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Thesis for the Degree of Master of Science

**Effects of Light Emitting Diode (LED) Spectra  
on the Expression of Vertebrate Ancient Long  
Opsin Gene and Maturation-related Hormones  
in the Goldfish, *Carassius auratus***



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Advisor: Prof. Cheol Young Choi



by  
Jin Ah Song

A dissertation submitted in partial fulfillment of the requirements for the  
degree of

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the Graduate School of Korea Maritime and Ocean University,  
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## List of Abbreviations

BW	body weight
cDNA	complementary deoxyribonucleic acid
cGnRH-II	chicken gonadotropin-releasing hormone-II
Ct	threshold cycle
E <sub>2</sub>	17β-estradiol
ELISA	enzyme-linked immunosorbent assay
ERs	estrogen receptors
FSH	follicle-stimulating hormone
GTH	Gonadotropin hormone
GnRH	gonadotropin-releasing hormone
HPG	hypothalamic-pituitary-gonad
LED	light-emitting diode
LH	luteinizing hormone
mRNA	mitochondrial ribonucleic acid
PCR	polymerase chain reaction
QPCR	quantitative real-time polymerase chain reaction
sGnRH	salmon gonadotropin-releasing hormone
TBS	tris-buffered saline
TTBS	Tris-buffered saline with Tween
VA	vertebrate ancient
VAL	vertebrate ancient long
ZT	Zeitgeber time

## Abstract (in Korean)

# 금붕어, *Carassius auratus*의 옵신 유전자 발현 및 성 호르몬 분비 조절에 미치는 발광다이오드(LED) 파장의 영향

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요약

본 연구는 형광등과 2가지의 파장의 LED 조명등(적색, 620 nm; 녹색, 530 nm)이 설치된 수조에서 금붕어, *Carassius auratus*를 사육하면서 빛 파장이 금붕어의 성 성숙에 미치는 영향을 파악하고자 수행하였다. 특히, 본 연구에서는 빛에 민감하게 반응하는 광수용체의 일종인 옵신(opsin) 중에서 뇌심부에 존재하면서 녹색 파장대의 빛에 민감하게 반응하는 것으로 알려져 있는 vertebrate ancient long opsin (VAL-옵신) 유전자와 성 성숙 호르몬 분비와의 관련성에 대해서도 조사하였다.

적색과 녹색 LED 파장 및 형광등의 조사에 따른 옵신 유전자(Red-옵신, Green-옵신 및 VAL-옵신) 및 성숙관련 유전자(GnRHs, GTHs 및 ERs)/단백질의 발현량 차이를 비교하였다. 또한, VAL-옵신과 성 성숙호르몬인 생식소자극호르몬(GTH)과의 관련성을 확인하기 위하여 생식소자극호르몬

을 금붕어에 복강 주사한 후 VAL-옵신 유전자의 발현량 변화를 관찰하였다. 그 결과, 녹색 과장 실험구에서 유의적으로 높은 발현량을 보인 옵신은 Green-옵신과 VAL-옵신이었으나, 생식소자극호르몬에 반응을 보여 발현량이 유의적으로 증가한 옵신은 VAL-옵신이 유일하였으며, 뇌 배양 실험(*in vitro*)에서도 비슷한 경향이 관찰되었다( $P < 0.05$ ). 또한, 녹색 과장 실험구에서는 타 실험구에 비하여 2가지 타입의 생식소자극호르몬방출호르몬(GnRH; sGnRH, cGnRH-II)과 에스트로겐수용체(ER) 유전자 및 단백질 발현량, 혈중  $17\beta$ -estradiol 농도 모두 유의적으로 증가하는 경향이 관찰되었다( $P < 0.05$ ).

이상의 연구 결과, 녹색 과장은 금붕어에서 생식소자극호르몬방출호르몬과 에스트로겐수용체 유전자/단백질 발현 및 활성을 유도하는 동시에, VAL-옵신의 발현량 또한 증가시키는 것으로 나타났다. 즉, 녹색 과장은 금붕어의 성 성숙을 유도할 뿐만 아니라, VAL-옵신 유전자의 발현 및 활성을 유도하여 기존의 생식소자극호르몬의 작용 경로와는 다른 VAL-옵신을 통한 별도의 성 성숙 촉진 기작을 가지고 있을 가능성이 제기된다.

**Effects of Light Emitting Diode (LED) Spectra  
on the Expression of Vertebrate Ancient Long  
Opsin Gene and Maturation-related Hormones  
in the Goldfish, *Carassius auratus***

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**Abstract**

This study was investigated the effect of light spectra on vertebrate ancient long opsin (VAL-opsin) and sex hormones in the goldfish, *Carassius auratus* using red and green light-emitting diodes (LEDs). In addition, this study was investigated the effect of *in vivo* injection of exogenous gonadotropin hormone (GTH; 5  $\mu\text{g/g}$  body weight) on VAL-opsin levels. It was measured changes in the expression levels of gonadotropin-releasing hormone (GnRH: sGnRH and cGnRH-II), GTHs (GTH: GTH $\alpha$ , LH $\beta$ , and FSH $\beta$ ), and estrogen receptor (ER: ER $\alpha$ , ER $\beta$ 1, and ER $\beta$ 2) mRNAs, and GnRH and GTH proteins. Furthermore, it was measured changes in plasma 17 $\beta$ -estradiol (E $_2$ ) levels. In fish exposed to green light spectra and 5  $\mu\text{g/g}$  GTH injection, VAL-opsin levels were significantly higher than the control (white) and red light-exposed groups. In fish exposed to green light spectra, the expression levels of GnRHs, ERs, and plasma E $_2$  were significantly higher than those of the control and red light-exposed groups ( $P < 0.05$ ).

These results indicate that GTH injection activates VAL-opsin and sex hormones and that green light spectra influence VAL-opsin in goldfish. In addition, red light is rapidly absorbed by water molecules and cannot be detected by fish. Thus, green light appears to trigger VAL-opsin to promote the expression of sexual maturation-related genes in this specie. These results provide insight into their environmental, physiological, and molecular processes underlying fish development.

**Keywords:** Gonadotropin hormone, Reproductive hormone, Sex maturation, VAL-opsin



## 1. Introduction

Light directly or indirectly affects the circadian rhythm, growth, and sexual maturation of fish (Doyle and Menaker, 2007; Shin et al., 2014). As the light is passed through the retina, it sends a signal to the neurons in the brain (Fisher et al., 2013). To date, research into the biological mechanisms associated with light has generally focused on the visual light path (retina); however, the effects of light on non-visual pathways (i.e., the deep brain, pineal complex, and skin) have been relatively ignored, despite their important roles in animal behavior and physiology (Hattar et al., 2003; Nosedá et al., 2010).

Vertebrates, including fish and birds, have photoreceptor cells in the retina (rods, cones and ganglion cells) as well as in various organs such as the pineal complex, deep brain, and skin, which regulate activation of the hypothalamus-pituitary-gonad (HPG) axis (Foster and Soni, 1998; Migaud et al., 2010; Shin et al., 2014b). Initial studies conducted in birds identified the role of opsin genes in promoting maturation through photoperiodic control, demonstrating the important role of deep-brain photoreceptors in the hypothalamus, such as opsin-like proteins, in regulation of the HPG axis (Foster and Soni, 1998; Halford et al., 2009; Nakane et al., 2010).

The pioneering study on vertebrate deep-brain photoreception (von Frisch, 1911) demonstrated that a light-induced change in skin color of the European minnow, *Phoxinus laevis* is not abolished by removal of the eyes and pineal complex, and the light sensitivity was instead ascribed to the “deep-brain photoreceptor” located at the ependyma of the diencephalic ventricle. The involvement of deep-brain photoreceptors in the photoperiodic response of gonadal development was also suggested in sweetfish, *Plecoglossus altivelis*, channel catfish, *Ictalurus punctatus* and stinging catfish, *Heteropneustes fossilis* (Davis et al., 1986; Garg, 1989; Masuda et al., 2005).

