlaming, 1980), and melatonin implants increased the body weight of Atlantic salmon parr *Salmo salar* (Porter et al., 1998).

GH, which is required for the normal growth of vertebrates, is synthesized in the pituitary gland and functions to control growth through the complex modulation of various metabolic processes (Canosa et al., 2007). GH also promotes growth in vertebrates, including fish, through various endocrine and environmental factors (i.e., salinity, pH, oxygen availability, light) that play a role in the physiological environment (Chen et al., 1994). Another hormone that promotes growth is melatonin (Zeman et al., 1993). This hormone modulates GH synthesis, and it is primarily controlled by light. Melatonin may also modulate the central neural pathways involved in the regulation of GH synthesis, as well as control the circadian rhythm of organisms (John et al., 1990). It has also been shown to affect the physiological processes involved in the growth and development of goldfish *C. auratus* (Bromage et al., 2001).

In some cases, color is added to rearing environments by painting the tanks or using colored lights in an attempt to increase the visibility of food and thereby increase growth rates by increasing food intake (Ullmann et al., 2011). For example, the survival rate of haddock larvae *Melanogrammus aeglefinus* L. is higher under blue and green light conditions (Downing, 2002), and the growth rate of silver carp larvae *Hypophthalmichthys molitrix* Val. and young carp *Cyprinus carpio* L. increases under green light conditions (Radenko and Alimov, 1991; Ruchin et al., 2002).

LED represent a new form of lighting technology that is still being developed. LED can be manufactured to output specific wavelengths (Migaud et al., 2007). Consequently, by emitting narrow bandwidth light, especially short high-energy wavelengths, LED might provide a much more efficient lighting system than those currently used by the fish farming industry because they can be adjusted to the environmental sensitivity of a given species (Villamizar et al., 2009). The spectral composition of incident light changes

differentially in underwater environments, with the short or blue end of the visible spectrum becoming predominant in deeper waters and the long or red end of the visible spectrum predominantly penetrating shallow waters (Lythgoe et al., 1994; Myrberg et al., 2002). From a practical perspective, because there is a rapid attenuation in light intensity with increasing depth, a particular LED wavelength can be applied according to the species of interest and the water environment. Yamanome et al. (2009) reported that barfin flounder *V. moseri* reared under light consisting of long-wavelengths, such as green and blue light (fluorescent lamp), showed rapid growth, while in Nile tilapia *O. niloticus*, the blue light spectrum was found to prevent stress (Volpato and Barreto, 2001). In contrast, compared to short wavelengths, certain long wavelengths, such as red light, have been found to induce stress in yellowtail clownfish (Shin et al., 2011). However, LED studies have only recently begun to address the effects of photoperiod and light intensity in fish (Pierce et al., 2008; Villamizar et al., 2009; Boeuf and Le Bail, 1999), and the number of investigations carried out to date on the effects of LED on the growth of fish is limited (Villamizar et al., 2009; Blanco-Vives et al., 2010).

The clownfish is a popular ornamental seawater fish (Tissot et al., 2010). The rapid growth of ornamental fish aquaculture, such as clownfish production, is an important component of aquaculture in Korea. To understand the effects of different LED spectra on growth and GH mRNA expression levels, yellowtail clownfish were reared under different LED lights (i.e., red, green, and blue) and investigated growth and diurnal changes in GH mRNA expression levels under the different light conditions. The effects of LED lights on GH mRNA expression levels were examined under different LED spectra following melatonin injection in order to understand the growth-promoting effect of melatonin.

2. Materials and methods

2.1. Experimental fish and conditions

Yellowtail clownfish (body length 3.5 ± 0.3 cm, body weight 2.1 ± 0.5 g) were purchased from the Center of Ornamental Reef and Aquarium (CCORA, Jeju, Korea) and then allowed to acclimate for 2 weeks in twelve 300-l circulation filter black-frosted tanks in the laboratory. The fish were exposed to a SNP. Fish exposed to a white fluorescent bulb (27 W) constituted the control group; light intensity near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 27.0 ± 1.1°C and 12/12 h (light/dark), respectively, with light supplied between 07:00 h and 19:00 h. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h) at a rate of 4-5 % of wet body weight per day. pH was 7.8～7.9, and ammonia was not detected (0 ppm) in the water. For the experimental groups, the fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LED (Daesin LED Co. Kyunggi, Korea) (Fig. 18). The LED were set 50 cm above the water surface, and the irradiance at the water surface was maintained at approximately 0.9 W/m^2. The fish were reared under these conditions for 6 months. Prior to each experiment, the fish were anesthetized with 200 mg/l MS-222 (Sigma, St. Louis, MO) and then euthanized by spinal transection at 4 h intervals in order to collect the pituitary under dim light.

2.2. Isolation of GH cDNA

Primers for GH were designed using highly conserved regions from fire clownfish *Amphiprion melanopus* GH (GenBank accession no. ADJ57589) and green sunfish *Lepomis cyanellus* GH (AAS20461) sequences. GH forward (5′ -TTT GCA GAC GGA GGA ACA AC-3′) and reverse (5′-GGC GAC AGT

CGA CAT TTA GC-3′) primers were used. Total RNA was extracted from the pituitary gland using a TRIzol kit (Gibco/BRL, Gaithersburg, MD). RT was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. PCR amplification was performed using a Takara Taq DNA polymerase (Takara, Tokyo, Japan) with the following cycling conditions: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min; a final extension at 72°C for 7 min. The PCR product was separated in 1% agarose gels, purified, and ligated into a pGEM-T Easy Vector (Promega, Madison, WI). A colony that formed after transformation was cultivated in DH5α (RBC Life Sciences, Seoul, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification kit (Cosmo, Seoul, Korea). The GH cDNA sequence data were analyzed using an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA).

2.3. 3′- and 5′-RACE of GH

For the GH RACE reaction, total RNA was extracted from the pituitaries using a TRIzol kit (Gibco/BRL). Using 2.5 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Seoul, Korea).

GH-specific primers were designed based on the sequences of the PCR product amplified by the RT-PCR. For 3′ RACE, the PCR reaction mixture (50 μl) contained 5 μl of 3′ RACE cDNA, 1 μl of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 mM 3′ RACE GH-specific primer (5′-CAC ATC CGA TCA CGG TGG AAA CAT

GTA C-3′), and 25 μl of SeeAmp Taq Plus Master Mix (Seegene). The PCR was performed as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min; a final extension at 72°C for 5 min. For 5′ RACE, the PCR reaction mixture (50 μl) contained 5 μl of 5′ RACE cDNA, 1 μl of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 mM 5′ RACE GH-specific primer (5′-TGA ACG TGG CTG CAG CGT TCT CTC TC-3′), and 25 μl of SeeAmp Taq Plus Master Mix. The PCR was carried out as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min; a final extension at 72°C for 5 min. The PCR product was processed by electrophoresis in 1% agarose gels. Transformation and sequencing were performed as described above.

2.4. Sequence comparisons

The BLAST algorithm (Blastp) of the National Center for Biotechnology Information was used to compare sequences between the yellowtail clownfish and other teleosts.

2.5. QPCR

QPCR was conducted to determine the relative expression levels of GH mRNA using total RNA extracted from the pituitary. The following QPCR primers were designed with reference to known sequences of the yellowtail clownfish: GH forward (5′-CTC CAG TTG GCT CCT TAT G-3′) and reverse (5′-CAC CGT CAA GTA TGT CTC C-3′) primers; β-actin forward (5′-CCA ACA GGG AGA AGA TGA C-3′) and reverse (5′-TAC GAC CAG AGG CAT ACA-3′) primers. The GH forward primer position was at 448-466 and the reverse position was at 555-573; the β-actin forward primer

position was at 128-146 and the reverse position was at 212-229. PCR amplification was conducted using a Bio-Rad iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA) and an iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. The cycling program for the QPCR was: 95°C for 5 min, followed by 35 cycles each of 95°C for 20 s and 55°C for 20 s. As an internal control, the experiments were duplicated with β-actin, with all the data expressed as the change relative to the corresponding calculated β-actin Ct levels. The efficiencies were found to be 95.3 % for β-actin 97.0% for GH. All data were expressed as change with respect to the corresponding calculated β-actin ΔCt levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$].

2.6. Melatonin injection

Melatonin (Sigma) was dissolved and diluted in 0.9% physiological saline. After being anesthetized, the yellowtail clownfish (3.5 ± 0.3 g) were intraperitoneally injected with 200 μg melatonin/g BW at a volume of 10 μl/g BW. The fish were injected at 07:00 h, with five fish subsequently being sacrificed by decapitation at 11:00 h (ZT4), 15:00 h (ZT8), 19:00 h (ZT12), 23:00 h (ZT16), 03:00 h (ZT20), 07:00 h (ZT24), and 11:00 h (ZT28), respectively. After injection, the pituitary was sampled from five fish at 4 h intervals from ZT4 to ZT28, respectively. All of the fish survived the experimental period.

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (ver. 10.0; SPSS, Chicago, IL). ANOVA followed by Tukey's post hoc test was used to assess statistically significant differences among the different time-points of

daily and circadian variation and the different light spectra. A value of $P<0.05$ was considered to be statistically significant. All values are presented as means ± SD (n=5).

3. Results

3.1. Identification of full-length GH cDNA

A single PCR product of the expected size (428 bp) was obtained by RT-PCR. A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was then used to clone the full-length cDNA encoding GH. The full-length GH cDNA contained a reading frame that was predicted to encode a protein of 204 aa (JN008015). On comparing the amino acid sequence of yellowtail clownfish GH with those deduced from the GH cDNAs of other teleost species, The yellowtail clownfish GH sequence was determined and it contained the conserved signal peptide (Met^{1}-Ser^{17}) and the three putative N-glycosylation sites (Asn^{201}-Cys^{202}-Thr^{203}). The four Cys residues in yellowtail clownfish GH are located at conserved positions (69, 177, 194, and 202). The yellowtail clownfish GH aa sequence showed 99% similarity with the fire clownfish GH (ADJ57589), 94% with the gilthead seabream GH (AAA03329), 93% with the green sunfish GH (AAS20461), and 93% with the ballyhoo GH (AAV48597) (Fig. 28).

Fig. 28. Comparison of the GH aa sequence of yellowtail clownfish *A. clarkii*, fire clownfish *A. melanopus*, green sunfish *Lepomis cyanellus*, gilthead seabream *S. aurata*, and ballyhoo *Hemiramphus brasiliensis* optimally aligned to match identical residues, as indicated by the shaded box. Line-box Signal peptide of the GH-encoding sequence. Putative N-glycosylation sites (Asn-X-Ser/Thr) are marked by asterisks, Cys residues are marked by closed inverted triangles. The sequences were taken from the GenBank/EMBL/DDBJ sequence database. The GenBank accession numbers for the GH sequences used for alignment are as follows: yellowtail clownfish (ycGH, JN008015), fire clownfish (fcGH, ADJ57589), green sunfish (gsGH, AAS20461), gilthead seabream (gsbGH, AAA03329), and ballyhoo (bhGH, AAV48597).

3.2. Expression of GH mRNA in the pituitary during the daily rhythm

The effect of the different LED light spectra on the expression of GH mRNA in the pituitary by QPCR were examined (Fig. 29). GH mRNA was expressed at significantly higher levels during the scotophase than during the photophase for all light spectra. In addition, the expression of GH mRNA was significantly higher in the pituitary of fish in the green and blue LED groups than in those in the red and SNP groups (Fig. 29A).

Following the injection of melatonin into living fish, the GH expression patterns of the different LED groups during the daily rhythm were similar to those of the control (i.e., without injection) LED groups. However, the expression levels of the GH gene in the melatonin-injected LED groups were significantly higher than that of the control LED groups (Fig. 29B).

3.3. Total length

The total lengths of the fish under green and blue LED conditions were significantly higher compared to those under other light conditions (Table 3). At the end of the experiment (6 months), the green and blue LED groups had the greatest total body length (6.1 ± 0.4 and 6.2 ± 0.2 cm, respectively), while the red LED and SNP groups had the shortest total body length (4.9 ± 0.3 and 4.8 ± 0.5 cm, respectively).

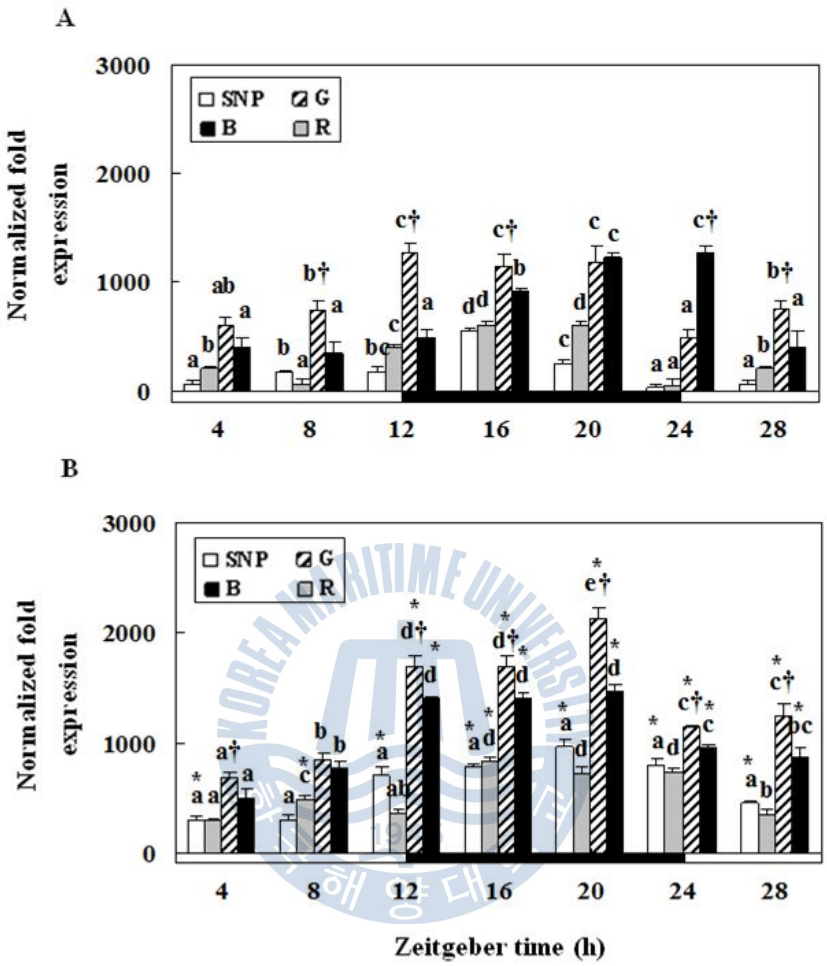

Fig. 29. Changes in the expression levels of GH mRNA in the pituitary before (A) and after injection with melatonin (B) under lighting conditions using red (R), green (G), blue (B) LED, and SNP, as measured by QPCR. The fish were reared under a LD cycle (12:12). White bar Photophase, black bar scotophase. The dagger symbol indicates significant differences between different light spectra within the same ZT (P<0.05), asterisks above the letters indicate significant differences between the control (i.e., without melatonin injection) and melatonin-injected group (P<0.05). All values represent means ± SD (n=5).

