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The environmental regulation of maturation in goldfish, *Carassius auratus*: Effects of various LED light spectra

Hyun Suk Shin [a], Hamid R. Habibi [b,*], Cheol Young Choi [a,**]

[a] Division of Marine Environment & BioScience, Korea Maritime and Ocean University, Busan 606-791, Republic of Korea
[b] Department of Biological Sciences, University of Calgary, 2500 University Drive N.W. Calgary, Alberta T3B 2V4, Canada

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ABSTRACT

While there have been a number of studies on the effects of photoperiod and duration of light and dark exposure, much less information is available on the importance of light intensity. This study investigated the effects of exposure of goldfish, *Carassius auratus* exposed to white fluorescent bulbs, and red (peak at 630 nm), and green (530 nm) light emitting diodes (LEDs) at approximately 0.9 W/m^2 (12-h light:12-h dark) for four months on a number of hormones of the hypothalamus–pituitary–gonad (HPG) axis, *in vivo* and *in vitro*. We investigated the effects of native GnRH molecules (gonadotropin-releasing hormones; salmon GnRH, sGnRH; and chicken GnRH-II, cGnRH-II), gonadotropin hormones (GTHα; follicle-stimulating hormone, FSH-β; luteinizing hormone, LH-β2), kisspeptin 1 (Kiss1) and G protein-coupled receptor 54 (GPR54) mRNA levels. Furthermore, we measured LH and 17α-hydroxypregnenolone levels in plasma and we performed gonad histological observations. GnRHs, Kiss1, GPR54 and GTH mRNA and plasma LH and 17α-hydroxypregnenolone levels in the *in vivo* and *in vitro* groups exposed to green LEDs were significantly higher than the other groups. Histological analysis revealed the presence of oocytes in the yolk stage in fish exposed to green light. These results suggest that green wavelengths regulate the HPG axis and enhance sexual maturation in goldfish.

1. Introduction

Sexual development and gonadal maturation in fish are regulated by hormones of the hypothalamus–pituitary–gonad (HPG) axis including gonadotropin-releasing hormone (GnRH) and other neurohormones, gonadotropins, and gonadal steroid hormones and peptides (Lee et al., 2001).

Light is a potent environmental factor with several informative characteristics – quality (spectra or wavelength), quantity (intensity), and periodicity (photoperiod) – that have a profound effect on the physiological function in fish (Boeuf and Le Bail, 1999). Among these characteristics, periodicity is a crucial determinant of reproductive success in fish and its importance in the initiation and termination of gonadal development has been studied extensively (de Vlaming, 1975; Bromage et al., 2001; Pankhurst and Porter, 2003).

A precise balance in hormones of HPG is possible because of positive and negative feedback mechanisms. Estrogen is one of the key gonadal hormones that regulates GnRH production *via* brain Kisspeptins (Kiss) thereby regulates gametogenesis and spawn time (Funes et al., 2003; Seminara et al., 2003; Colledge, 2009). There are two types of Kiss isoforms present in the brain of teleosts and other vertebrate species (Lee et al., 2009; Um et al., 2010). Kiss1 is an important neuroendocrine factor that regulates sexual maturation in teleosts and other species (Colledge, 2009; Roa et al., 2011). Kiss 1 has been cloned in fish models (Biran et al., 2008), and its involvement in gonadotropin release (Felip et al., 2009) and signaling of sexual maturation/puberty (Filby et al., 2008) has been confirmed (e. g., medaka and zebrafish). The two paralogous genes of kisspeptin (Kiss1 and Kiss2) have been reported in a number of teleost species including zebrafish, *Danio rerio* (Biran et al., 2008), medaka, *Oryzias latipes* (Kanda et al., 2008), goldfish, *Carassius auratus* (Li et al., 2009) and European seabass, *Dicentrarchus labrax* (Felip et al., 2009). Kiss 1 regulates GnRH expression *via* specific receptors known as GPR54 (Roa et al., 2011). The Kiss1–GPR54 signal system is one of the circuits regulating reproduction by controlling GnRH secretion from the hypothalamus. As in other vertebrates, GnRH is a key regulator of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production, and plays an important role in the control of reproduction in teleosts (van der Kraak et al., 1998; Habibi and Andreu-Vieyra, 2007). LH and FSH, in turn stimulate the maturation of the ovary and testis and induce the synthesis of gonadal hormones (Andrews et al., 1988). LH and FSH stimulate conversion of cholesterol to 17α-hydroxypregnenolone which is converted to the main gonadal steroids, estradiol and testosterone (Yamato et al., 2010).