To date, several opsins, including rhodopsin, cone-like opsin, vertebrate

ancient (VA) opsin, VA-long (VAL) opsin, and melanopsin, have been found in the brain of fishes (Kojima et al., 2000; Minamoto and Shimizu, 2002; Drivenes et al., 2003; Masuda et al., 2003; Shin et al., 2012). Melanopsin is responsible for the regulation of the circadian rhythms, not maturation (Hattar et al., 2002). Also, among these, VA-opsin and VAL-opsin share a common core sequence in the membrane-spanning domains, although VAL-opsin has a much longer C-terminal tail than that of VA-opsin. Functional reconstitution experiments on the recombinant proteins showed that VAL-opsin bound with 11-cis-retinal is a green-sensitive pigment ( $\lambda_{\max} \sim 500$  nm), whereas VA-opsin exhibited no photosensitivity even in the presence of 11-cis-retinal (Kojima et al., 2000). Furthermore, it was reported that the deep-brain stimulator VAL-opsin is closely related to maturation in birds (Halford et al., 2009). Also, García-Fernández et al. (2015) shows that a high level of co-expression of VA opsin with GnRH neurosecretory cells across the avian brain. Although it may be suggest relation of VAL-opsin and sexual development and maturation, it is not clearly known that mutual expression of VAL-opsin and maturation-related hormone. So, this study was investigated VAL-opsin expression by GTH injection.

Sexual development and maturation in teleosts are regulated by various sex hormones in the HPG axis, including gonadotropin-releasing hormone (GnRH), gonadotropin hormone (GTH), and steroid hormones, and are regulated elsewhere by neuroendocrine materials and gonadal hormones (Lee et al., 2001; Pati and Habibi, 2002).

To date, 15 GnRH isoforms have been isolated from vertebrates (Fernald and White, 1999). Using the goldfish, *Carassius auratus* as experimental fish, two distinct forms of GnRH, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), have been characterized in the brain (Yu et al., 1988). GTHs are pituitary hormones that are secreted by GnRH stimulation, and are reported to play important roles in the regulation of gonadal development and sexual differentiation and to stimulate spawning times and steroid hormone regulation



in vertebrates, including fish (Colombo and Chicca, 2003). In general, in fish, follicle-stimulating hormone (FSH) is involved in early gametogenesis, vitellogenin synthesis, and spermatogenesis, whereas luteinizing hormone (LH) is known to regulate final gonad maturation, ovulation, ejaculation, and steroid hormone synthesis (Nagahama et al., 1995; Kobayashi et al., 2006).

Estrogen is a steroid hormone that plays various roles in reproductive processes such as sexual development and maturation, including oogenesis, spermatogenesis, secretion of GTH, and testis development, alone and in combination with estrogen receptors (ERs), a specific receptor in vertebrates, including fish (Ishibashi and Kawashima, 2001). There are two nuclear ER forms, designated as ER $\alpha$  and ER $\beta$ , and ray-finned fish species, *Actinopterygii* have at least three distinct subtypes, including ER $\alpha$ , ER $\beta$ 1, and ER $\beta$ 2. Their functional roles have been suggested to be related to maturation in various vertebrates, including fish (Choi and Habibi, 2003).

Various wavelengths of light induce or inhibit the photoreceptors in organisms, influencing not only physiological processes but also the reproduction and behavior of an organism (Duston and Saunders, 1990; Baroiller et al., 1999; Biswas et al., 2002). In particular, light regulates sexual maturation by strongly affecting neuroendocrine control and the HPG axis (Rodríguez et al., 2001a,b; Biswas et al., 2002).

Recent research has provided novel insights into the effect of light on fish maturation (Karigo et al., 2013; Shin et al., 2014a,b); however, understanding of the effect of the various wavelengths of light on photoreceptor activation and photoreceptor-related maturation remains incomplete.

A light-emitting diode (LED) has the dual advantage of being able to emit light within a specific wavelength range with easily adjustable sensitivity. Thus, an LED is very effective for light-related research (Villamizar et al., 2009; Shin et al., 2013). In addition, Migaud et al. (2006) reported that most of the light energy is wasted in the long wavelengths such as red light, which is rapidly absorbed by water molecules. Thus, a fish can generally

detect short wavelengths such as green light better than longer wavelengths such as red light.

Light is a potent environmental factor with several informative characteristics that have a profound effect on physiological function in fish, such as in reproduction and growth. In particular, VAL-opsin is a green-sensitive pigment affected by specific spectra, and is closely associated with fish maturation (Kojima et al., 2008). Therefore, this study was investigated the correlation of VAL-opsin and maturation-related hormones in goldfish by exposing the fish to irradiation of two kinds of LED light (red and green) and a white fluorescent bulb (control), and the difference in VAL-opsin and maturation-related gene/hormone expression was investigated with and without GTH injection.

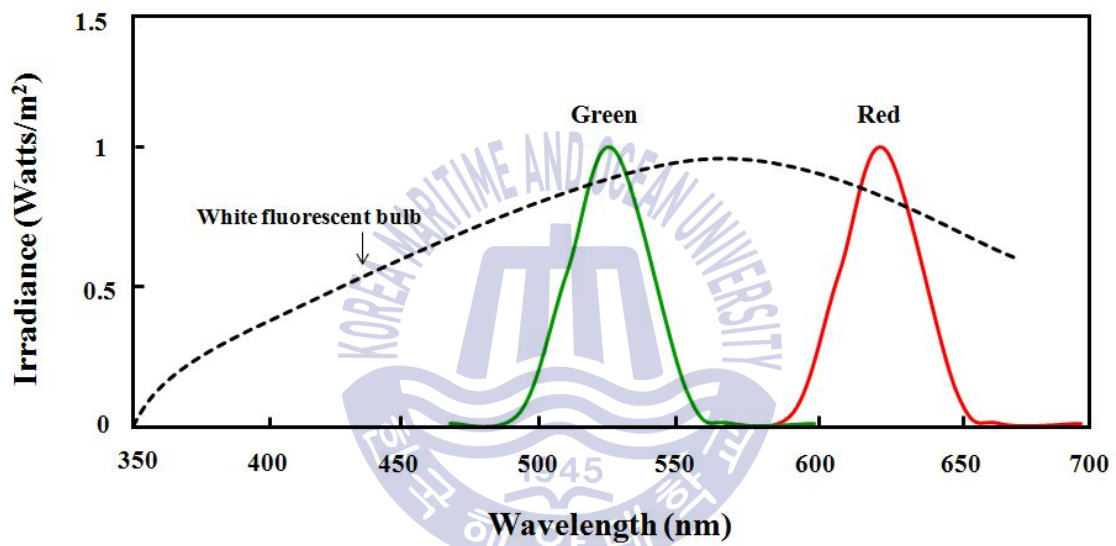


## 2. Materials and methods

### 2.1. Experimental fish and conditions

For each experiment, immature goldfish, *Carassius auratus* ( $n = 320$ , total length,  $6.1 \pm 0.5$  cm; body weight,  $12.5 \pm 0.4$  g) were purchased from a commercial aquarium (Busan, Korea) and maintained in four 300-L circulation filter tanks prior to experiments in the laboratory. The four experimental conditions were reared in duplicate and with 40 fish per tank. The goldfish were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Buchoen, Korea), with the water temperature maintained at  $22^{\circ}\text{C}$ , and were allowed to acclimate to the experimental conditions for 24 h.