Table 3. Changes in the total length of yellowtail clownfish reared under a SNP and red, blue, and green LED

Months	Total length of yellowtail clownfish (cm)			
	SNP	Red	Blue	Green
0	3.5 ± 0.3^{a1}	3.5 ± 0.3^{a1}	3.5 ± 0.3^{a1}	3.5 ± 0.3^{a1}
2	3.7 ± 0.2^{a1}	3.8 ± 0.4^{ab1}	4.2 ± 0.3^{b2}	4.1 ± 0.2^{ab1}
4	3.9 ± 0.3^{a1}	4.0 ± 0.3^{a1}	5.2 ± 0.4^{b3}	5.3 ± 0.3^{b2}
6	4.8 ± 0.5^{a2}	4.9 ± 0.3^{a2}	6.2 ± 0.2^{b4}	6.1 ± 0.4^{b2}

Different lowercase letters indicate significant differences between lights of different wavelengths; different numbers indicate significant differences between the start of the experiment and after 6 months of rearing ($P<0.05$) SNP and LED.

4. Discussion

In this study, the growth of yellowtail clownfish under different LED spectra was examined. Also, the effect of melatonin on the growth effects induced by specific LED wavelengths wes determined by examining diurnal changes in GH mRNA expression levels following the intraperitoneal injection of melatonin.

Analysis of the full-length GH cDNA isolated from the pituitary of yellowtail clownfish revealed that it had a high identity (93-99%) with those of other species (Fig. 28). Li et al. (2005) reported that the first 17 amino acid residues (Met^{1}-Ser^{17}) at the N terminus are highly hydrophobic in GH of the orange-spotted grouper *Epinephelus coioides* and that this sequence has a high degree of homology to the signal peptide sequence of other fish GHs. The positions of four Cys residues in yellowtail clownfish GH (Cys^{69}, Cys^{177}, Cys^{194}, and Cys^{202}) were nearly the same as those in other vertebrate GHs, whereby two disulfide bonds were formed to contribute to the tertiary structure/hormone binding/hormone receptor interaction (Li et al., 2005). One possible site for N-glycosylation (Asn-X-Ser/Thr motif) is present at Asn^{201} in the predicted amino acid sequence of the yellowtail clownfish GH (Law et al., 1996). Therefore, yellowtail clownfish GH was examined with respect to the GHs of other teleosts for the presence of the signal peptide and the putative N-glycosylation sites and found them to be present.

This study showed that the expression levels of GH RNA in the pituitary of yellowtail clownfish were significantly higher in the green and blue LED groups than in the red LED and control groups during the diurnal rhythm (Fig. 29A). In addition, yellowtail clownfish reared for 6 months under these different light regimes showed a significantly rapid growth rate about 74 and 77% under the green and blue LED conditions, respectively, whereas the

growth rates were 37 and 40% under control and red LED conditions, respectively (Table 3). Blanco-Vives et al. (2010) reported that the exposure of Senegal sole *Solea senegalensis* larvae to red light resulted in a delay in growth, with the yolk sac remaining visible, whereas under the blue light treatment the yolk sac was completely absorbed. Based on these results, blue light appeared to be the most efficient light spectrum for the development of Senegal sole larvae. Using a similar experimental design for European sea bass *D. labrax*, Villamizar et al. (2009) observed that the growth rate and feeding performance of larvae were significantly higher under the blue treatment than under to red or white treatments. These researchers suggested that blue wavelengths provide sufficient stimulus to the visual system for the larvae to feed well. The difference in photoperiodic response under the same intensity of light at different wavelengths is due to a difference in the number of photons received by the photoreceptors (Vriend and Lauber, 1973). In another study, Migaud et al. (2007) reported that LED of the blue-green spectrum might be the most suitable for fishes because these wavelengths generally penetrate seawater more efficiently. For example, the maximal growth rate of crucian carp and rotan occurs under both green and blue light (Ruchin, 2004). This process is primarily regulated by the eyes and pineal organ because only these organs can detect color (Levin and McNicol, 1982; Ekström and Meissl, 1997). It is known, for example, that crucian carp are able to detect colors at an early stage of development. However, it is more difficult to distinguish between blue and green colors than green and red colors. In other words, if there is greater contrast between colors, detection is easier. Ruchin (2004) suggested that the negative effect of red light on growth rate was induced through changes in energy metabolism, endocrinology, and/or physiology. These various findings support these results that the rapid growth rate was rapid under green and blue LED spectra, leading to the conclusion that short wavelengths, such as green and blue,

effectively enhance the growth of adult fish as well as larvae.

In this study, although changes in the GH mRNA expression patterns were similar between melatonin-injected and non-injected fish, the GH mRNA expression levels were significantly higher in the melatonin-injected group (Fig. 29B). These results support the findings of John et al. (1990), who reported the stimulation of GH secretion by melatonin in Japanese quail *Coturnix japonica*. The cause of these changes is still unclear, but de Vlaming (1980) reported a concomitant increase in growth with increasing doses of melatonin in goldfish, leading him to theorize a functional relationship between growth and melatonin. In addition, the localization of 2-[^{125}I]-iodomelatonin binding sites (melatonin binding site) in the brain revealed high levels of melatonin binding within the preoptic area, which has also been shown to contain neurons that are immunoreactive for GH-releasing factor (Porter et al., 1998; Falcón et al., 2010). The effects of melatonin on growth may thus result from the differential impact of the hormone on GH, and perhaps on other pituitary hormones. In addition to melatonin having a direct effect on the pituitary, it might also modulate fish feeding and growth through controlling the production of releasing and inhibiting factors by neurons from the preoptic area and hypothalamic nuclei, as well as by directly targeting peripheral tissues (Schreck, 1993). Furthermore, salmonid fish are susceptible to certain wavelengths at which the pineal photoreceptor cell integrates the light signal, which allows melatonin production to be controlled in an on/off manner (Falcón, 1999; Bayarri et al., 2002; Iigo et al., 2007). Melatonin also contributes to the control of GH secretions (Falcón et al., 2003). These findings led us to hypothesize that light of different wavelengths controls melatonin secretion, with melatonin production subsequently affecting GH.

Shin et al. (2011) reported that green and blue LED spectra reduce stress relative to red LED spectra in yellowtail clownfish, while Schlenk and Rice

(1998) reported that stress inhibits the growth of fish. These results indicate that the stress-reducing effect of short wavelengths, such as green and blue LED, promotes the growth of fish.

In conclusion, this study showed a component of the relationship among different light wavelengths, GH expression, and melatonin in ornamental yellowtail clownfish by using LED, which are a new form of lighting technology. This study indicated that green and blue spectra promote growth in yellowtail clownfish and that melatonin stimulates GH secretion, consequently playing a role in promoting fish growth. Therefore, this study results might be applied to an artificial photic system aimed at effectively enhancing the growth of fish reared under captive conditions.

Chapter 7

Effects of LED light spectral sensitivity on the ovarian maturation in yellowtail damselfish *Chrysiptera parasema*

1. Introduction

The influence of environmental factors on the growth and reproduction of fish has been extensively studied (Boeuf and Le Bail, 1999). It is well known that light and temperature are among the most important natural environmental factors that regulate reproduction in fish. Lighting characteristics including wavelength (quality), intensity (quantity), and periodicity (daily cycle) are among factors that regulate seasonally dependent changes in reproductive and growth physiology of fish (Boeuf and Le Bail, 1999). The reproductive physiology of fish is closely related with the perception of environmental factors by the sensory systems and the transduction of suitable signals to the hypothalamo-pituitary-gonadal axis (Bromage et al., 2001; Pankhurst and Porter, 2003). The spectral composition (quality) of incident light are key properties affecting the physiological response of teleosts with, among others, effects on growth, reproduction, behaviour and stress documented (Boeuf and Le Bail, 1999).

In various reproductive hormones, estrogen is an essential steroid hormone in reproduction, playing an important role in sexual maturation and differentiation, including oogenesis, vitellogenesis, and testicular development (Ishibashi and Kawashima, 2001). Estrogen activity is mediated by nuclear ERα and ERβ, and ERα is a member of a superfamily of transcription factors that induce target gene expression by binding cis-acting enhancer

elements located in the promoter region of their responsive genes (Green et al., 1986). Furthermore, the induction of hepatic VTG, which is a precursor yolk protein, in response to estrogens by an ER-mediated pathway has been well documented in several oviparous fish species (Ryffel, 1978; Nelson and Habibi, 2010). Thus, VTG and ER might serve as indicators of reproduction and maturation in fish.

The application of artificial lighting in recirculating aquaculture systems requires appropriate combination of light hours (photoperiod), intensity and spectrum. There are numerous data related to photoperiod and light intensity effects on several farmed fishes and life stages (Boeuf and Le Bail, 1999). In most studies fluorescent lamps are used, resulting in what humans perceive as white light, despite the fact that in natural fish habitat, wavelength of light penetrating water varies greatly, fish vision and spectrum perception are strongly adapted to each species natural habitat and living ethology (Neumeyer, 1992), and recent studies indicate that light spectrum affects farmed fish growth performance (Karakatsouli et al., 2007), behaviour (Volpato et al., 2004) and physiological status (Karakatsouli et al., 2007).

To date, it has been shown that periodicity is a crucial determinant of reproductive success in fish, with extensive studies on its importance in the initiation and termination of gonadal development (de Vlaming, 1975; Bromage et al., 2001; Pankhurst and Porter, 2003). Also, the effects of light-intensity have been well studied over recent years and findings clearly suggest that exposure to threshold intensity levels is required to manipulate physiological functions in various teleosts (Oppedal et al., 1997; Porter et al., 1999; Taylor et al., 2005; Migaud et al., 2006). Hence, it is important to evaluate the impact of different types of lighting on reproduction.

Metal halide bulbs are the present source of underwater artificial lighting used in the industry, but in many aspects they are not suitable for fish

farming as they are neither environment nor species specific. They create a bright point source of light, involve high running costs and much of their light energy is wasted in the form of unsuitable wavelengths (i.e. longer wavelength yellow-red light) which are rapidly absorbed in the water column and therefore cannot be detected by fish (Migaud et al., 2006; Loew and McFarland, 1990). LED can output light at specific wavelengths (Migaud et al., 2007). Furthermore, LED have lower power requirements, electrical running costs and a longer life span than standard metal halide bulbs (Migaud et al., 2007). Narrow bandwidth high-energy short wavelength light may improve the efficiency of lighting systems compared to those currently used in the fish farming industry since it can be tuned more specifically in line with sensitivity of a target species (Villamizar et al., 2009). Furthermore, it is known that the spectral composition of incidental light is differentially affected in underwater environments, and that rapid attenuation occurs with increasing depth (Lythgoe, 1979).

Recently, several studies have investigated the utility of LED lights as photo-environmental factors, using different LED light wavelength light sources for aquaculture. For instance, Shin et al. (2011) reported that green and blue LED, which have short wavelengths, increased the level of antioxidants in response to oxidative stress in the yellowtail clownfish *A. clarkii*. In addition, Volpato and Barreto (2001) reported that blue spectrum prevents stress in Nile tilapia *O. niloticus*. Meanwhile, red LED wavelength affects physiological function, and was found to induce oxidative stress in yellowtail clownfish (Shin et al., 2011). However, studies on the effect of LED light wavelengths on fish reproduction remain very limited (Bapary et al., 2009; Bapary et al., 2011).

For energy-savings and the way to enhance the gonad development, in the present study, the effects of LED on sexual maturation and development were

examined in yellowtail damselfish. This species is a reef-associated damselfish that is widely distributed in shallow waters. It has commercial value as an ornamental fish and is widely used as a scientific experimental model. The effect of different types of lighting on ovarian maturation was investigated in this species. Fish were reared for 4 months under 3 LED wavelengths and 3 lighting intensities. Changes in the expression of VTG and ER mRNA, as well as expression of VTG and ER proteins were investigated. In addition, ovarian development was evaluated by measuring steroid hormone (estradiol-17β; E_2) levels, and by determining oocyte development in relation to histological indices of gonadal maturation.

2. Materials and methods

2.1. Experimental fish

The immature yellowtail damselfish (body length, 2.1 ± 0.4 cm; body weight, 1.1 ± 0.2 g) were purchased from a commercial store. Fish length and weight were measured quickly during divide to each experimental tanks, and then fish were allowed to acclimate for 1 week in 300 l circulation filter tanks with circular filtration prior to laboratory-based experiments under LD photoperiod (lights on at 07:00 h and light off at 19:00 h) using white fluorescent bulb at 27°C. The water temperature and photoperiod were 27.0 ± 1.1°C, with a 12L:12D photoperiod, and fed commercial feed twice daily (at 09:00 h and 17:00 h).

The fish were exposed to a white fluorescent bulb (27 W; SNP) was used for the control group. In the experimental groups, fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LED (Daesin LED Co. Kyunggi, Korea) for 4 months (Fig. 30). The LED were set 50 cm above the water surface, and irradiance at the water surface was maintained at approximately 0.3, 0.6, and 0.9 W/m^2, respectively (Fig. 30).