It is well established that reproductive cycle in fish is regulated by environmental cues, including temperature and photoperiod. The reproductive physiology of fish is influenced by the perception of

* Corresponding author. Tel.: +1 403 220 5270; fax: +1 403 289 9311.
** Corresponding author. Tel.: +82 51 410 4756; fax: +82 51 404 4750.
E-mail addresses: habibi@ucalgary.ca (H.R. Habibi), choic@kmou.ac.kr (C.Y. Choi).

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environmental factors *via* the sensory systems and the transduction of suitable signals (Bromage et al., 2001; Pankhurst and Porter, 2003). Wavelength, intensity and photoperiod have been shown to influence the physiological function of fish (Boeuf and Le Bail, 1999). While there is considerable information on the effect of photoperiod on reproduction (de Vlaming, 1975; Bromage et al., 2001; Pankhurst and Porter, 2003), much less information is available on the importance of light wavelengths and light intensity on the regulation of sexual maturation.

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 friendly 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 (Loew and McFarland, 1990; Migaud et al., 2006).

Light-emitting diodes (LEDs), which is a new form of lighting technology can be designed to output specific wavelengths (Migaud et al., 2007) such as narrow bandwidth light which is important because it can be tuned to the environmental sensitivity of a target species (Villamizar et al., 2009). There is evidence that the spectral composition of incidental light is differentially affected in underwater environments, and rapid attenuation occurs with increasing depth (Lythgoe, 1979).

In the present study we investigated the relationship between specific LED wavelengths and sexual maturation and physiological regulation mechanism affecting fish sexual maturation and development. Fish were reared for 4 months under a fluorescent bulb and 2 LED wavelengths (red and green). Changes in the expression of endogenous GnRH molecular forms, Kiss1, Kiss receptor GPR54, LH-β2 and FSH-β subunits were investigated. In addition, changes in plasma LH and 17α-hydroxypregnenolone concentrations were investigated.

2. Materials and methods

2.1. Experimental fish and conditions

For each experiment, common goldfish (n = 65; length, 6.1 ± 0.5 cm; mass, 12.5 ± 0.4 g) were purchased from the commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in three 300-L circulation filter tanks in the laboratory. Each tank (each experimental group) contained 15 fish. The control fish were exposed to a white fluorescent bulb (27 W), and the light intensity near the water surface of the tanks was approximately 0.96 W/m^2. The water temperature and photoperiod were 20 ± 1 °C and a 12-h light (L):12-h dark (D) period (lights on 07:00 h and lights off 19:00 h), respectively. The fish were fed a commercial feed twice daily (09:00 h and 17:00 h). For the three experimental groups, the fish were exposed to either red (peak at 630 nm) or green (530 nm) LEDs (Daesin LED Co. Kyunggi, Korea) in addition to a white fluorescent bulb (Control). The LEDs were placed 40 cm above the surface of water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2, and average light intensities from the difference light sources in the water tanks were indicated to Table 1. The fish were reared under these conditions with a daily feeding of commercial feed until the day prior to the sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD, CO, USA). The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) to minimize stress prior to blood collection. Blood was collected rapidly 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 euthanized by spinal transection at 2-month sampling intervals (*i.e.*, at 2, 4 and 6 months) to collect the brain and blood under dim white light using an attenuated white fluorescent bulb.