The light control group was exposed to light from a white fluorescent bulb (27 W, wavelength range 350-650 nm); the light intensity at the water surface was approximately  $0.96 \text{ W/m}^2$ . The experimental groups were exposed to red (peak at 620 nm) and green (530 nm) LEDs (Daesin LED Co. Kyunggi, Korea) (Fig. 1). The fish in the control and experimental groups were exposed to a 12-h light:12-h dark photoperiod (lights on at 07:00 h and lights off at 19:00 h). The LEDs were placed 50 cm above the surface of the water, and the irradiance at the surface of the water was maintained at approximately  $0.9 \text{ W/m}^2$ . The fish were reared under these conditions with daily feeding of a commercial feed until the day prior to the sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec<sup>®</sup>, ASD, CO, USA). The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) to minimize stress prior to blood collection. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin (Acros organics, USA). Plasma samples were separated by centrifugation ( $4^{\circ}\text{C}$ ,  $10,000 \times g$ , 10 min) and stored at  $-80^{\circ}\text{C}$  until analysis. The fish were euthanized by spinal transection (first sampling at 11:00 h) at 4-h sampling intervals to collect the hypothalamus, pituitary, retina, gonads, and blood under dim light using an attenuated white fluorescent bulb.



**Fig. 1.** Spectral profiles of the red (620 nm) and green (530 nm) light-emitting diodes (LEDs) used in this study. The dotted line shows the spectral profile of white fluorescent bulb (Control).

## 2.2. GTH injection

To investigate the effects of gonadotropin (GTH) on VAL-opsin expression, the fish were anesthetized with MS-222 prior to injection. Human chorionic gonadotropin (hCG; WAKO, Osaka, Japan) was dissolved in 0.9% physiological saline, and each fish was injected with GTH (5  $\mu\text{g/g}$ , body weight [BW]) at a volume of 10  $\mu\text{L/g}$  BW. The sham group was injected with an equal volume of 0.9% physiological saline (10  $\mu\text{L/g}$  BW). The control groups are same as the control of light experiment. Each tank (each experimental group) included 40 fish. Four hours after the injection, the retina and hypothalamus samples were removed from the fish at 4-h sampling intervals for 2 days (first sampling at 11:00 h).

## 2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using the TRI reagent<sup>®</sup> (Molecular Research Center, Inc., Ohio, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 nm and 280 nm. Total RNA (2  $\mu\text{g}$ ) was reverse-transcribed in a total volume of 20  $\mu\text{L}$ , using an oligo-d(T)<sub>15</sub> anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4°C for use in polymerase chain reaction (PCR) and quantitative (q) PCR.

## 2.4. qPCR

In this study, we have considered the recommendations of the minimum Information for publication of quantitative real-time PCR experiments guidelines (Bustin et al., 2009). qPCR was conducted to determine the relative expression levels of Red opsin, Green opsin, VAL-opsin, cGnRH-II, sGnRH, GTH $\alpha$ , FSH $\beta$ , LH $\beta$ , ER $\alpha$ , ER $\beta$ 1, ER $\beta$ 2, and  $\beta$ -actin mRNA using

cDNA reverse-transcribed from the total RNA extracted from the hypothalamus, pituitary, retina, and gonads. The primers used for qPCR are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure their specificity. PCR amplification was conducted using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The qPCR program was as follows: 95°C for 5 min, followed by 50 cycles of 95°C for 20 sec and 55°C for 20 sec. Amplification of a single product from PCR was confirmed by melt curve analysis, and representative samples were electrophoresed to verify that only a single product was present. As internal controls, experiments were duplicated with  $\beta$ -actin, and all data are expressed relative to the corresponding  $\beta$ -actin threshold cycle ( $\Delta C_t$ ) levels. The calibrated  $\Delta C_t$  value ( $\Delta\Delta C_t$ ) for each sample and internal controls ( $\beta$ -actin) was calculated using the  $2^{-\Delta\Delta C_t}$  method: [ $\Delta\Delta C_t = 2^{-(\Delta C_{t\text{sample}} - \Delta C_{t\text{internal control}})$ ].

## 2.5. Western blot analysis

The fish were euthanized at 4-h sampling intervals (first sampling at 11:00 h) to collect the brain, pituitary, and gonads for western blot analysis. Total protein isolated from the brains of goldfish was extracted using a T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. A total of 25  $\mu$ g of protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad), and a protein ladder (Bio-Rad) was used for reference. Samples were electrophoresed at 180 V, and the gels were immediately transferred to a 0.2- $\mu$ m polyvinylidene difluoride membrane (Bio-Rad) at 85 V for 3 min using the Trans-Blot® Turbo™ Transfer System. Thereafter, the membranes were blocked with 5% skim milk in 0.04% Tris-buffered saline with Tween

(TTBS) for 45 min and subsequently washed in TTBS. The membranes were incubated with GnRH antibody [LRH13, a monoclonal mouse antiserum that recognizes most vertebrate GnRH forms; dilution, 1:5000; courtesy of M.K. Park (Park and Wakabayashi, 1986)], followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (dilution, 1:5,000; Bio-Rad) for 60 min. In addition, membranes were incubated with a polyclonal rabbit antibody to GTH $\alpha$  antibodies [anti-goldfish GTH $\alpha$ ; dilution, 1:4,000; courtesy of M. Kobayashi (Kobayashi et al., 2006)], followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution, 1:5,000; Bio-Rad) for 60 min. The membranes were also incubated with ER antibodies (ER $\alpha$ , dilution 1:1,000, Sigma; ER $\beta$ , dilution 1:4,000, Santa Cruz Biotech, Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated anti-rabbit ER $\alpha$  and mouse ER $\beta$  IgG secondary antibodies (dilution 1:5,000; Bio-Rad) for 60 min. The internal control was  $\beta$ -tubulin (dilution, 1:4,000; ab6046, Abcam, Cambridge, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:5,000; Bio-Rad) for 60 min. Bands were detected using a sensitive electrochemiluminescence system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager<sup>®</sup> ChemiDoc<sup>™</sup> XRS+ Systems (Bio-Rad).

## **2.6. *In vitro* culture of cells**

Culture of the goldfish brain was performed using enzymatic and mechanical procedures. The brain tissue was quickly removed at 07:00 h (lights-on time) and placed in 3 mL of ice-cold dispersion buffer (pH 7.4, Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride, containing 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2.5  $\mu$ g/mL fungizone; Gibco-BRL, Rockville, MD, USA). The isolated brain tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25%



**Table 1.** Primers used for amplification of PCR

Genes	DNA sequences
Red opsin-F	5'-CTG CCT ACT TTG CCA AGA G-3'
Red opsin-R	5'-TCG GAA CCA TCA TCT ACC TT-3'
Green opsin-F	5'-CTG TCA TCT ATG TGC TGC TAA-3'
Green opsin-R	5'-GAC TCC TCA TCG CCA AGA-3'
VAL-opsin-F	5'-CAC CAC CTG CTT CAT CTT-3'
VAL-opsin-R	5'-TCA TCA CAA CCA CCA TAC G-3'
cGnRH-II-F	5'-TTC AGA GGT TTC AGA AGA AAT CAA-3'
cGnRH-II-R	5'-GCG TCC AGC AGT ATT GTC-3'
sGnRH-F	5'-CCA ACA GAC GAG GAA GAG-3'
sGnRH-R	5'-CGA TTC AGG ACG CAA ACT-3'
GTH $\alpha$ -F	5'-TAT CGG TGG TGC TGG TTA-3'
GTH $\alpha$ -R	5'-GCT GTC CTC AAA GTC GTT A-3'
FSH $\beta$ -F	5'-CCT GGA AAG TGA GGA ATG-3'
FSH $\beta$ -R	5'-GTT CTG GTA AGA CAG CAT CA-3'
LH $\beta$ -F	5'-TGT CCT ATT CTC TGT AAT TGT CC-3'
LH $\beta$ -R	5'-GTC TCA TTA ACT GGC TCA CA-3'
ER $\alpha$ -F	5'-ACA GCC CTT CTA GCA CC-3'
ER $\alpha$ -R	5'-GCT CCT CAC ACA AAC CAA CA-3'
ER $\beta$ 1-F	5'-ACC GAC TGA CAC GAT TCT-3'
ER $\beta$ 1-R	5'-GTA ACC ACC TTA TTT CCA CTG A-3'
ER $\beta$ 2-F	5'-TCC TCA CAT CAA CAG TCC AT-3'
ER $\beta$ 2-R	5'-CAT ACA GCA GCA CCA CAT T-3'
$\beta$ -actin-F	5'-TTC CAG CCA TCC TTC CTA T-3'
$\beta$ -actin-R	5'-TAC CTC CAG ACA GCA CAG-3'



trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the brain tissues were chopped into small pieces with a pair of scissors. The brain cells and minced brain tissue were transferred to a flask and incubated for 10 min at room temperature with slow stirring. The mixture of dispersed brain cells and tissues was filtered, and the culture medium (neuro basal medium, without L-glutamine, containing 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2.5  $\mu$ g/mL fungizone, and 1% fetal bovine serum, Gibco-BRL; osmolarity adjusted to match the goldfish plasma osmolarity of 353 mOs) was added. The cell suspension was centrifuged at  $800 \times g$  for 10 min, and the cells were then resuspended in fresh culture medium.