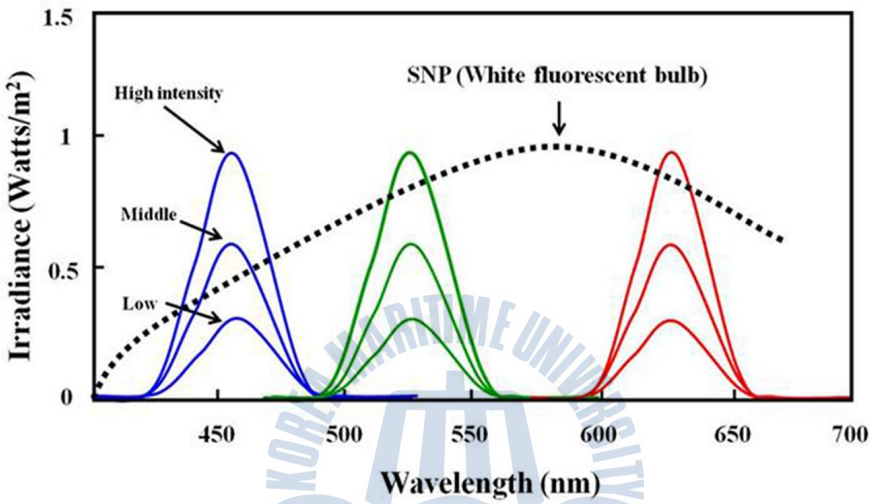

Fig. 30. Spectral profiles of the blue (B), green (G), and red (R) LED. Low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) light intensities were used for each type of LED in this study. Square dotted line shows the spectral profile of a white fluorescent light (SNP).

2.2. Sampling

At the end of the 4 month experimental period, blood was collected from the 30 fish per tanks (fluorescent bulb, red, green, and blue LED at 3 light intensities) using a 3-ml syringe coated with heparin from caudal vein after anesthetization. Plasma samples were separated by centrifugation (4°C, 10,000×g, 5 min) and stored at -80°C.

The fish were euthanized by spinal transection for the collection of liver and gonads under white dim light using attenuated white fluorescent bulb. Immediately after collection, the liver and gonads were frozen in liquid nitrogen and stored at -80°C until total RNA extraction was performed. No mortalities were observed.

2.3. QPCR

QPCR was conducted to determine the relative expression of VTG (accession no. JQ906787) and ERα (JQ906788) mRNA, using total RNA extracted from the liver and gonads of yellowtail damselfish, respectively. Primers for QPCR were designed in reference to known yellowtail damselfish sequences as follows: VTG forward primer (5′- ACC CGT CAG TGC TCA GTA -3′), VTG reverse primer (5′- TCG CTG CTG GTC TTA ATC A -3′), ERα forward primer (5′- TGA CTA GCA TGT CTC CTG AT -3′), ERα reverse primer (5′- ATG GTG ACC TCG GTG TAA -3′), β-actin forward primer (5′- GCA AGA GAG GTA TCC TGA CC -3′), and β-actin reverse primer (5′- CTC AGC TCG TTG TAG AAG G -3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. QPCR was performed as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 20 s and 55°C for 20 s (An et al., 2008). As internal controls, the experiments were

duplicated with β-actin calculated Ct levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated using the formula: [$\Delta\Delta Ct = 2^{-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})}$] (Livak and Schmittgen, 2001).

2.4. Western blot analysis

Total protein isolated from the liver and gonads of yellowtail damselfish was extracted using protein extraction buffer [5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 0.15 mg/ml leupeptin]. It was then sonicated, and quantified using the Bradford method (Bio-Rad, CA, USA). In each lane, total protein (30 μg) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. For reference, a protein ladder (Fermentas) was used. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye front had run off the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad, CA, USA) at 85 V for 1.5 h at 4ºC. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min followed by washing in TBS. The membranes were incubated with VTG (ABIN326357, 1:2,000 dilution, Antibodies-online, USA), followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (dilution 1:2,000; Bio-Rad, CA, USA). A separate set of membranes were incubated with ERα (E1528 1:2,000 dilution; Sigma, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1:2,000; Bio-Rad, CA, USA) for 60 min. The internal control was β-tubulin (dilution 1:5000, ab6046; abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5,000; Bio-Rad, CA, USA) for 60 min. Bands were detected using a standard ECL system, in addition to the more sensitive ECL system (ECL Advance; GE Life Sciences, Sweden),

and were exposed to autoradiography-sensitive film for 2 min.

2.5. Gonadosomatic index (GSI) and gonadal histology

After dissecting and weighing, the GSI [GSI = (gonad mass/body mass) × 100] was calculated for each fish.

To analyze the gonads exposed to LED, the 5 gonads of each experimental groups were fixed in Bouin's solution, and subjected to histological observation. The samples were dehydrated in increasing concentrations of ethanol solution, clarified in xylene, and embedded in paraffin. Sections (5-μm thick) were selected and stained with hematoxylin-eosin for observation under a light microscope (Leica DM 1000; Leica, Germany), and the images were captured using a digital camera (Leica DM 1000, Leica, Germany).

In accordance with oocyte staging of the white-spotted spinefoot *Siganus canaliculatus* and sapphire devil *C. cyanea* oocytes in the ovary of yellowtail damselfish were classified into the following stages: peri-nucleolus (PNS), primary yolk stage (PYS), secondary yolk stage (SYS), and tertiary yolk stages (TYS) (Bapary et al., 2011; Hoque et al., 1998).

2.6. Analysis of plasma parameters

Plasma E_2 levels were analyzed by the immunoassay technique using the E_2 ELISA kit (Cusabio Biotech, Hubei, China).

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). Two-way ANOVA followed by Tukey's post hoc test was used to assess statistically significant differences among the different light spectra and different light intensities. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Total body length

The total body lengths of fish reared under green and blue LED conditions were significantly greater compared to those of fish reared under other light conditions (Fig. 31). The green (4.6 ± 0.2 cm; low) and blue (4.6 ± 0.3 cm; medium) LED groups exhibited the greatest total body lengths, while the red (3.4 ± 0.2 cm; medium) LED and SNP (3.2 ± 0.3 cm) groups exhibited the shortest total body lengths.

3.2. VTG and ERα expression in the liver and gonads

In the green and blue LED groups, the expression levels of VTG and ERα mRNA were significantly higher than those in fish exposed to other light spectrums (Fig. 32). Especially, the expressions under low and medium intensity were significantly higher than those under high intensity. Western blot analysis identified two protein bands VTG and ERα-immunoreactive proteins corresponding to predicted mass for yellowtail damselfish VTG (43 kDa) and ERα (64 kDa). The expression pattern of the immunoreactive proteins resembled that of VTG and ERα transcript levels in the yellowtail damselfish liver and gonads (Fig. 32A, C).

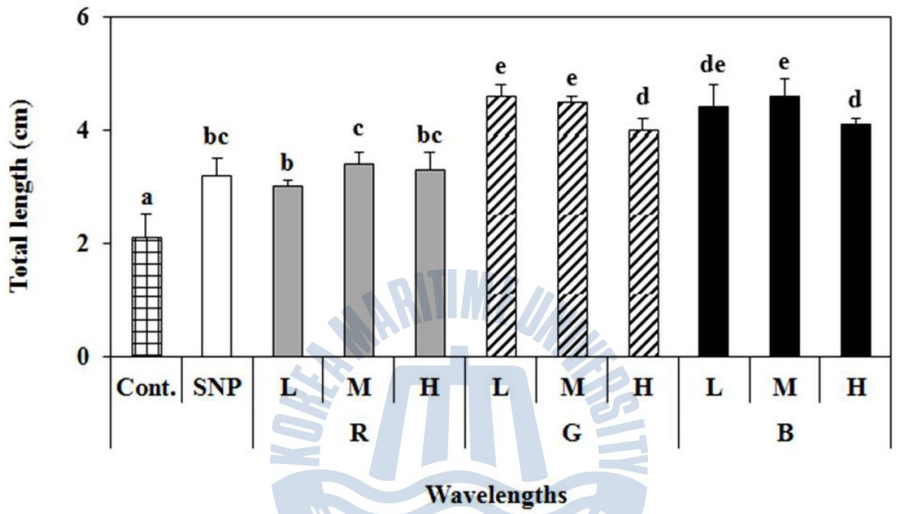

Fig. 31. Changes in the total body length of yellowtail damselfish, which were reared for 4 months under simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LED lights. Low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) light intensities were used for each different LED light type. Cont. (control) indicates the initial total body length at the start of experiment.

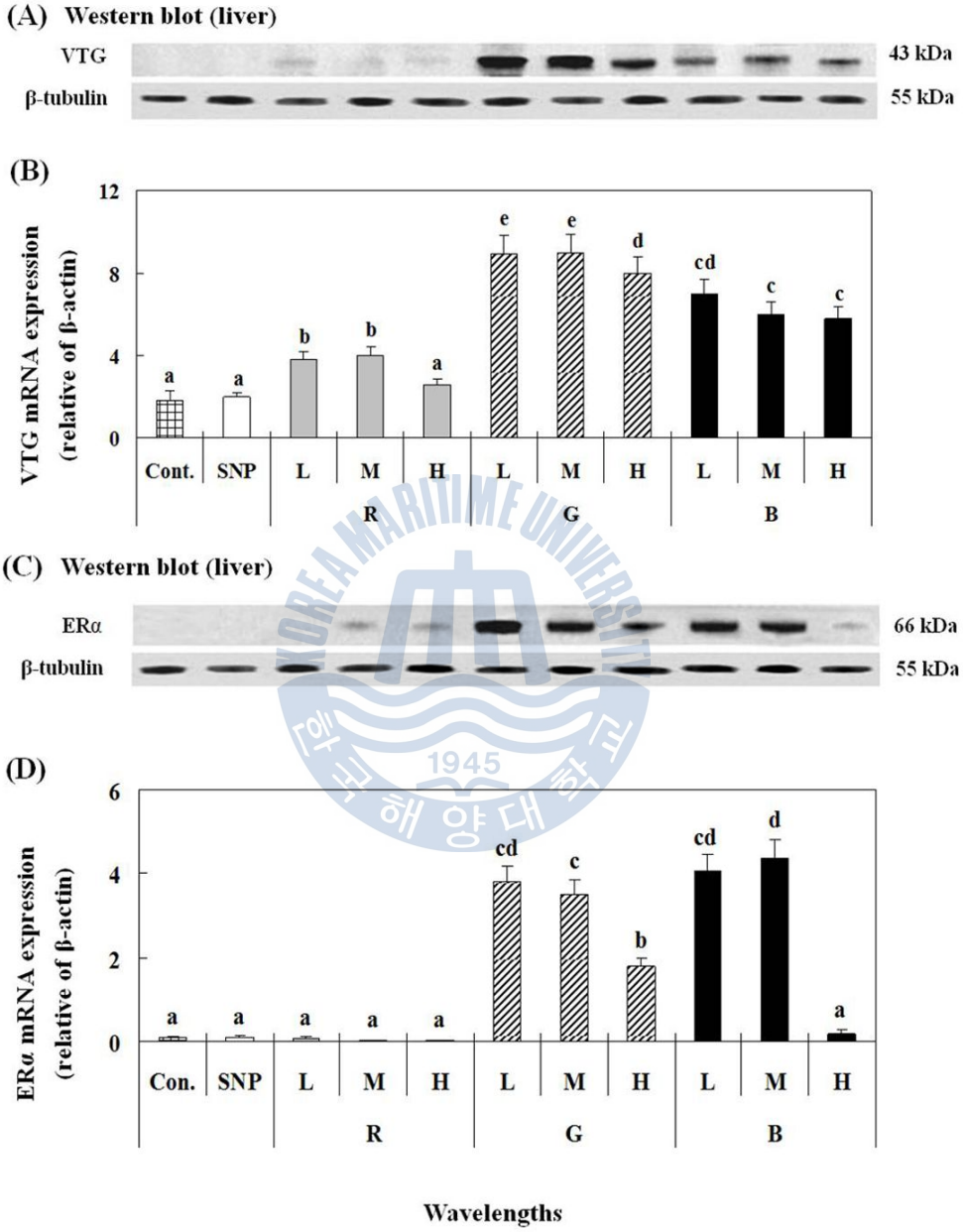

Fig. 32. VTG and ERα mRNA expression levels in the liver of yellowtail damselfish under lighting conditions using simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LED at low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) light intensities. Cont. (control) indicates initial VTG and ERα mRNA levels at the start of the experiment. Western blotting using VTG (43 kDa) (A) and ERα (66 kDa) (C) to examine protein expression in the liver of yellowtail damselfish. The 55 kDa β-tubulin was used as the internal control. VTG (B) and ERα (D) mRNA levels relative to β-actin mRNA levels in the liver and gonads of yellowtail damselfish under lighting conditions using SNP, as well as red (R), green (G), and blue (B) LED at low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) lighting intensities, as measured by QPCR. The fish were reared under a LD cycle (12:12). Values with letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum ($P<0.05$). All values are means ± SD ($n=30$).

3.3. GSI and histological observation

The GSI values of fish exposed to green (4.5 ± 0.3; low) and blue (3.1 ± 0.3; low) LED lights were significantly higher than that of the SNP group (0.5 ± 0.1). Furthermore, the GSI values at low intensity were significantly higher than those observed at medium or high intensity lights in the LED groups (Fig. 33). To investigate gonadal morphology, performed histological studies of gonadal samples as shown in Fig. 34. The gonads of all fish in the SNP group contained only immature oocytes at PNS (Fig. 34A), and red LED group contained oocytes at PYS and SYS (Fig. 34B). In contrast, well-developed vitellogenic oocytes and most of them were at TYS in the green and blue LED groups (Fig. 34C, D).

3.4. Plasma E_2 levels

Positive correlations were observed between circulating E_2 levels and gonadal development in the yellowtail damselfish. The plasma concentration of E_2 in fish exposed to green (503.1 ± 35.0 pg/ml; low) and blue (504.2 ± 16.7 pg/ml; low) LED lights were significantly higher than that of the SNP group (202.1 ± 20.2 pg/ml) (Fig. 35).