The brain cells ( $1.2 \times 10^6$  cells/800  $\mu$ L per well) were applied to a 24-well tissue culture plate. The cell culture was started at 07:00 h and then the cells were sampled at Zeitgeber time (ZT)4, ZT8, ZT12, ZT16, ZT20, ZT24, ZT36, and ZT48. ZT4, ZT8, ZT12 and ZT36 are photophase with light. ZT16, ZT20, ZT24 and ZT48 are scotophase without light. The control groups were exposed to a white fluorescent bulb. For the experimental groups, the cells were exposed to red and green LEDs. The lights were set 50 cm above the surface of the cell culture plate; the irradiance at the surface of the plate was maintained at approximately  $0.9 \text{ W/m}^2$  with a 12-h light:dark period (lights on 07:00 h and lights off 19:00 h). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec<sup>®</sup>, ASD).

## 2.7. Plasma $17\beta$ -estradiol level

Plasma  $17\beta$ -estradiol ( $E_2$ ) levels were analyzed by the immunoassay technique using the  $E_2$  ELISA kit (Cusabio Biotech, Hubei, China).

## 2.8. 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, were used to compare differences in the data ( $P < 0.05$ ). Values are expressed as mean  $\pm$  standard error (SE).



### **3. Results**

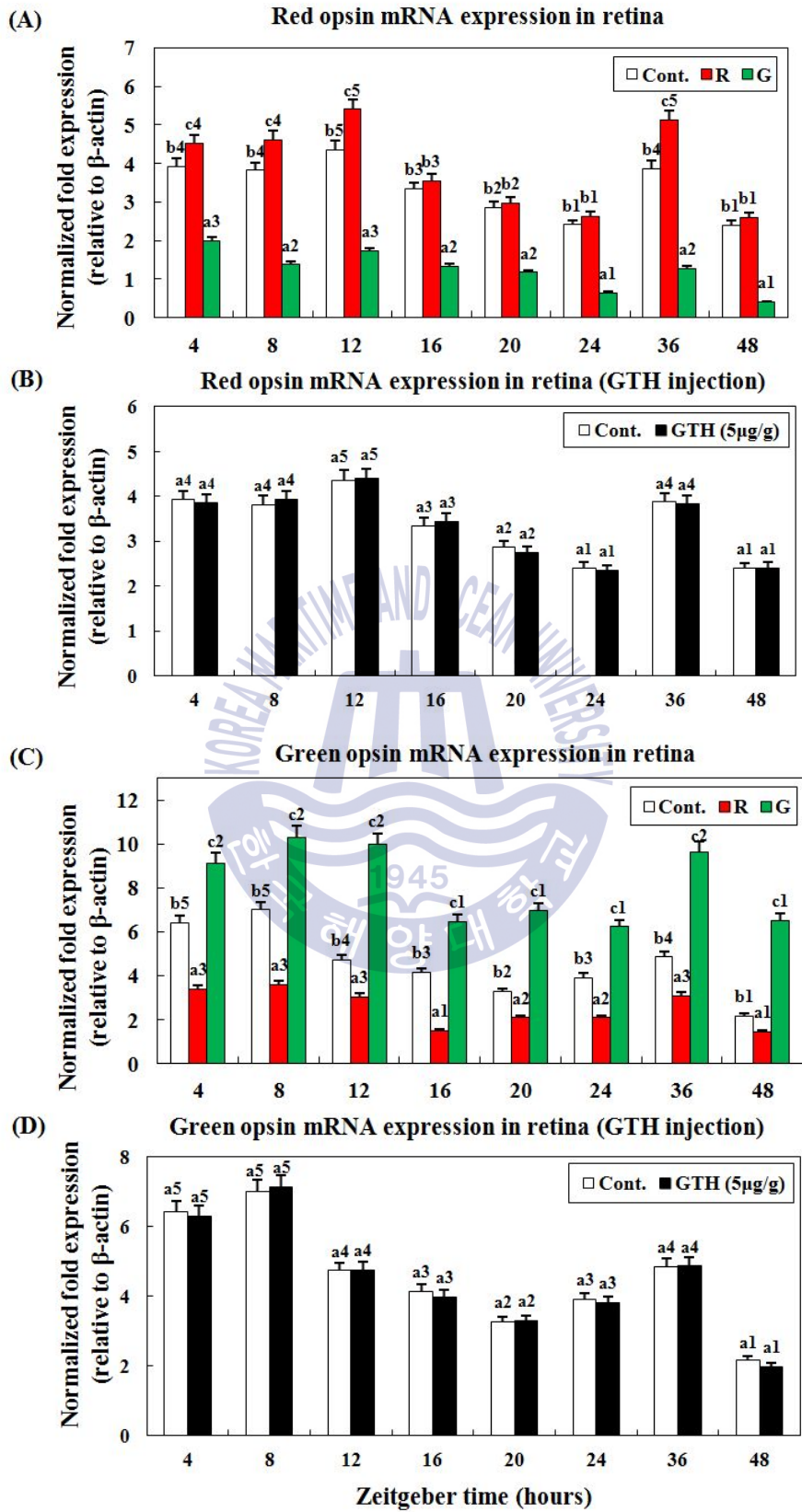
#### **3.1. Changes in Red opsin and Green opsin mRNA expression levels**

The expression of Red opsin mRNA using cDNA extracted from the goldfish, *Carassius auratus* retina in the red LED group and the expression of Green opsin mRNA using cDNA extracted from the goldfish retina in the green LED group was significantly higher compared with the other groups. These levels were particularly higher in the photophase than in the scotophase.