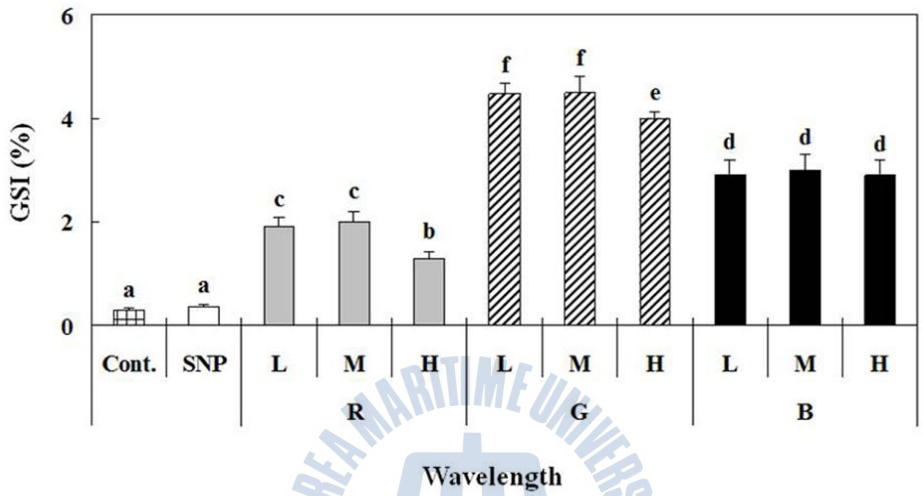

Fig. 33. Changes in the GSI of yellowtail damselfish under lighting conditions using SNP, as well as red (R), green (G), and blue (B) LED at low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) light intensities. Cont. (control) indicates initial total fish body length at the start of the experiment. Values with letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum (P<0.05). All values are means ± SD (n=30).

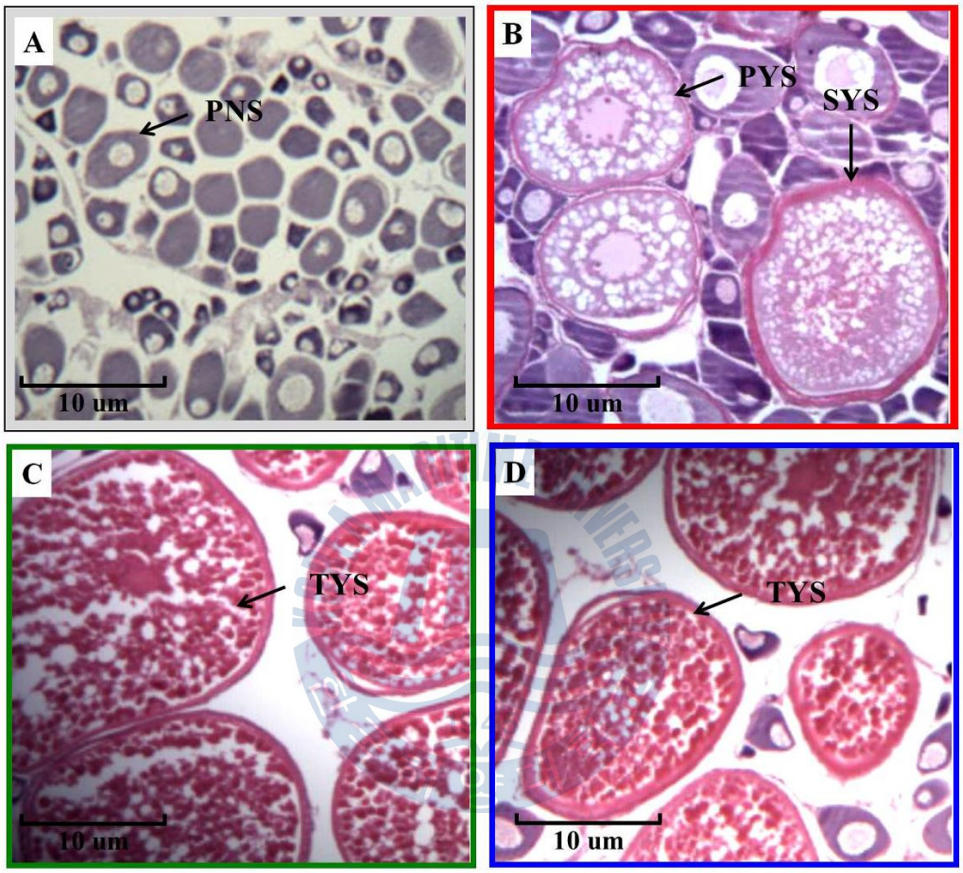

Fig. 34. Histological changes in cross section of the ovary histology of yellowtail damselfish under different lighting conditions using simulated natural photoperiod (SNP) (A), as well as red (B), green (C), and blue (D) LED lights at low (L, 0.3 W/m^2) light intensity. Peri-nucleolus (PNS), primary yolk stage (PYS), secondary yolk stage (SYS), and tertiary yolk stages (TYS). Experimental groups were indicated by colored box line. Scale bar=10 μm.

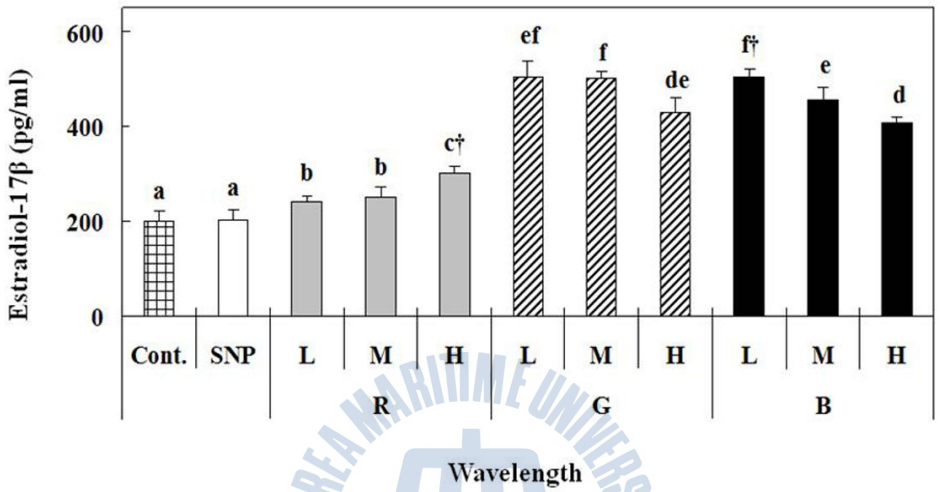

Fig. 35. Plasma E_2 levels of yellowtail damselfish under lighting conditions using SNP, as well as red (R), green (G), and blue (B) LED lights at low (L, 0.3 W/m^2), medium (M, 0.6 W/m^2), and high (H, 0.9 W/m^2) light intensities. Cont. (control) indicates initial E_2 level at the start of the experiment. Values with letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum (P<0.05). All values are means ± SD (n=30).

4. Discussion

In this study, the expression levels of VTG (a precursor yolk protein), ERα (mediates the effects of E_2) were examined by means of measuring mRNA levels and immunoreactive proteins. Also, GSI (measure of gonadal development), histology and circulating E_2 levels were studied. The present study investigated possible utility of LED wavelengths and intensities to improve growth and gonadal maturation during early stages of yellowtail damselfish development.

The results demonstrate that total yellowtail damselfish body length can be increased significantly when exposed to green and blue wavelengths for 4 months. These results are consistent with a report demonstrating increased growth in barfin flounder *V. moseri* exposed to green and blue lights (fluorescent lamp) (Yamanome et al., 2009). In addition, Shin et al. (2012) reported significantly higher levels of growth hormone and total body length in yellowtail clownfish reared under green and blue LED light compared to fluorescent light and red LED.

In the present study, showed that significantly higher expressions of VTG and ERα in yellowtail damselfish under green and blue LED groups compared to the other groups. This is consistent with observed higher circulating level of E_2, GSI and histological characteristics in the same groups. The present results provide a strong support for the hypothesis that exposure of fish to short green and blue wavelengths enhance ovarian maturation. This hypothesis is further supported by the observed mature oocytes (TYS) in the green and blue LED groups. The present results are in accordance with previous report by Volpato (2000), the study demonstrated that enhancing reproductive performance of hormone-induced Matrinxa fish *Brycon cephalus* and increasing spawning rate in female fish reared under

green light. These results collectively demonstrate that exposure to short wavelength lights increase reproductive capacity in cultured fish.

However, little information is available on the mechanisms underlying short wavelength light-induced enhancement of ovarian maturation. It is possible that a single pigment with wide spectral sensitivity, or several photopigments, may be involved in the transduction of exogenous photic stimuli in fish. Furthermore, specific photoreceptors may be involved in this process. It is interesting to note that Urasaki (1976) and Garg (1988) reported that the extent of gonadal development is lower in blind and pinealectomized fish, and suggest possible mediation of eyes and extra-retinal photoreceptors.

These result suggest that a physiological response is involved in the mechanisms linking specific light wavelengths with fish gonadal maturation. It should also be noted that light is closely connected with stress response in fish. Studies by Shin et al. (2011) demonstrated lower LPO and H_2O_2 levels in fish exposed to green and blue lights compared to fish exposed to other spectra. It was suggested that short green and blue LED wavelengths may inhibit oxidative stress in fish compared to those exposed to other light spectra. Furthermore, Volpato and Barreto (2001) observed lower cortisol levels in fish exposed to blue spectra. In stressed Nile tilapia, lower level of cortisol was observed in fish exposed to blue light, for 48 h, compared to fish exposed to other light spectra. The findings suggest that certain light spectra may affect the activity of pituitary-adrenal axis, and related physiological parameters.

In conclusion, exposure of fish to short wavelength light lower stress response, enhance the immune function, and enhance gonadal development. In the present study, these results demonstrated that low intensity LED lighting significantly increased the expressions of VTG and ERα, increase plasma levels of E_2, and enhance body growth and oocyte maturation. These results

support the hypothesis that the use of green and blue wavelengths LED would be valuable by improving immunity and reproductive ability in cultured fish. Further studies will be required to understand the mechanisms regulating the reproductive response in fish by analyzing photoreceptors connecting reproduction to different light spectra and intensities.

Chapter 8

General Discussion

Fish live in aquatic environments with varying light intensity, and they often possess visual pigments with the absorption spectrum adapting to their habitats (Yokoyama et al., 1999). Fish that migrate from the pelagic zone to the benthic habitat, or vice versa, during ontogeny change their visual receptors (Helvik et al., 2001). The retina at the larval stages consists of only single cone cells; later during metamorphosis double cones and rods develop (Helvik et al., 2001). Because fish use environmental light signals for multiple physiological functions such as vision, photoentrainment of circadian rhythms, regulation of body colour and detection of seasonal changes in the photoperiod (Cole and Youson, 1982), they are useful model species to study the adaption of animals to the environmental light conditions. Among the factors that influence circadian rhythms (physiological and behavioural changes within a period of 24 h), light is the most important one (Simonneaux and Ribelayga, 2003; Pierce et al., 2008). The biological circadian rhythm usually has a diurnal rhythm that is controlled by the external illumination time often with seasonal periodicity (Bolliet et al., 2001; Pierce et al., 2008).

Recently, studies of light effects have been performed by using LED instead of natural light or metal halide lights (Shin et al., 2011). LED are a new form of lighting technology that is still being developed and can be manufactured to output specific wavelengths (Migaud et al., 2007). Furthermore, LED have lower power requirements, lower electrical running costs, and longer life spans than standard metal halide bulbs (Migaud et al., 2007). However, LED studies have only recently examined the effects of photoperiod and light intensity in fish (Pierce et al., 2008). Although studies have documented the responses of fishes to various spectra (Villamizar et al., 2009; Shin et al., 2011, 2012), investigations into changes in the

control of fish circadian rhythms, oxidative stress, growth and reproduction due to exposure to various light spectra remain very limited.

First of all, the changes of genes related with circadian rhythm of fish were investigated to understand fish diurnal and circadian rhythm by lights.

I. The changes in the mRNA expression of RH and Exo-RH were investigated for examining the part of the photic regulation molecular mechanism in the olive flounder using the photoreceptors, RH and Exo-RH. Also, the relationship between the detection of external light and circadian rhythm by cloning full length RH and Exo-RH cDNA and examining the changes in the expression of the RH family genes encoding these pigments. Importantly, the expression of Exo-RH mRNA was investigated in cultured pineal glands to determine whether it is specifically expressed in the pineal gland during the circadian cycle. Molecular phylogenetic analysis indicated that olive flounder RH, expressed in the retina, and Exo-RH, expressed in the pineal gland, clustered with the vertebrate RH and Exo-RH groups, respectively. However, Exo-RH, phylogenetically separated from the RH groups as a member of the RH family (a family including RH and Exo-RH), has a similar aa structure to RH. This tree agrees with the findings of Minamoto and Shimizu (2003), who showed that RH and Exo-RH, separated from the RH groups were phylogenetically very similar, according to phylogenetic analysis of ayu RH and Exo-RH. In this study, the differential expression of RH and Exo-RH mRNA were observed in the nervous and peripheral tissues of olive flounder by RT-PCR analysis: RH is expressed predominantly in the retina, while Exo-RH is strongly expressed in the pineal gland. However, these genes were slightly expressed in the peripheral tissues, it indicated that RH and Exo-RH were specific to retina and pineal gland, respectively. When the olive flounder was exposed to LD in this study, RH

was expressed at a significantly higher level during the photophase and was expressed at a lower level during the scotophase. Under the DD and LL conditions, RH mRNA expression exhibits circadian rhythmicity as under LD condition. To examine the roles played by Exo-RH as a putative photoreceptor of the pineal gland and as a controller of circadian rhythm, its expression levels in the pineal gland were assessed during diurnal rhythm. Exo-RH expression was significantly increased at the start of the scotophase, ZT12 and peaked during the night at ZT20. Exo-RH mRNA expression was also examined in pineal glands (*in vivo* and *in vitro*) under DD condition and found that its expression is rhythmic, as under LD condition, with peaks at ZT20. The difference of expression levels were observed between *in vivo* and *in vitro* (*In vitro* group, expression levels were significantly higher about 3-5 times than *in vivo* group), I hypothesised that Exo-RH mainly expresses in pineal gland and there are very close relationship between Exo-RH and pineal gland. Plasma melatonin levels showed rhythmic changes under DD and LL conditions like they did under LD condition, with a peak at ZT20. In conclusion, this result suggests that RH and Exo-RH potentially mediates the effects of environmental photocycle on pineal circadian rhythms and melatonin synthesis. This study showed that Exo-RH controls circadian rhythm by increasing its levels in the pineal gland, an important organ for the generation and maintenance of circadian rhythm.

II. The changes in MT mRNA expression were observed in the retina, pineal gland, and optic tectum during diurnal and circadian variations to examine the role of MTs in circadian rhythm control in olive flounder. Specifically, the changes in MT mRNA expression were investigated during diurnal and circadian variations by treating cultured pineal gland samples with melatonin. In terms of their tissue distribution, all three genes were strongly expressed in the pineal gland, pituitary,

optic tectum, diencephalon, cerebellum, telencephalon, and retina. The expressions of these genes in parts of the visual system, such as the retina, confirm that they play a role in visual processing (Ikegami et al., 2009). All three MTs were also expressed in the central nervous systems, such as the optic tectum and diencephalon. These results are in good agreement with the high number of melatonin-binding sites in the optic tectum-thalamus region. The pineal gland receives light information from the retina and acts as the central organ secreting melatonin. I therefore hypothesize that melatonin targets are mainly present in central areas integrating the visual/light information. These findings also suggest that the three types of MTs participate in mediating the melatonin activity involved in processing light information in the brain, and their distribution suggests that they may be involved in diverse functions of melatonin, such as circadian and annual control of behavioral and physiological rhythms, regulation of sleep, and neuronal apoptosis (Iigo et al., 1994a). The expression of three genes in the retina, pineal gland, and optic tectum varied diurnally. Specifically, they showed significantly high expression levels in the scotophase, with increasing expression at the start of nighttime (ZT12) and peak expression at ZT16 or ZT20 or ZT24 of approximately 3～18-fold. Under the DD conditions, the mRNA expression for the three genes exhibited similar circadian rhythmicity as under LD conditions. The expression of the MTs peaked at scotophase during the diurnal rhythm in the cultured pineal gland samples which was similar to the *in vivo* results.

In conclusion, MT1, MT2, and MT3 show high expression levels in the brain regions receiving light information, particularly the optic tectum-thalamus region as well as the retina, the organ that primarily handles light information. These results suggest that MTs play a central role in controlling the diurnal and circadian rhythms in the brain and retina of olive flounder.

III. The expression of the MT1, *Per2*, and *Cry1* genes was examined in yellowtail

clownfish exposed to different LED spectral groups (red, green, and blue). The effects of specific LED spectra on the circadian rhythm of the fish were also investigated by measuring plasma melatonin levels.

First, MT1 gene expression was significantly higher in the group exposed to red LED than in groups exposed to either green or blue LED. Migaud et al. (2006) reported that most light energy is wasted in the form of unsuitable wavelengths (longer wavelengths, red light) that are rapidly absorbed by water molecules and therefore cannot be detected by fishes. I hypothesized that MT1 expression would be significantly higher in the group of fish exposed to red LED than in the groups exposed to other LED. This study showed that, at night, the levels of MT1 mRNA were significantly higher in these fish than in those in the SNP group and the groups exposed to blue-green LED. I hypothesized that fish cannot detect red light and thus perceive it as night; therefore, MT1 mRNA expression levels would be significantly higher in the group exposed to red LED than to other light spectra. Furthermore, studies of the circadian system in fish using various spectra are very limited. Therefore, expression of clock genes was measured in fish exposed to various LED spectra. Expressions of the clock genes, *Per2* and *Cry1*, were significantly higher in groups of fish exposed to blue-green LED than in those exposed to red LED. While MT1 expression increased significantly during periods of darkness, clock gene expression increased significantly during periods of light. *Per2* and *Cry1* are induced by light exposure (Okamura et al., 2002). The expressions of *Per2* and *Cry1* were induced by environmental factors and light. These observations are consistent with the involvement of clock genes and the light switch pattern of the circadian clock (Pando et al., 2001; Kim et al., 2012). In the present study, plasma glucose levels were significantly higher throughout the entire photoperiod in fish exposed to a red LED spectrum than in those exposed to other light spectra, and that *Cry1* expression levels showed the opposite trend. Hirota et al. (2012) reported that *Cry1* inhibits gluconeogenesis, and these results suggest that *Cry1* mRNA expression was

significantly lower under red LED than under blue or green LED. On the other hand, plasma glucose was significantly higher under red LED than under blue or green LED. In this study, plasma melatonin levels were significantly higher during the scotophase than during the photophase for all LED spectra. Moreover, melatonin levels were significantly higher throughout the entire photoperiod when fish were exposed to a red LED spectrum than to other spectra. I hypothesized that fish cannot detect red light; therefore, they perceive illumination by red light as darker than illumination by other spectra, and melatonin levels remain high. The expression of the clock genes *Per2* and *Cry1* was significantly higher in fish exposed to blue-green LED than in those exposed to red LED, suggesting that red light inhibits the circadian rhythms and is a stressor.

IV. The mRNA levels of the *AANAT2* enzymes, which synthesizes the precursor of melatonin, the mRNA expression and activities of the antioxidant enzymes SOD, CAT, plasma H_2O_2, and melatonin levels were examined to investigate the effects of different LED spectra (red, green, and blue) on oxidative stress in order to investigate the antioxidant response mechanisms. In addition, this study showed that the *AANAT2* mRNA expression levels in the pineal organ and SOD/CAT mRNA expression and activity changes in the liver of the yellowtail clownfish during the daily rhythm to examine the antioxidant capacity of melatonin.

First, the *AANAT2* mRNA expression were significantly higher for the red LED spectrum than other spectra for both the pineal organ (*in vivo*) and cultured pineal organ (*in vitro*). Furthermore, SOD and CAT mRNA expression and SOD and CAT activities were significantly higher for the red LED spectrum than for the other spectra. Hence, these results suggest that red LED spectra produce oxidative stress in the yellowtail clownfish. Therefore, *AANAT2* mRNA expression and SOD and CAT expression and activity were analyzed in yellowtail clownfish injected (*in vivo*) or treated (*in vitro*) with melatonin to examine whether melatonin plays a role as an

antioxidant against oxidative stress induced by specific LED spectra. Blanco-Vives et al. (2010) study reported that larvae exposed to red light exhibited a delay in growth and thus the yolk sac was visible, while in the blue light the yolk sac was completely absorbed. Larvae responded best to blue light, which appeared to be the most efficient light spectrum on the development on Senegal sole larvae (Blanco-Vives et al., 2010). Also, the expression levels for *AANAT2*, SOD, and CAT were significantly lower for the melatonin treated or -injected groups than for the uninjected or untreated ones. These results indicated that melatonin reduces oxidative stress and are in agreement with those of Reiter et al.'s (2007) study, which reported that melatonin reduces oxidative stress. Therefore, I hypothesized that red LED light induced oxidative stress in yellowtail clownfish, and melatonin injection or treatment removed the ROS, as a strong antioxidant, that had occurred in fish. Consistent with this model for melatonin function, the plasma H_2O_2 levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~2 fold in melatonin-injected fish than the uninjected ones. These results are in agreement with the studies of Allegra et al. (2003) and Martinez et al. (2005), which reported that melatonin scavenges ROS induced by red spectrum. In addition, the plasma H_2O_2 levels in the rat *Rattus norvegicus* treated with the environmental toxicants, PCB, were significantly higher than those in rats that intraperitoneally received melatonin (Venkataraman et al., 2008). Induced ROS from red LED spectrum can oxidatively damage the cellular elements in its target organ such as muscle. In this study, LPO levels in various LED spectra were significantly higher for the red LED spectrum than for the other ones, but for all spectra, these levels were significantly less by ~100 fold in melatonin-injected fish than the uninjected ones. Red LED spectrum induced oxidative stress and then caused oxidative damage in tissue of yellowtail clownfish. Plasma melatonin levels were measured and they were at a significantly higher level during the scotophase than during the photophase for all LED spectra.

Moreover, the melatonin levels were significantly higher for the red LED spectrum than for the other spectra over the course of the entire photoperiod. These results indicated that the red spectrum induced oxidative stress in yellowtail clownfish and that more melatonin was produced under the red LED to scavenge for increased ROS. In addition, plasma melatonin levels rapidly increased after melatonin injection. This study showed that melatonin levels were temporarily increased by the injection of the highest dose of melatonin. In conclusion, the red spectrum is a factor in inducing oxidative stress in yellowtail clownfish. Also, melatonin likely plays a strong antioxidant role to reduce oxidative stress in fish. Light is known as a factor that affects various physiological changes in fish.

V. The growth of yellowtail clownfish was examined under different LED spectra. The effect of melatonin on the growth effects induced by specific LED wavelengths was also determined by examining diurnal changes in GH mRNA expression levels following the intraperitoneal injection of melatonin.

Analysis of the full-length GH cDNA isolated from the pituitary of yellowtail clownfish revealed that it had a high identity (93-99%) with those of other species. Li et al. (2005) reported that the first 17 amino acid residues (Met^{1}-Ser^{17}) at the N terminus are highly hydrophobic in GH of the orange-spotted grouper *E. coioides* and that this sequence has a high degree of homology to the signal peptide sequence of other fish GHs. This study showed that the expression levels of GH RNA in the pituitary of yellowtail clownfish were significantly higher in the green and blue LED groups than in the red LED and control groups during the diurnal rhythm. In addition, yellowtail clownfish reared for 6 months under these different light regimes showed a significantly rapid growth rate about 74 and 77 % under the green and blue LED conditions, respectively, whereas the growth rates were 37 and 40% under control and red LED conditions, respectively. In this study, although changes in the GH mRNA expression patterns were similar between melatonin-injected and

non-injected fish, the GH mRNA expression levels were significantly higher in the melatonin-injected group. These results support the findings of John et al. (1990), who reported the stimulation of GH secretion by melatonin in Japanese quail *Coturnix japonica*. In conclusion, these results showed a component of the relationship among different light wavelengths, GH expression, and melatonin in ornamental yellowtail clownfish by using LED, which are a new form of lighting technology. This study indicated that green and blue spectra promote growth in yellowtail clownfish and that melatonin stimulates GH secretion, consequently playing a role in promoting fish growth.

VI. The expression levels of VTG (a precursor yolk protein), ERα (mediates the effects of E_2) were examined by means of measuring mRNA levels and immunoreactive proteins. GSI (measure of gonadal development), histology and circulating E_2 levels were also studied. The present study investigated possible utility of LED wavelengths and intensities to improve growth and gonadal maturation during early stages of yellowtail damselfish development. The results demonstrate that total yellowtail damselfish body length can be increased significantly when exposed to green and blue wavelengths for 4 months. In the present study, significantly higher expressions of VTG and ERα in yellowtail damselfish under green and blue LED groups compared to the other groups. This is consistent with observed higher circulating level of E_2, GSI and histological characteristics in the same groups. The present results provide a strong support for the hypothesis that exposure of fish to short green and blue wavelengths enhance ovarian maturation. This hypothesis is further supported by the observed mature oocytes (TYS) in the green and blue LED groups. These results suggest that a physiological response is involved in the mechanisms linking specific light wavelengths with fish gonadal maturation. It should also be noted that light is closely connected with stress response in fish. Shin et al. (2011)'s study

demonstrated lower LPO and H_2O_2 levels in fish exposed to green and blue lights compared to fish exposed to other spectra. It was suggested that short green and blue LED wavelengths may inhibit oxidative stress in fish compared to those exposed to other light spectra. The findings suggest that certain light spectra may affect the activity of pituitary–adrenal axis, and related physiological parameters. In conclusion, exposure of fish to short wavelength light lower stress response, enhance the immune function, and enhance gonadal development. The present study demonstrated that low intensity LED lighting significantly increased the expressions of VTG and ERα, increase plasma levels of E_2, and enhance body growth and oocyte maturation. These results support the hypothesis that the use of green and blue wavelengths LED would be valuable by improving immunity and reproductive ability in cultured fish.

In conclusion, this study showed that changes in the expression of circadian, growth, sex maturation and oxidative stress-related genes as well as in the levels of plasma melatonin, H_2O_2, glucose, E_2, and LPO levels and antioxidant enzymes activities as fish were exposed to fluorescent light and various LED spectra. As the results, light is known as a factor that affects various physiological changes in fish. One of the these results suggest that MTs play a central role in controlling the diurnal and circadian rhythms in the brain and retina of olive flounder. Furthermore, one of these results suggests that RH and Exo-RH potentially mediates the effects of environmental photocycle on pineal circadian rhythms and melatonin synthesis. Also, Exo-RH, as a member of the RH family, is expressed depending on the light dependent increase in RH expression. I hypothesise that Exo-RH controls circadian rhythm by increasing its levels in the pineal gland, an important organ for the generation and maintenance of circadian rhythm. And, the expression of the clock genes *Per2* and *Cry1* was significantly higher in fish exposed to blue-green LED than in those exposed to red LED, suggesting that red light inhibits the circadian rhythms and is a stressor.

The red spectrum is a factor in inducing oxidative stress in yellowtail clownfish. Also, melatonin likely plays a strong antioxidant role to reduce oxidative stress in fish. Also, these study indicated that green and blue spectra promote growth in yellowtail clownfish and that melatonin stimulates GH secretion, consequently playing a role in promoting fish growth. Also, these results support the hypothesis that the use of green and blue wavelengths LED would be valuable by improving immunity and reproductive ability in cultured fish.

Therefore, the results of this study might be applied to an artificial photic system aimed at effectively enhancing the growth and reproduction and regulating stress and circadian rhythm of fish reared under captive conditions (Fig. 36).

Furthermore, skin color is one of the most important things to ornamental fish, thus additional studies about skin color will be necessary to understand relationship between various LED wavelengths and fish skin color.