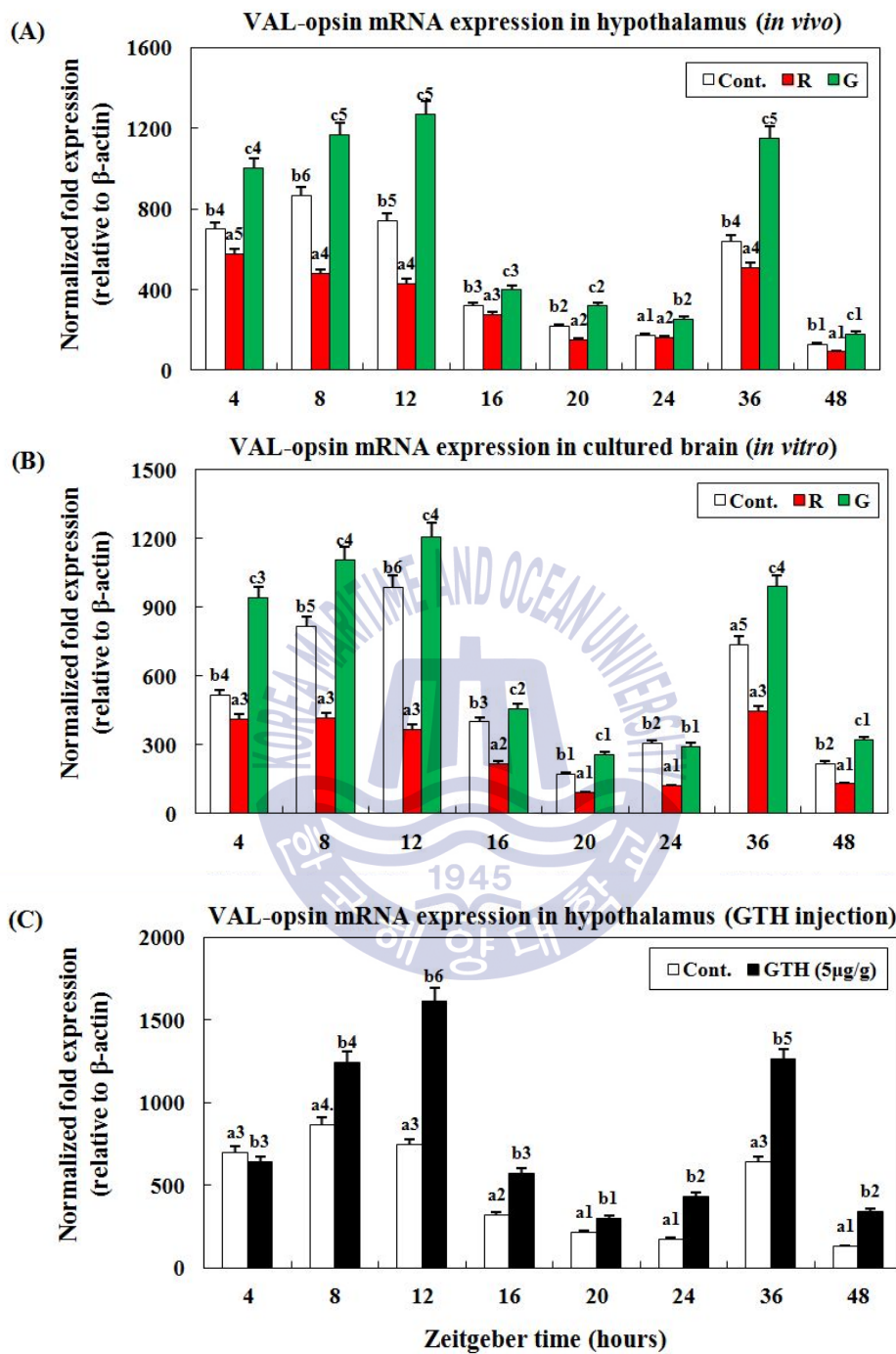
In addition, the Red opsin and Green opsin mRNA expression levels were not significantly different between the corresponding GTH injection group and non-injection group (Fig. 2).

#### **3.2. Changes in VAL-opsin mRNA expression levels**

The expression of VAL-opsin mRNA using cDNA extracted from the goldfish hypothalamus was significantly higher in the photophase than in the scotophase in the control, red, and green LED groups. In particular, the VAL-opsin expression level in the green LED group was higher compared with that of the other groups. Moreover, VAL-opsin mRNA expression levels in the GTH injection group were significantly higher than those in the non-injection group (Fig. 3).



**Fig. 2.** Changes in the expression levels of Red opsin (A) and Green opsin (C) mRNA in the retina of goldfish, *Carassius auratus*, under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control). Expression levels of Red opsin (B) and Green opsin (D) mRNA in the retina after injection of gonadotropin hormone (GTH; 5  $\mu\text{g/g}$ , body weight) under a simulated natural photoperiod (control), as measured by quantitative real-time PCR. Total retina RNA (2.0  $\mu\text{g}$ ) was reverse-transcribed and amplified. The white bar represents the photophase and the black bar represents the scotophase. Results are expressed as the normalized fold-change in expression levels with respect to the  $\beta$ -actin levels in the same sample. Different letters indicate significant differences among treatments exposed to different LED spectra or GTH injection at the same time ( $P < 0.05$ ). Different numbers indicate significant differences among time points within the same experimental treatment group (LED spectra or GTH injection status) ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).



**Fig. 3.** Changes in the expression levels of vertebrate ancient long opsin (VAL-opsin) mRNA under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control) in the hypothalamus (A), brain cell culture (in vitro) (B), and the hypothalamus (C) after gonadotropin hormone (GTH; 5  $\mu\text{g/g}$ , body weight) injection, as measured by quantitative real-time PCR in goldfish, *Carassius auratus*. Total hypothalamus and cultured brain RNA (2.0  $\mu\text{g}$ ) was reverse-transcribed and amplified. The white bar represents the photophase and the black bar represents the scotophase. Results are expressed as the normalized fold-change in expression levels with respect to the  $\beta$ -actin levels in the same sample. Different letters indicate a significant difference among treatments exposed to different LED spectra at the same time ( $P < 0.05$ ). Different numbers indicate a significant difference among time points for the same LED spectra exposure ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).



### **3.3. Changes in sGnRH and cGnRH-II mRNA and GnRH protein expression levels**

In the control group, sGnRH and cGnRH-II mRNA expression levels using cDNA extracted from the goldfish hypothalamus in the photophase at ZT36 were significantly higher than those in the other groups. However, in the scotophase, sGnRH and cGnRH-II mRNA expression levels in the green LED group were higher than those in the other groups, and expression levels in the red LED group were lower compared to the control levels (Figs. 4B and 4C).

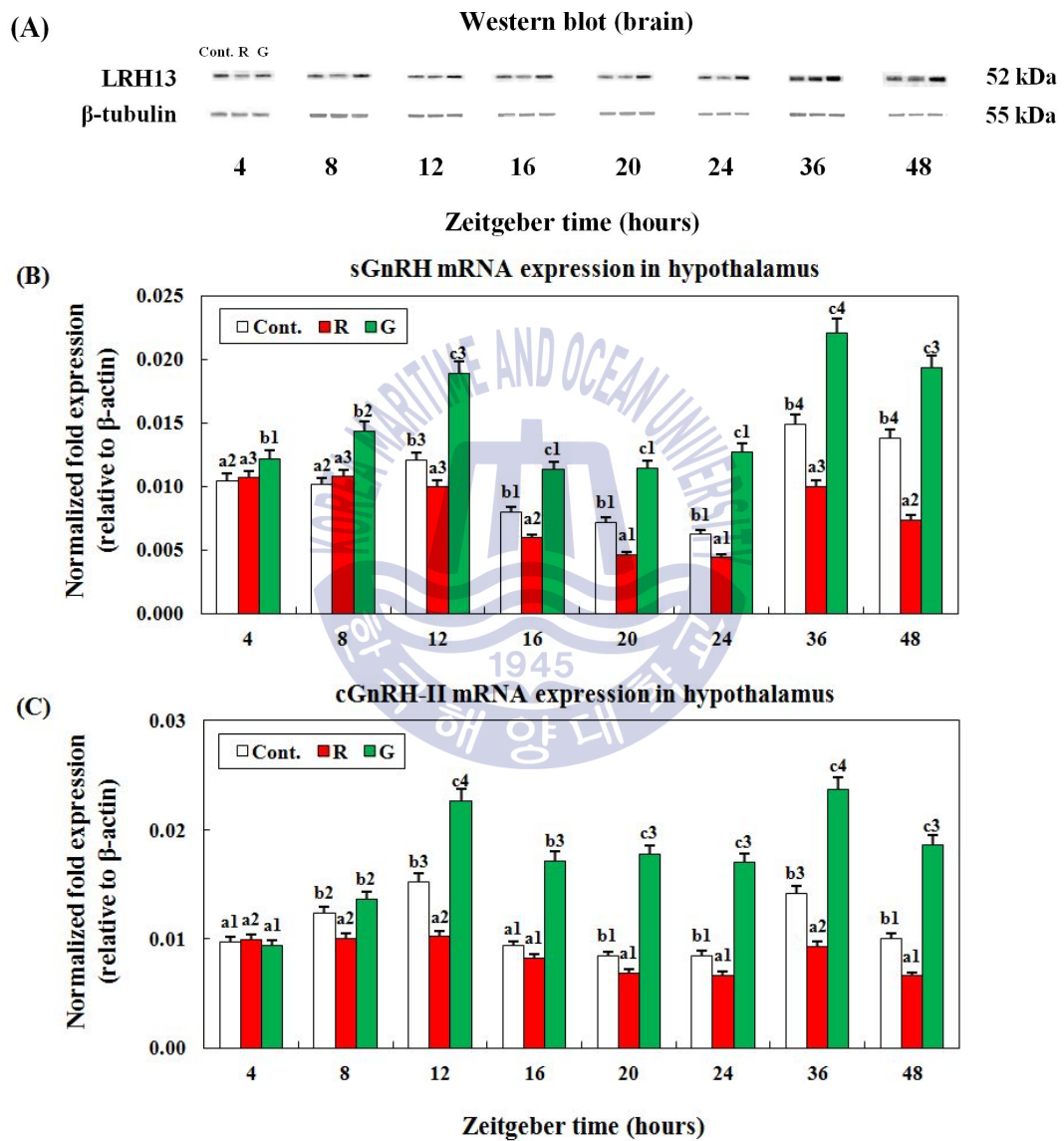
GnRH protein expression (LRH13) showed similar patterns to mRNA expression levels; in particular, levels in the green LED group were higher compared with the other groups (Fig. 4A).