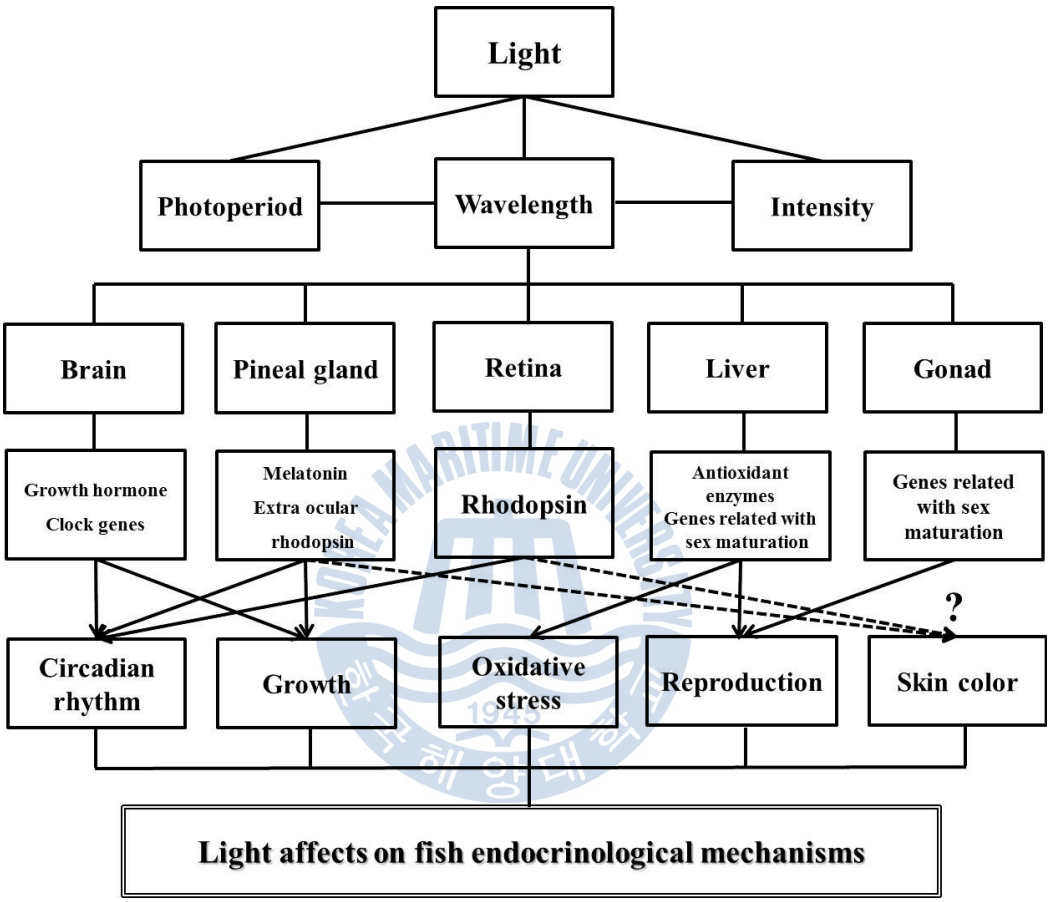

Fig. 36. Schematic diagram showing multiple pathways following on various factors.

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학부 2년차에 연구실에 입실하여 10년이 가까운 세월 동안 관심과 조언을 주신 분들께, 박사과정을 마치면서 진심으로 감사의 마음을 전하고자 합니다. 학부 시절부터 분자생물학을 접하며 많은 과학적 지식을 가르쳐 주시고 아낌없는 조언을 주신 최철영 지도교수님께 깊은 감사를 드립니다. 전공 분야와 관련하여 다양한 학문적 소양뿐만 아니라 따뜻한 조언을 주신 조성환 교수님과 박인석 교수님, 공사다망하신 중에도 부족한 논문을 애정으로 봐주시고 세심한 부분까지 다듬어 주신 부경대학교 장영진 교수님과 많은 조언을 아끼지 않으신 국립수산과학원 민병화 박사님께도 감사의 말씀 올립니다. 대학교 입학 후, 학문적 기초를 만들어 주신 강효진, 노일, 서영완, 이호진, 안종웅, 임선영, 이경은 교수님들께도 진심으로 감사드립니다. 해양분자환경생리학 연구실에 입실하여 오랜 시간 같이 생활하며 의지할 수 있었던 김나나 선배님, 실험과 여러 가지로 도움을 준 최영재 오빠, 송진아, 임지례, 최지용 후배님들과, 졸업 후에도 많은 관심과 격려를 아끼지 않은 조필규 선배님과 박찬흠 선배님께도 감사드립니다.

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References

Allegra, M., Reiter, R.J., Tan, D.X., Gentile, C., Tesoriere, L., and Livrea, M.A., 2003. The chemistry of melatonin's interaction with reactive species. J. Pineal Res. 34, 1-10.

An, K.W., Nelson, E.R., Jo, P.G., Habibi, H.R., Shin, H.S., Choi, C.Y., 2008. Characterization of estrogen receptor β2 and expression of the estrogen receptor subtypes a, β1, and β2 in the protandrous black porgy (*Acanthopagrus schlegeli*) during the sex change process. Comp. Biochem. Physiol. 150, 284-291.

Bagnyukova, T.V., Lushchak, Storey, K.B., Lushchak, V.I., 2007. Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23ºC. J. Therm. Biol. 32, 227-234.

Baldwin, J.M., Schertler, G.F., Unger, V.M., 1997. An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. J. Mol. Biol. 272, 144-164.

Bapary, M.A.J., Fainuulelei, P., Takemura, A., 2009. Environmental control of gonadal development in the tropical damselfish *Chrysiptera cyanea*. Mar. Biol. Res. 5, 462-469.

Bapary, M.A.J., Amin, M.N., Takeuchi, Y., Takemura, A., 2011. The stimulatory effects of long wavelengths of light on the ovarian development in the tropical damselfish, *Chrysiptera cyanea*. Aquaculture 314, 188-192.

Barahona-Fernandes, M.H., 1979. Some effects of light intensity and photoperiod on the sea bass larvae (*Dicentrarchus labrax*) reared at the Centre Oceanologique de Bretagne. Aquaculture 17, 311-321.

Basha Siraj, P., Rani Usha, A., 2003. Cadmium-induced antioxidant defense

mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxical. Environ. Saf. 56, 218-221.

Bayarri, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., 2002. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. J. Pineal Res. 32, 34-40.

Bayarri, M.J., Iigo, M., Muñoz-Cueto, J.A., Isorna, E., Delgado, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., Alonso-Gómez, A.L., 2004. Binding characteristics and daily rhythms of melatonin receptors are distinct in the retina and the brain areas of the European sea bass (*Dicentrarchus labrax*). Brain Res. 1029, 241-250.

Baydas, G., Yilmaz, O., Celik, S., Yasar, A., Ferit Gursu, M., 2002. Effects of certain micronutrients and melatonin on plasma lipid, lipid peroxidation, and homocysteine levels in rats. Arch. Med. Res. 33, 515-519.

Begay, V., Falcon, J., Cahill, G.M., Klein, D.C., Coon, S.L., 1998. Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebrafish, but not in trout. Endocrinology 139, 905-912.

Bellingham, J., Morris, A.G., Hunt, D.M., 1998. The rhodopsin gene of the cuttlefish *Sepia officinalis*: sequence and spectral tuning. J. Exp. Biol. 201, 2299-2306.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O., Sánchez-Vázquez, F.J., 2010. Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. Aquaculture 306, 137-145.

Boeuf, G., Le Bail, P.Y., 1999. Does light have an influence on fish growth? Aquaculture 177, 129-152.

Bolliet, V., Aranda, A., Boujard, T., 2001. Demand-feeding rhythm in rainbow trout and European catfish: synchronisation by photoperiod and

food availability. Physiol. Behav. 73, 625-633.

Bromage, N.R., Porter, M.J.R., Randall, C.F., 2001. The environmental regulation of maturation in farmed fish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-98.

Cahill, G.M., Besharse, J.C., 1991. Resetting the circadian clock in cultured *Xenopus* eyecups: regulation of retinal melatonin rhythms by light and D2 dopamine receptors. J. Neurosci. 11, 2959-2971.

Cahill, G.M., Besharse, J.C., 1995. Circadian rhythmicity in vertebrate retina: regulation by a photoreceptor oscillator. Prog. Retin. Eye Res. 14, 268-291.

Cahill, G.M., 1996. Circadian regulation of melatonin production in cultured zebrafish pineal and retina. Brain Res. 708, 177-181.

Canosa, L.F., Chang, J.P., Peter, R.E., 2007. Neuroendocrine control of growth hormone in fish. Gen. Comp. Endocrinol. 151, 1-26.

Chen, T.T., Marsh, A., Shamblott, M., Chan, K.M., Tang, Y.L., Cheng, C.M., Yang, B.Y., 1994. Structure and evolution of fish growth hormone and insulin-like growth factors genes. In: Sherwood, N.M., Hew, C.L. (Eds.), Fish Physiology.

Cole WC, Youson JH. 1982. Morphology of the pineal complex of the anadromous sea lamprey, *Petromyzon marinus* L. Am. J. Anat. 165, 131-163.

Collin, J.P., Voisin, P., Falcón, J., Faure, J.P., Brisson, P., Defaye, J.R., 1989. Pineal transducers in the course of evolution: molecular organization, rhythmic metabolic activity and role. Arch. Histol. Cytol. 52, 441-449.

Confente, F., Rendón, M.C., Besseau, L., Falcón, J., Muñoz-Cueto, J.A., 2010. Melatonin receptors in a pleuronectiform species, *Solea senegalensis*: cloning, tissue expression, day-night and seasonal variations. Gen. Comp. Endocrinol. 167, 202-214.

de Vlaming, V.L., 1975. Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas*. Biol. Bull. 148, 402-415.

de Vlaming, V.D., 1980. Effects of pinealectomy and melatonin treatment on growth in the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 40, 245-250.

Deguchi, T., 1979. Circadian rhythm of serotonin N-acetyltransferase activity in organ culture of chicken pineal gland. Science 203, 1245-1247.

Deguchi, T., 1981. Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature 290, 706-707.

Delaunay F., Thisse C., Marchand O., Laudet V., Thisse B., 2000. An inherited functional circadian clock in zebrafish embryos. Science 289, 297-300.

Dowling, J.E., Ehinger, B., 1978. The interplexiform cell system. I. Synapses of the dopaminergic neurons of the goldfish retina. Proc. R. Soc. Lond. B 201, 7-26.

Downing, G., 2002. Impact of spectral composition on larval haddock, *Melanogrammus aeglefinus* L., growth and survival. Aquacult. Int. 33, 251-259

Dubocovich, M.L., 1995. Melatonin receptors: are there multiple subtypes? Trends Pharmacol. Sci. 16, 50-56.

Dubocovich, M.L., Rivera-Bermudez, M.A., Gerdin, M.J., Masana, M.I., 2003. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front. Biosci. 8, d1093-d1108.

Ebisawa, T., Karne, S., Lerner, M.R., 1994. Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. Proc. Natl. Acad. Sci. U.S.A. 91, 6133-6137.

Ekström, P., Meissl, H., 1997. The pineal organ of teleost fishes. Rev. Fish

Biol. Fish 7, 199-284.

Eskin, A., 1979. Identification and physiology of circadian pacemakers. Fed. Proc. 38, 2570-2572.

Esterbauer, H., Schaur, R.J., Zoliner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 81-128.

Falcón, J., Meissl, H., 1981. The photosensory function of the pineal organ of the pike (*Esox lucius* L.). Correlation between structure and function. J. Comp. Physiol. 144, 127-137.

Falcón, J., Molina-Borja, M., Collin, J.-P., Oaknin, S., 1996. Age-related changes in 2- [^{125}I]-iodomelatonin binding sites in the brain of sea bass breams (*Sparus aurata* L). Fish Physiol. Biochem. 15, 401-411.

Falcón, J., 1999. Cellular circadian clocks in the pineal. Prog. Neurobiol. 8, 121-162.

Falcón, J., Besseau, L., Fazzari, D., Attia, J., Gaildrat, P., Beauchaud, M., Boeuf, G., 2003. Melatonin modulates secretion of growth hormone and prolactin by trout pituitary glands and cells in culture. Endocrinology 144, 4648-4658.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab. 18, 81-88.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469-482.

Ganguly, S., Coon, S.L., Klein, D.C., 2002. Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res. 309, 127-37.

Garg, S.K., 1988. Role of pineal and eyes in the regulation of ovarian activity and vitellogenin levels in the catfish exposed to continuous light

or continuous darkness. J. Pineal Res. 5, 1-12.

Gauer, F., Masson-Pévet, M., Skene, D.J., Vivien-Roels, B., Pévet, P.,1993. Daily rhythms of melatonin binding sites in the rat pars tuberalis and suprachiasmatic nuclei; evidence for a regulation of melatonin receptors by melatonin itself. Neuroendocrinology 57, 120-126.

Gothilf, Y., Coon, S.L., Toyama, R., Chitnis, A., Namboodiri, M.A., Klein, D.C., 1999. Zebrafish serotonin N-acetyltransferase-2: marker for development of pineal photoreceptors and circadian clock function. Endocrinology 140, 4895-4903.

Green, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., Shine, J., 1986. Sequence and expression of human estrogen receptor complementary DNA. Science 231, 1150-1154.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Head, A.B., Malison, J.A., 2000. Effects of lighting spectrum and disturbance level on the growth and stress responses of yellow perch *Perca flavescens*. J. World Aquacult. Soc. 31, 73-80.

Helvik, J.V., Drivenes, Ø., Naess, T.H., Fjose, A., Seo, H.C., 2001. Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*). Vis. Neurosci. 18, 767-780.

Hirota, T., Lee, J.W., St John, P., Sawa, M., Iwaisako, K., Noguchi, T., Pongsawakul, P.Y., Sonntag, T., Welsh, D.K., Brenner, D.A., Doyle, F.J.3rd., Schultz, P.G., Kay, S.A., 2012. Identification of small molecule activators of cryptochrome. Science 338, 1094-1097.

Hope, A.J., Partridge, J.C., Hayes, P.K., 1998. Switch in rod opsin gene expression in the European eel, *Anguilla anguilla* (L.). Proc. Roy. Soc.

Lond. B 265, 869-874.

Hoque, M.M., Takemura, A., Takano, K., 1998. Annual changes in oocyte development and serum vitellogenin level in the rabbitfish *Siganus canaliculatus* (Park) in Okinawa, Southern Japan. Fish. Sci. 64, 44-51.

Hur, S.P., Takeuchi, Y., Itoh, H., Uchimura, M., Takahashi, K., Kang, H.C., Lee, Y.D., Kim, S.J., Takemura, A., 2012. Fish sleeping under sandy bottom: Interplay of melatonin and clock genes. Gen. Comp. Endocrinol. 177, 37-45.

Iigo, M., Kezuka, H., Suzuki, T., Tabata, M., Aida, K., 1994a. Melatonin signal transduction in the goldfish, *Carassius auratus*. Neurosci. Biobehav. Rev. 18, 563–569.

Iigo, M., Kobayashi, M., Ohtani-Kaneko, R., Hara, M., Hattori, A., Suzuki, T., Aida, K., 1994b. Characteristics, day-night changes, subcellular distribution and localization of melatonin binding sites in the goldfish brain. Brain Res. 644, 213-220.

Iigo, M., Furukawa, K., Hattori, A., Hara, M., Ohtani-Kaneko, R., Suzuki, T., Tabata, M., Aida, K., 1995. Effects of pinealectomy and constant light exposure on day-night changes of melatonin binding sites in the goldfish brain. Neurosci. Lett. 197, 61-64.

Iigo, M., Sánchez-Vázquez, F.J., Hara, M., Ohtani-Kaneko, R., Hirata, K., Shinohara, H., Tabata, M., Aida, K., 1997. Characterization, guanosine 5′-O-(3-thiotriphosphate) modulation, daily variation, and localization of melatonin-binding sites in the catfish (*Silurus asotus*) brain. Gen. Comp. Endocrinol. 108, 45-55.

Iigo, M., Furukawa, K., Tabata, M., Aida, K., 2003. Circadian variations of melatonin binding sites in the goldfish brain. Neurosci. Lett. 347, 49-52.

Iigo, M., Abe, T., Kambayashi, S., Oikawa, K., Masuda, T., Mizusawa, K., Kitamura, S., Azuma, T., Takagi, Y., Aida, K., Yanagisawa, T., 2007.

Lack of circadian regulation of in vitro melatonin release from the pineal organ of salmonid teleosts. Gen. Comp. Endocrinol. 154, 91-97.

Ikegami T, Motohashi E, Doi H, Hattori A, Ando H. 2009. Synchronized diurnal and circadian expressions of four subtypes of melatonin receptor genes in the diencephalon of a puffer fish with lunar-related spawning cycles. Neurosci. Lett. 462, 58-63.

Im, L.H.J., Isoldi, M.C., Scarparo, A.C., Visconti, M.A., Castrucci, A.M., 2007. Rhythmic expression, light entrainment and alpha-MSH modulation of rhodopsin mRNA in a teleost pigment cell line. Comp. Biochem. Physiol. A 147, 691-696.

Ishibashi, O., Kawashima, H., 2001. Cloning and characterization of the functional promoter of mouse estrogen receptor β gene. Biochim. Biophys. Acta. 1519, 223-229.

Iuvone, P.M., Tosini, G., Pozdeyev, N., Haque, R., Klein, D.C., Chaurasia, S.S., 2005. Circadian clocks, clock networks, arylalkylamine N-acetyltransferase, and melatonin in the retina. Prog. Retin. Eye Res. 24, 433-456.

John, T.M., Viswanathan, M., George, J.C., Scanes, C.G., 1990. Influence of chronic melatonin implantation on circulating levels of catecholamines, growth hormone, thyroid hormones, glucose, and free fatty acids in the pigeon. Gen. Comp. Endocrinol. 79, 226-232.

Karaganis, S.P., Bartell, V.R., Shende, V.R., Moore, A.F., Cassone, V.M., 2009. Modulation of metabolic and clock gene mRNA rhythms by pineal and retinal circadian oscillators. Gen. Comp. Endocrinol. 161, 179-192.

Karakatsouli, N., Papoutsoglou, S.E., Pizzonia, G., Tsatsos, G., Tsopelakos, A., Chadio, S., Kalogiannis, D., Dalla, C., Polissidis, A., Papadopoulou-Daifoti, Z., 2007. Effects of light spectrum on growth and physiological status of gilthead seabream *Sparus auratus* and rainbow trout

Oncorhynchus mykiss reared under recirculating system conditions. Aquacult. Eng. 36, 302-309.

Khorana, H.G., Reeves, P.J., Kim, J.M., 2002. Structure and mechanism in G protein-coupled receptors. Pharmaceut. Rev. 9, 287-294.

Kim, M.O., Phyllis, E.B., 1998. Oxidative stress in critical care: is antioxidant supplementation beneficial? J. Am. Diet. Assoc. 98, 1001-1008.

Kim, J.M., Kim, S.W., Kim, S.K., 2007. Molecular cloning and characterization of the rod opsin gene in olive flounder *Paralichthys olivaceus*. J. Fish. Sci. Technol. 10, 8-15.

Kim, N.N., Shin, H.S., Lee, J., Choi, C.Y., 2012. Diurnal gene expression of *Period2*, *Cryptochrome1*, and *arylalkylamine N-acetyltransferase-2* in olive flounder, *Paralichthys olivaceus*. Anim. Cells Syst. 16, 27-33.

King, D.P., Takahashi, J.S., 2000. Molecular genetics of circadian rhythms in mammals. Annu. Rev. Neurosci. 23, 713-742.

Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Bégay, V., Falcón, J., Cahill, G.M., Cassone, V.M., Baler, R., 1997. The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog. Horm. Res. 52, 307-357.

Klein, D.C., 2007. Arylalkylamine N-acetyltransferase: “the Tim enzyme”, J. Biol. Chem. 282, 4233-4237.

Kojima, D., Mano, H., Fukada, Y., 2000. Vertebrate ancientlong opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J. Neurosci. 20, 2845-2851.

Law, M.S., Cheng, K.W., Fung, T.K., Chan, Y.H., Yu, K.L., Chan, K.M., 1996. Isolation and characterization of two distinct growth hormone cDNAs from the goldfish, *Carassius auratus*. Arch. Biochem. Biophys.

330, 19-23.

Levin, J., McNicol, E., 1982. Color vision in fishes. Sci. Am. 246, 108-117.

Li, W.S., Chen, D., Wong, A.O.L., Lin, H.R., 2005. Molecular cloning, tissue distribution, and ontogeny of mRNA expression of growth hormone in orange-spotted grouper (*Epinephelus coioides*). Gen. Comp. Endocrinol. 144, 78-89.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25, 402-408.

Loew, E.R., McFarland, W.N., 1990. The underwater visual environment. In: Douglas, R.H., Djamgoz (Eds.), The Visual System of Fish. Chapman and Hall, London.

Lythgoe, J.N., 1979. The Ecology of Vision. Clarendon Press, Oxford.

Lythgoe, J.N., Muntz, W.R.A., Partridge, J.C., Shand, J., Williams, D. McB, 1994. The ecology of visual pigments of snappers (Lutjanidae) on the Great Barrier Reef. J. Comp. Physiol. 174A, 461-467.

MacKenzie, D.S., Vanputte, C.M., Leiner, K.A., 1998. Nutrient regulation of endocrine function in fish. Aquaculture 161, 3-25.

Mailliet, F., Ferry, G., Vella, F., Berger, S., Coge, F., Chomarat, P., Mallet, C., Guenin, S.P., Guillaumet, G., Viaud-Massuard, M.C., Yous, S., Delagrange, P., Boutin, J.A., 2005. Characterization of the melatoninergic MT3 binding site on the NRH: quinine oxidoreductase 2 enzyme. Biochem. Pharmacol. 71, 74-88.

Mano, H., Kojima, D., Fukada, Y., 1999. Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Mol. Brain Res. 73, 110-118.

Martinez, G.R., Almeida, E.A., Klitzke, C.F., Onuki, J., Prado, F.M., Medeiros, M.H., Di Mascio, P., 2005. Measurement of melatonin and its metabolites: importance for the evaluation of their biological roles.

Endocrine 27, 111-118.

Masana, M.I., Benloucif, S., Dubocovich, M.L., 2000. Circadian rhythm of mt1 melatonin receptor expression in the suprachiasmatic mucleus of the C3H/HeN mouse. J. Pineal Res. 28, 185-192.

Max, M., Menaker, M., 1992. Regulation of melatonin production by light, darkness, and temperature in the trout pineal. J. Comp. Physiol. A 170, 479-489.

Mazurais, D., Brierley, I., Anglade, I., Drew, J., Randall, C., Bromage, N., Michel, D., Kah, O., Williams, L.M., 1999. Central melatonin receptors in the rainbow trout: comparative distribution of ligand binding and gene expression. J. Comp. Neurol. 409, 313-324.

McFarland, W.N., 1991. The visual world of coral reef fishes. In: Sale, P.F. (Ed.), The Ecology of Fishes on Coral Reefs. Academic Press, San Diego, pp. 16-38.

McFarland, V.A., Inouye, L.S., Lutz, C.H., Jarvis, A.S., Clarke, J.U., McCant, D.D., 1999. Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown bullhead, *Ameiurus nebulosus*. Arch. Environ. Contam. Toxicol. 37, 236-241.

Migaud, H., Taylor, J.F., Taranger, G.L., Davie, A., Cerdá-Reverter, J.M., Carrillo, M., Hansen, T., Bromage, N.R., 2006. Pineal gland sensitivity to light intensity in salmon (*Salmo salar*) and sea bass (*Dicentrarchus labrax*): an in vivo and ex vivo study. J. Pineal Res. 41, 42-52.

Migaud, H., Cowan, M., Taylor, J., Ferguson, H.W., 2007. The effect of spectral composition and light intensity on melatonin, stress and retinal damage in post-smolt Atlantic salmon, *Salmo salar*. Aquaculture 270, 390-404.

Minamoto, T., Shimizu, I., 2003. Molecular cloning and characterization of rhodopsin in a teleost (*Plecoglossus altivelis*, Osmeridae). Comp. Biochem.

Physiol. B 134, 559-570.

Munz, F.W., McFarland, W.N., 1977. Evolutionary adaptations of fishes to the photic environment. In: Crescitell F, (editor) The visual system in vertebrates. New York: Springer. p. 193-274.

Myrberg A.A. Jr., Fuiman, L.A., 2002. The sensory world of coral reef fishes. In: Sale, P.F. (Eds.), Coral Reef Fishes. Academic Press, San Diego, pp. 123-148.

Nakayama, T.A., Khorana, H.G., 1991. Mapping of the amino acids in membrane-embedded helices that interact with the retinal chromophore in bovine rhodopsin. J. Biol. Chem. 266, 4269-4275.

Namboodiri, M.A., Dubbels, R., Klein, D.C., 1987. Arylalkylamine N-acetyltransferase from mammalian pineal gland. Methods Enzymol. 142, 583-90.

Nelson, E.R., Habibi, H.R., 2010. Functional significance of estrogen receptor subtypes in the liver of goldfish. Endocrinology 151, 1668-1676.

Neumeyer, C., 1992. Tetrachromatic color vision in goldfish: evidence from color mixture experiments. J. Comp. Physiol. A 171, 639-649.

Nouroozzadeh, J., Tajaddinisarmadi, J., Wolff, S.P., 1994. Measurement of plasma hydroperoxide concentrations by ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 200, 403-409.

O'Brien, J., Ripps, H., Al-Ubaidi, M.R., 1997. Molecular cloning of a rod opsin cDNA from the skate retina. Gene 193, 141-150.

Okamura, H., Yamaguchi, S., Yagita, K., 2002. Molecular machinery of the circadian clock in mammals. Cell Tissue Res. 309, 47-56.

Oppedal, F., Taranger, G.L., Juell, J.-E., Fosseidengen, J.E., Hansen, T., 1997. Light intensity affects growth and sexual maturation of Atlantic salmon (*Salmo salar*) postsmolts in sea cages. Aquat. Living Resour. 10, 351-357.

Ovchinnikov, Y.A., Abdulaev, N.G., Bogachuk, A.S., 1988. Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated. FEBS Lett. 230, 1-5.

Pandey, S., Parvez, S., Sayeed, I., Haques, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (BI & Schn.). Sci. Total Environ. 309, 105-115.

Pando M.P., Pinchak A.B., Cermakian N,, Sassone-Corsi P., 2001. A cell-based system that recapitulates the dynamic lightdependent regulation of the vertebrate clock. Proc. Natl. Acad. Sci. 98, 10178-10183.

Pankhurst, N.W., Porter, M.J.R., 2003. Cold and dark or warm and light: variations on the theme of environmental control of reproduction. Fish Physiol. Biochem. 28, 385-389.

Park, Y.J., Park, J.G., Kim, S.J., Lee, Y.D., Rahman, M.S., Takemura, A., 2006. Melatonin receptor of a reef fish with lunar-related rhythmicity: cloning and daily variations. J. Pineal Res. 41, 166-174.

Park, Y.J., Park, J.G., Hiyakawa, N., Lee, Y.D., Kim, S.J., Takemura, A., 2007a. Diurnal and circadian regulation of a melatonin receptor, MT1, in the golden rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 150, 253-262.

Park, Y.J., Park, J.G., Jeong, H.B., Takeuchi, Y., Kim, S.J., Lee, Y.D., Takemura, A., 2007b. Expression of the melatonin receptor Mel1c in neural tissues of the reef fish *Siganus guttatus*. Comp. Biochem. Physiol. A 147, 103-111.

Philp, A.R., Bellingham, J., Garcia-Fernandez, J.M., Forster, R.G., 2000. A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. FEBS Lett. 468, 181-188.

Pierce, L.X., Noche, R.R., Ponomareva, O., Chang, C., Liang, J.O., 2008. Novel function for period 3 and Exo-rhodopsin in rhythmic transcription

and melatonin biosynthesis within the zebrafish pineal organ. Brain Res. 1223, 11-24.

Porter, M.J.R., Randall, C.F., Bromage, N.R., Thorpe, J.E., 1998. The role of melatonin and the pineal gland on development and smoltification of Atlantic salmon (*Salmo salar*) parr. Aquaculture 168, 139-155.