### **3.4. Changes in GTH mRNA and GTH $\alpha$ protein expression levels**

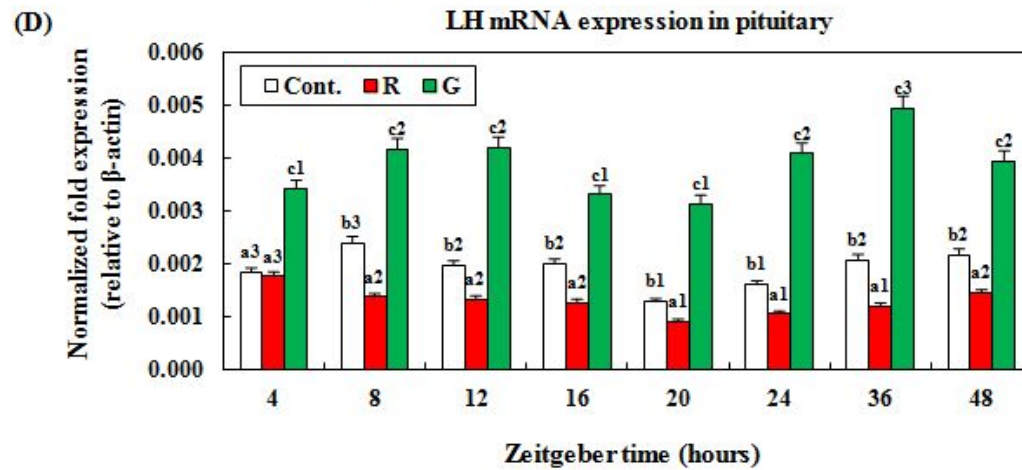
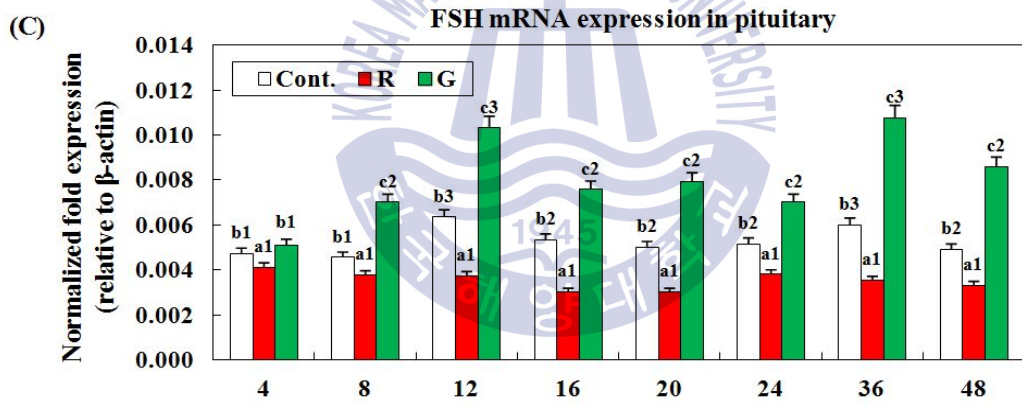
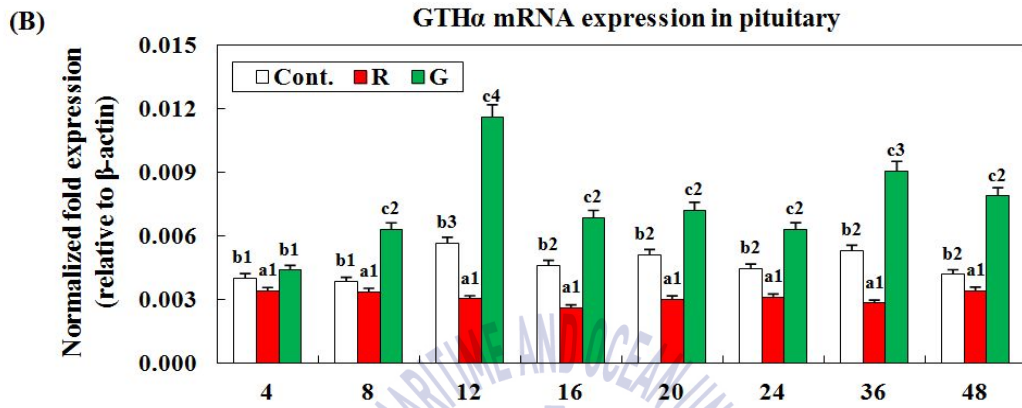
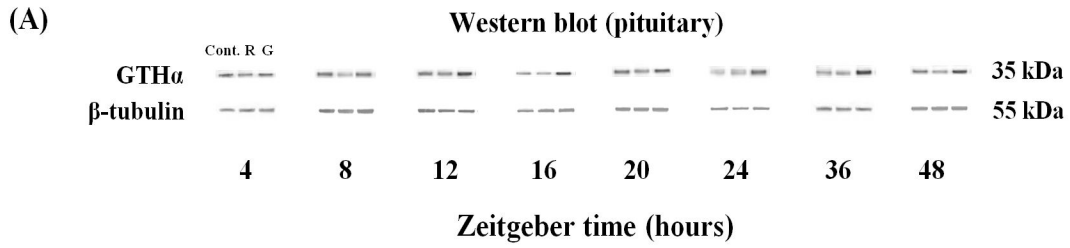
In the control group, the mRNA expression levels of GTHs (GTH $\alpha$ , LH $\beta$ , and FSH $\beta$ ) using cDNA extracted from the goldfish pituitary at ZT12 and ZT36 were significantly higher than those of the other groups. In addition, expression levels in the green LED group were higher compared with those in the other groups (Figs. 5B, 5C, and 5D).

GTH $\alpha$  protein expression was similar to the mRNA expression levels; in particular, levels in the green LED group were higher compared with those in the other groups (Fig. 5A).