Porter, M.J.R., Duncan, N.J., Mitchell, D., Bromage, N.R., 1999. The use of cage lighting to reduce plasma melatonin in Atlantic salmon (*Salmo salar*) and its effects on the inhibition of grilsing. Aquaculture 176, 237-244.

Reddy P., Zehring W.A., Wheeler D.A., Pirrotta V., Hadfield C., Hall J.C., Rosbash M., 1984. Molecular analysis of the period locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell 38, 701-10.

Radenko, V.N., Alimov, I.A., 1991. Importance of temperature and light to growth and survival of larval silver carp, *Hypophthalmichthys molitrix*. Vopr. Ikhtiol. 31, 655-663.

Reiter, R.J., 1991. Melatonin: the chemical expression of darkness. Mol. Cell. Endocrinol. 79, C153-C158.

Reiter, R.J., Carneiro, R.C., Oh, C.S., 1997. Melatonin in relation to cellular antioxidative defense mechanisms. Horm. Metab. Res. 29, 363-372.

Reiter, R.J., Tan, D.X., Pilar, T.M., Flores, L.J., Czarnocki, Z., 2007. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochim. Pol. 54, 1-9.

Renuka K., Joshi B.N, 2010. Melatonin-induced changes in ovarian function in the freshwater fish *Channa punctatus* (bloch) held in long days and continuous light. Gen. Comp. Endocrinol. 165, 42-46

Reppert, S.M., Godson, C., Mahle, C.D., Weaver, D.R., Slaugenhaupt, S.A., Gusella, J.F., 1995a. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin

receptor. Proc. Natl. Acad. Sci. U.S.A. 92, 8734-8738.

Reppert, S.M., Weaver, D.R., Cassone, V.M., Godson, C., Kolakowski Jr., L.F., 1995b. Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in chick brain. Neuron 15, 1003-1015.

Reppert, S.M., Weaver, D.R., Godson, C., 1996. Melatonin receptors step into the light: cloning and classification of subtypes. Trends Pharmacol. Sci. 17, 100-102.

Roca, A.L., Godson, C., Weaver, D.R., Reppert, S.M., 1996. Structure, characterization, and expression of the gene encoding the mouse Mel1a melatonin receptor. Endocrinology 137, 3469-3477.

Rosa Nogués, M., Giralt, M., Romeu, M., Mulero, M., Sánchez-Martos, V., Rodríguez, E., Acuña-Castroviejo, D., Mallol, J., 2006. Melatonin reduces oxidative stress in erythrocytes and plasma of senescence-accelerated mice. J. Pineal Res. 41, 142-149.

Ruchin, A.B., Vechkanov, V.S., Kuznetsov, V.A., 2002. Growth and feeding intensity of young carp *Cyprinus carpio* under different constant and variable monochromatic illuminations. J. Ichthyol. 42, 191-199.

Ruchin, A.B., 2004. The effect of light regime on feeding intensity and growth rate in fishes (in Russian). Hydrobiologichesky Zhurn (Kiev) 40, 48-52.

Ryffel, G.U., 1978. Synthesis of vitellogenin, an attractive model for investigation hormone-induced gene activation. Mol. Cell. Endocrinol., 12, 237-246.

Sakamar, T.P., Franke, R.R., Khorana, H.G., 1989. Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Proc. Natl. Acad. Sci. USA 86, 8309-8313.

Samach, A., Coupland, G., 2000. Time measurement and the control of

flowering in plants. BioEssays 22, 38-47.

Sauzet, S., Besseau, L., Herrera Perez, P., Covès, D., Chatain, B., Peyric, E., Boeuf, G., Muñoz-Cueto, J.A., Falcón, J., 2008. Cloning and retinal expression of melatonin receptors in the European sea bass, *Dicentrarchus labrax*. Gen. Comp. Endocrinol. 157, 186-195.

Schantz, M., Lucas, R.J., Foster, R.G., 1999. Circadian oscillation of photopigment transcript levels in the mouse retina. Mol. Brain Res. 72, 108-114.

Schlenk, D., Rice, C.D., 1998. Effects of zinc and cadmium treatment on hydrogen peroxide mortality and expression of glutathione and metallothionein in a teleost hepatoma cell line. Aquat. Toxicol. 43, 121-129.

Schreck, CB., 1993. Glucocorticoids: metabolism, growth and development. In: The endocrinology of growth, development, and metabolism in vertebrates. San Diego: Academic Press; p. 367-392.

Shi, Q., Ando, H., Coon, S.L., Sato, S., Ban, M., Urano, A., 2004. Embryonic and post-embryonic expression of arylalkylamine N-acetyltransferase and melatonin receptor genes in the eye and brain of chum salmon (*Oncorhynchus keta*). Gen. Comp. Endocrinol. 136, 311-321.

Shin, H.S., Lee, J.H., Choi, C.Y., 2011. Effects of LED light spectra on oxidative stress and the protective role of melatonin in relation to the daily rhythm of the yellowtail clownfish, *Amphiprion clarkii*. Comp. Biochem. Physiol. A. 160, 221-228.

Shin, H.S., Lee, J.H., Choi, C.Y., 2012. Effects of LED light spectra on the growth of the yellowtail clownfish, *Amphiprion clarkii*. Fish. Sci. 78, 549-556.

Simonneaux, V., Ribelayga, C., 2003. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin

synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol. Rev. 55, 325-395.

Skene, D.J., Deacon, S., Arendt, J., 1996. Use of melatonin in circadian rhythm disorders and following phase shifts. Acta. Neurobiol. Exp. (Warsz) 56, 359-362.

Soni, B.G., Foster, R.G., 1997. A novel and ancient vertebrate opsin. FEBS Letters 406, 279-283.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M., Hall, J.C., 1998. The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95, 681-692.

Takemura, A., Susilo, E.S., Rahman, M.D., Morita, M., 2004. Perception and possible utilization of moonlight intensity for reproductive activities in a lunar-synchronized spawner, the golden rabbitfish. J. Exp. Zool. A 301, 844-851.

Takemura, A., Ueda, S., Hiyakawa, N., Nikaido, Y., 2006. A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish, *Siganus guttatus*. J. Pineal Res. 40, 236-241.

Tandler, A., Helps, S., 1985. The effects of photoperiod and water exchange on growth and survival of gilthead sea bream (*Sparus aurata*, Linnaeus; Sparidae) from hatching to metamorphosis in mass rearing systems. Aquaculture 48, 71-82.

Taylor, J.F., Migaud, H., Porter, M.J.R., Bromage, N.R., 2005. Photoperiod influences growth rate and insulin-like growth factor-I (IGF-I) levels in juvenile rainbow trout. Gen. Comp. Endocrinol. 142, 169-185.

Terakita, A., 2005. The opsins. Gen. Biol. 6, 213.

Tissot, B.N., Best, B.A., Borneman, E.H., Bruckner, A.W., Cooper, C.H., D'Agnes, H., Fitzgerald, T.P., Leland, A., Lieberman, S., Amos, A.M.,

Sumaila, R., Telecky, T.M., McGilvray, F., Plankis, B.J., Rhyne, A.L., Roberts, G.G., Starkhouse, B., Stevenson, T.C., 2010. How U.S. ocean policy and market power can reform the coral reef wildlife trade. Mar. Policy 34, 1385-1388.

Ullmann, J.F.P., Gallagher, T., Hart, N.S., Barnes, A.C., Smullen, R.P., Collin, S.P., Temple, S.E., 2011. Tank color increases growth, and alters color preference and spectral sensitivity, in barramundi (*Lates calcarifer*). Aquaculture 322-323, 235-240.

Underwood, H., 1989. The pineal and melatonin: regulators of circadian function in lower vertebrates. Experientia 45, 914-922.

Urasaki, H., 1976. The role of pineal and eyes in the photoperiodic effect on the gonad of the medaka, *Oryzias latipes*. Chronobiologia 3, 228-234.

Vallone D., Gondi S.B., Whitmore D., Foulkes N.S., 2004. E-box function in a period gene repressed by light. Proc. Natl. Acad. Sci. USA 101, 4106–4111.

Van der Salm, A.L., Martínez, M., Flik, G., Wendelaar Bonga, S.E., 2004. Effects of husbandry conditions on the skin colour and stress response of red porgy *Pagrus pagrus*. Aquaculture 241, 371-386.

Venkataraman, P., Krishnamoorthy, G., Vengatesh, G., Srinivasan, N., Aruldhas, M.M., Arunakaran, J., 2008. Protective role of melatonin on PCB (Aroclor 1254) induced oxidative stress and changes in acetylcholine esterase and membrane bound ATPases in cerebellum, cerebral cortex and hippocampus of adult rat brain. Int. J. Devl. Neurosci. 26, 585-591.

Vera, L.M., Davie, A., Taylor, J.F., Migaud, H., 2010. Differential light intensity & spectral sensitivities of Atlantic salmon, European sea bass and Atlantic cod pineal glands ex vivo. Gen. Comp. Endocrinol. 165, 25-33.

Villamizar, N., García-Alcazar, A., Sánchez-Vázquez, F.J., 2009. Effect of light spectrum and photoperiod on the growth, development and survival

of European sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 292, 80-86.

Volpato, G.L., 2000. Aggression among farmed fish. In: Flos, R., Creswell, L. (Eds), Aqua 2000: Responsible aquaculture in the new millennium: European Aquaculture Society Special publication, 28. Nice, France.

Volpato, G.L., Barreto, R.E., 2001. Environmental blue light prevents stress in the fish Nile tilapia. Braz. J. Med. Biol. Res. 34, 1041-1045.

Volpato, G.L., Duarte, C.R.A., Luchiari, A.C., 2004. Environmental color affects Nile tilapia reproduction. Braz. J. Med. Biol. Res. 37, 479-483.

Von Gall, C., Stehle, J.H., Weaver, D.H., 2002. Mammalian melatonin receptors: molecular biology and signal transduction. Cell. Tissue Res. 309, 151-162.

Vriend, J., Lauber, J.K., 1973. Effects of light intensity, wavelength and quanta on gonads and spleen of the deer mouse. Nature 244, 37-38.

Wagner, H., 1990. Retinal structure of fishes. In: Douglas, R.H., Djamgoz, M., editors, The visual system of fish. Springer Netherlands: Chapman and Hall; p. 109-157.

Wang, J.K., McDowell, J.H., Hargrave, P.A., 1980. Site of attachment of 11-cis-retinal in bovine rhodopsin. Mol. Biol. Evol. 12, 53-61.

Wiechmann, A.F., Smith, A.R., 2001. Melatonin receptor RNA is expressed in photoreceptors and displays a diurnal rhythm in *Xenopus* retina. Mol. Brain. Res. 91, 104-111.

Wu, Y.H., Swaab, D.F., 2005. The human pineal gland and melatonin in aging and Alzheimer's disease. J. Pineal Res. 38, 145-152.

Yamanome, T., Mizusawa, K., Hasegawa, E., Takahashi, A., 2009. Green light stimulates somatic growth in the barfin flounder, *Verasper moseri*. J. Exp. Zool. 311A, 73-79.

Yokoyama, S., Zhang, H., 1997. Cloning and characterization of the pineal

gland-specific opsin gene of marine lamprey (*Petromyzon marinus*). Gene 202, 89-93.

Yokoyama, S., Radlwimmer, F.B., 1998. The 'five-sites' rule and the evolution of red and green color vision in mammals. Mol. Biol. Evol. 15, 560-567.

Yokoyama, S., Zhang, H., Radlwimmer, F.B., Blow, N.S., 1999. Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). Proc. Natl. Acad. Sci. U.S.A. 96, 6279-6284.

Yokoyama, S., 2000. Molecular evolution of vertebrate visual pigments. Prog. Retin. Eye Res. 19, 385-419.

Yuan, H., Tang, F., Pang, S.F., 1990. Binding characteristics, regional distribution and diurnal variation of [^{125}I]-iodomelatonin binding sites in the chicken brain. J. Pineal Res. 9, 179-191.

Zachmann, A., Falcón, J., Knijff, S.C.M., 1992. Effects of photoperiod and temperature on rhythmic melatonin secretion from the pineal organ of the white sucker (*Catostomus commersoni*) in vitro. Gen. Comp. Endocrinol. 86, 26-33.

Zatz, M., Mullen, D.A., 1988. Two mechanisms of photoendocrine transduction in cultured chick pineal cells: pertussis toxin blocks the acute but not the phase-shifting effects of light on the melatonin rhythm. Brain Res. 453, 63-71.

Zawilska, J.B., Nowak, J.Z., 1992. Regulatory mechanisms in melatonin biosynthesis in retina. Neurochem. Int. 20, 23-36.

Zawilska, J.B., Nowak, J.Z., 1999. Melatonin: from biochemistry to therapeutic applications. Pol. J. Pharmacol. 51, 3-23.

Zawilska, J.B., Lorenc, A., Berezińska, M., Vivien-Roels, B., Pévet, P., Skene, D.J., 2006. Diurnal and circadian rhythms in melatonin synthesis in the turkey pineal gland and retina. Gen. Comp. Endocrinol. 145, 162-168.

Zeman, M., Výboh, P., Juráni, M., Lamošová, D., Košt' ál, B., Bilčík, B., Blažíček, P., Jurániová, E., 1993. Effects of exogenous melatonin on some endocrine, behavioral and metabolic parameters in Japanese quail *Coturnix coturnix japonica*. Comp. Biochem. Physiol. 105A, 323-328.

Zhang, T., Tan, Y.H., Fu, J., Lui, D., Ninh, Y., Jirik, F.R., Brenner, S., Venkatesh, B., 2003. The regulation of retina specific expression of rhodopsin gene in vertebrates. Gene 313, 189-200.

Zhang, E.E., Liu, Y., Dentin, R., Pongsawakul, P.Y., Liu, A.C., Hirota, T., Nusinow, D.A., Sun, X., Landais, S., Kodama, Y., Brenner, D.A., Montminy, M., Kay, S.A., 2010. Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. Nat. Med. 16, 1152-1156.

Ziv, L., Levkovitz, S., Toyama, R., Falcon, J., Gothilf, Y., 2005. Functional development of the zebrafish pineal gland: light-induced expression of period2 is required for onset of the circadian clock. J. Neuroendocrinol. 17, 314-320.