**Fig. 4.** Changes in the expression levels of gonadotropin-releasing hormone (GnRH) protein (anti-goldfish LRH13; a polyclonal rabbit antibody of 52 kDa) (A), and sGnRH (B) and cGnRH-II (C) mRNA in the hypothalamus under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control) (B and C), as measured by quantitative real-time PCR in goldfish, *Carassius auratus*. Total hypothalamus RNA (2.0  $\mu\text{g}$ ) was reverse-transcribed and amplified. The white bar represents the photophase and the black bar represents the scotophase. Results are expressed as the normalized fold-change in expression levels with respect to the  $\beta$ -actin levels in the same sample. Different letters indicate a significant difference among treatments exposed to different LED spectra at the same time ( $P < 0.05$ ). Different numbers indicate a significant difference among times within the same LED spectra exposure group ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).



**Fig. 5.** Changes in the expression levels of gonadotropin hormone-alpha (GTH $\alpha$ ) protein (anti-goldfish GTH $\alpha$ ; a polyclonal rabbit antibody of 35 kDa) (A), and GTH $\alpha$  (B), FSH $\beta$  (C), and LH $\beta$  (D) mRNA expression in the pituitary under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control), as measured by quantitative real-time PCR. Total pituitary RNA (2.0  $\mu$ g) was reverse-transcribed and amplified. The white bar represents the photophase and the black bar represents the scotophase. Results are expressed as the normalized fold-change in expression levels with respect to the  $\beta$ -actin levels in the same sample. Different letters indicate a significant difference among treatments exposed to different LED spectra at the same time ( $P < 0.05$ ). Different numbers indicate a significant difference among time points within the same LED spectra exposure group ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).

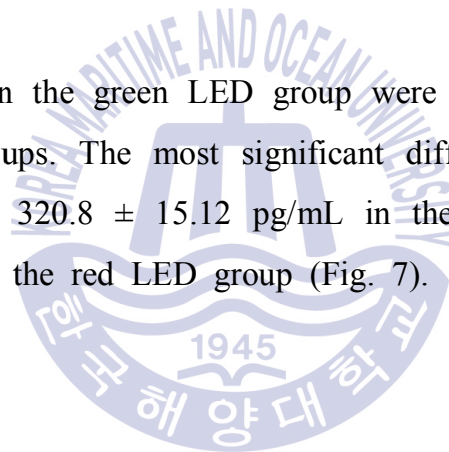
### **3.5. Changes in ER $\alpha$ , ER $\beta$ 1, and ER $\beta$ 2 mRNA and ER protein expression levels**

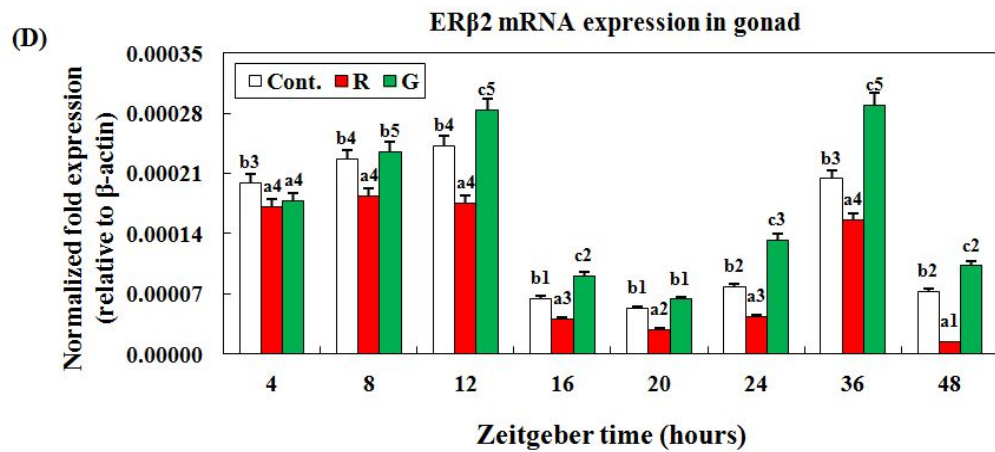
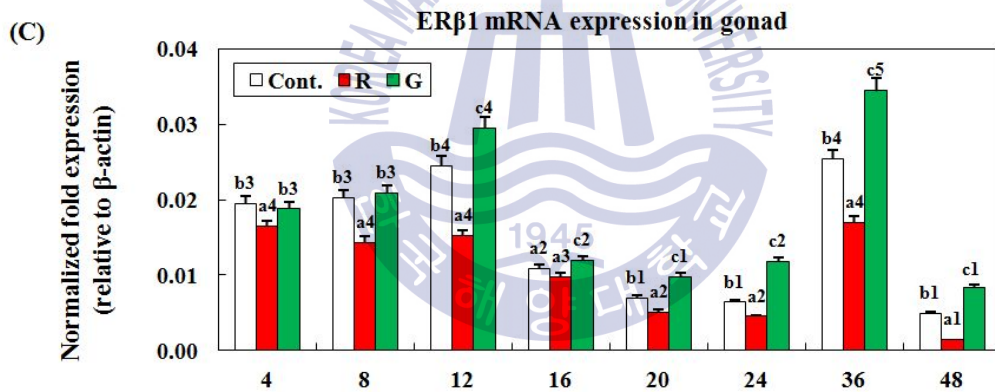
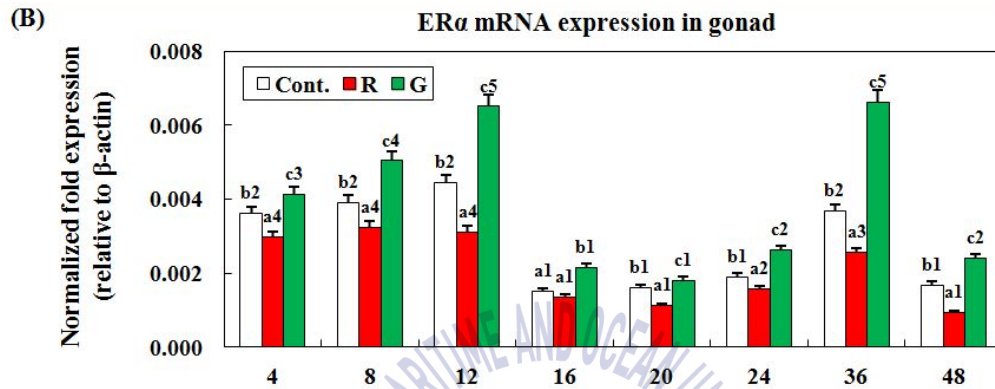
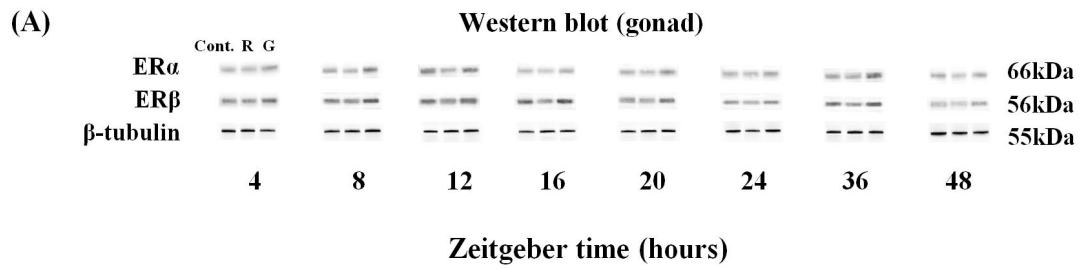
In the control group, the mRNA expression levels of ERs (ER $\alpha$ , ER $\beta$ 1, ER $\beta$ 2) using cDNA extracted from the goldfish gonads at ZT12 and ZT36 were significantly higher than those of the other groups. In addition, expression levels in the green LED group were higher compared with those in the other groups (Figs. 6B, 6C, and 6D).

Protein levels of ERs (ER $\alpha$ , ER $\beta$ ) showed a similar trend to mRNA expression levels; in particular, expression levels in the green LED group were higher compared with those of the other groups (Fig. 6A).

### **3.6. Plasma E<sub>2</sub> levels**

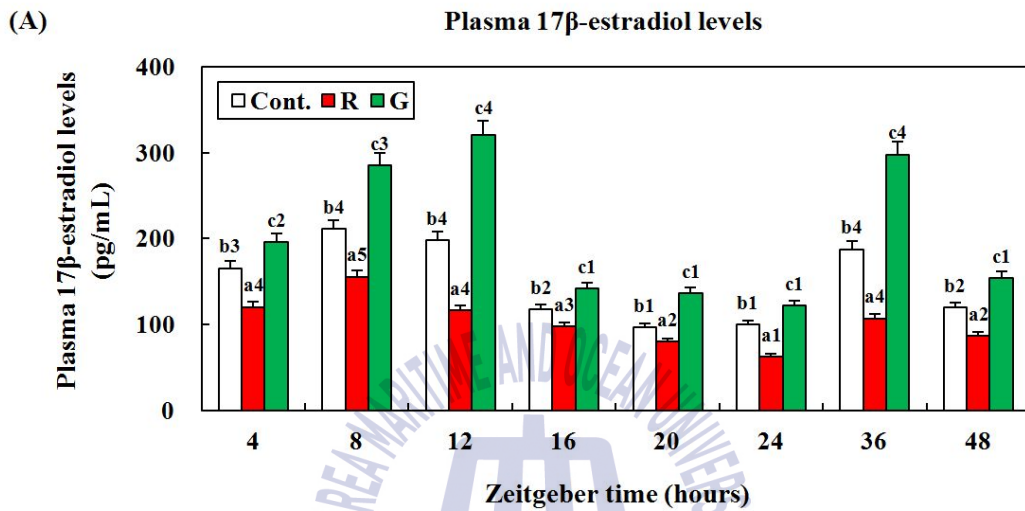
Plasma E<sub>2</sub> levels in the green LED group were significantly higher than those of the other groups. The most significant difference was observed at ZT12, with a level of  $320.8 \pm 15.12$  pg/mL in the green LED group and  $116.8 \pm 5.21$  pg/mL in the red LED group (Fig. 7).





**Fig. 6.** Changes in the protein expression levels of estrogen receptors (ER $\alpha$  and ER $\beta$ ) (A), and ER $\alpha$  (B), ER $\beta$ 1 (C), and ER $\beta$ 2 (D) mRNA expression in the gonad under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control), as measured by quantitative real-time PCR in goldfish, *Carassius auratus*. Total gonad RNA (2.0  $\mu$ g) was reverse-transcribed and amplified. The white bar represents the photophase and the black bar represents the scotophase. Results are expressed as the normalized fold-change in expression levels with respect to the  $\beta$ -actin levels in the same sample. Different letters indicate a significant difference among treatments exposed to different LED spectra at the same time ( $P < 0.05$ ). Different numbers indicate a significant difference among time points within the same LED spectra exposure group ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).





**Fig. 7.** Changes in the levels of plasma 17 $\beta$ -estradiol (E<sub>2</sub>) in goldfish, *Carassius auratus* under red (R) and green (G) light-emitting diodes (LEDs) and a white fluorescent bulb (control), as measured by an enzyme-linked immunoassay. Different letters indicate a significant difference among treatments exposed to different LED spectra at the same time ( $P < 0.05$ ). Different numbers indicate a significant difference among time points within the same LED spectra group ( $P < 0.05$ ). All values are means  $\pm$  SD ( $n = 5$ ).



## 4. Discussion

To investigate the effects of LED spectra on the sexual maturation of goldfish, *Carassius auratus*, as produced by different LED wavelengths (red and green), this study was examined the mRNA and protein expression levels of Red opsin, Green opsin, VAL-opsin, maturation-related genes (GnRHs, GTHs, ERs), and plasma E<sub>2</sub> levels. Furthermore, it was investigated the effects of exogenous GTH on VAL-opsin expression.

First, the goldfish were irradiated by white (control), red, and green LED, and the expression levels of Red opsin, Green opsin, and VAL-opsin were evaluated. Red opsin mRNA expression was highest in the red LED group, and Green opsin mRNA expression was highest in the green LED group compared to the other groups. The VAL-opsin mRNA expression level in the green LED spectra group was significantly higher than that in the other groups, and was significantly lower in the red LED spectra group than the control group. These results are in agreement with a previous report by Kojima et al. (2000), which demonstrated variety LED spectra, but a significant increase in VAL-opsin was detected in fish exposed to green LED spectra.

In addition, the expression levels of opsin genes over time were investigated in goldfish irradiated with white (control), red, and green LEDs. The three kinds of opsin genes investigated were found to increase in the photophase. These results are in agreement with a previous report by Li et al. (2005), which demonstrated that Red opsin mRNA expression levels in the photophase were significantly higher than those in the scotophase in zebrafish. Similarly, Moore et al. (2014) demonstrated that VAL-opsin mRNA expression levels in the photophase were significantly higher than those in the scotophase in zebrafish. In this study, VAL-opsin was highly expressed in the photophase, thereby confirming that this protein is sensitively controlled by light.

This study was further examined the expression of opsin genes after 5  $\mu$  g/g GTH injection to investigate the association between opsin and the expression of GnRHs (sGnRH, cGnRH-II mRNA) and GTHs (GTH $\alpha$ , LH $\beta$ , and FSH $\beta$ ) hormones secreted by the HPG axis, over time. The mRNA expression levels of Red and Green opsin were not significantly different between the GTH injection group and non-injection group. However, VAL-opsin mRNA expression in the GTH injection group was significantly higher than that in the non-injection group. This shows that between Green opsin and VAL-opsin sensitive to green light spectra, VAL-opsin is related to maturation-related hormones. The present results are in agreement with a previous report by Halford et al. (2009), which demonstrated that VAL-opsin mRNA expression levels increased in association with extension of the length of the day in chicken. Increased VAL-opsin stimulates hypothalamic GnRH neurons, and promotes the secretion of GnRH by activating the HPG axis (Halford et al., 2009). These effects were suggested to promote the maturation of the chicken by increasing VAL-opsin expression. In this study, the increase in VAL-opsin expression in GTH-treated in goldfish indicated an interaction between VAL-opsin and GTH.

This study was further investigated the change in mRNA and protein levels of GTHs and GnRHs over time in fish exposed to red and green LED spectra. GTH and GnRH mRNA and protein expression levels were significantly higher at ZT12 and ZT36 than at other time points. These results are in agreement with a previous report by Chai et al. (2013), which demonstrated that GnRH mRNA expression levels of orange-spotted grouper, *Epinephelus coioides* were significantly higher in the photophase than scotophase. In addition, the present results are in agreement with a previous report by Karigo et al. (2013), which showed that light stimulation increased the synthesis and secretion of GnRH, leading to HPG axis activation to promote maturation. In the present study, GTH and GnRH mRNA and protein expression levels were significantly higher in the green LED groups

than the other groups, and those in the red LED group were significantly lower than the control levels. Similarly, the mRNA expression of ERs and the plasma E<sub>2</sub> levels were significantly higher in the green LED groups than the other groups. The present results are in agreement with those of a previous report by Shin et al. (2014a, b), which demonstrated that goldfish bred for 4 months under different LED wavelengths had significantly higher GnRH and GTH mRNA expression levels in the green LED groups than in the other groups, and the levels in the red LED groups were significantly lower than those in the other groups. Our results are also in agreement with a previous report by Choi et al. (2015), who used the long-after glow phosphorescent pigment (luminous sheet) to emit green spectra, which accounts for both extended light conditions as well as green light, and found that mRNA expression levels of GTHs and ERs of yellowtail damselfish were increased and could promote sexual maturation.

In summary, this study was suggested that fish maturation is associated with light spectra in terms of the molecular mechanism and physiological response. Our findings support the hypothesis that VAL-opsin sensitively reacts to green spectra in goldfish. Furthermore, VAL-opsin and maturation-related hormones may interact to affect fish maturation. However, studies on the effect of wavelength intensity on fish.

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