Table 1
Average light intensities (W/m^2) from the different light sources in the water tanks.

Water depth	White fluorescent bulb	Red LED	Green LED
Surface (0 cm)	0.96	0.96	0.96
Middle (30 cm)	0.74	0.72	0.88
Bottom (60 cm)	0.63	0.60	0.77

2.2. In vitro culture of hypothalamus cells

The culture of goldfish hypothalamus neurons was performed using enzymatic and mechanical procedures. Hypothalamus tissue was quickly removed 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 μg/mL streptomycin and 2.5 μg/mL fungizone; GIBCOBRL, Rockville, MD, USA). The isolated hypothalamus tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma). The connective tissues and other impurities were removed, and the hypothalamus tissues were chopped into small pieces with a pair of scissors. Hypothalamus cells and the minced hypothalamus tissue were transferred to a flask and incubated for 10 min at room temperature with slow stirring. The mixture of dispersed hypothalamus cells and tissues was filtered, and the culture medium (neurobasal medium, without L-glutamine, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 1% fetal bovine serum, Gibco-BRL; to adjust the medium's osmolarity to goldfish plasma osmolarity, 353 mOs) was added. The cell suspension was centrifuged at 800 *g* for 10 min, and the cells were then resuspended in fresh culture medium. Hypothalamus cells (1.2 × 106 cells/800 μL/well) were applied to a 24-well tissue culture plate, which was started at 15:00 h and sampled at 6-, (21:00 h), 12- (03:00 h), 24- (15:00 h) and 48-hour (15:00 h) intervals. Fresh culture medium (600 μL) was added to the culture wells at 24 h, 27 °C and 0.5% CO_2.

For the experimental groups, hypothalamus cells were exposed to red (peak at 630 nm) and green (530 nm) LEDs (Daesin LED Co. Kyunggi, Korea) in addition to a white fluorescent bulb (Cont.). The LEDs were set 50 cm above the surface of the cell culture plate, and the irradiance at the surface of the plate was maintained at approximately 0.9 W/m^2, and a 12-h light (L):12-h dark (D) period (lights on 07:00 h and lights off 19:00 h). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD), and the temperature was maintained using heat prevent system of LEDs.

Table 2
Primers used for QPCR amplification.

Genes (accession no.)	Primer	DNA sequences
cGnRH-II	Forward	5′-TTC AGA GGT TTC AGA AGA AAT CAA-3′
(U40567)	Reverse	5′-GCG TCC AGC AGT ATT GTC-3′
sGnRH	Forward	5′-CCA ACA GAC GAG GAA GAG-3′
(U30301)	Reverse	5′-CGA TTC AGG ACG CAA ACT-3′
Kiss1	Forward	5′-TGA ACC TAC TTA CCA TAA TTT TGA TG-3′
(FJ236327)	Reverse	5′-CCTGAG ACC CTG GAG TGA-3′
GPR54	Forward	5′-AGT GGT CAT TGT TGT TCT CTT-3′
(FJ465139)	Reverse	5′-AGG AGT TGG CAT AGG ACA T-3′
GTHα	Forward	5′-TAT CGG TGG TGC TGG TTA-3′
(D86552)	Reverse	5′-GCT GTC CTC AAA GTC GTT A-3′
FSH-β	Forward	5′-CCT GGA AAG TGA GGA ATG-3′
(D88023)	Reverse	5′GTT CTG GTA AGA CAG CAT CA-3′
LH-β2	Forward	5′-TGT CCT ATT CTC TGT AAT TGT CC-3′
(D88024)	Reverse	5′-GTC TCA TTA ACT GGC TCA CA-3′
β-actin	Forward	5′-TTC CAG CCA TCC TTC CTA T-3′
(AB039726)	Reverse	5′-TAC CTC CAG ACA GCA CAG-3′

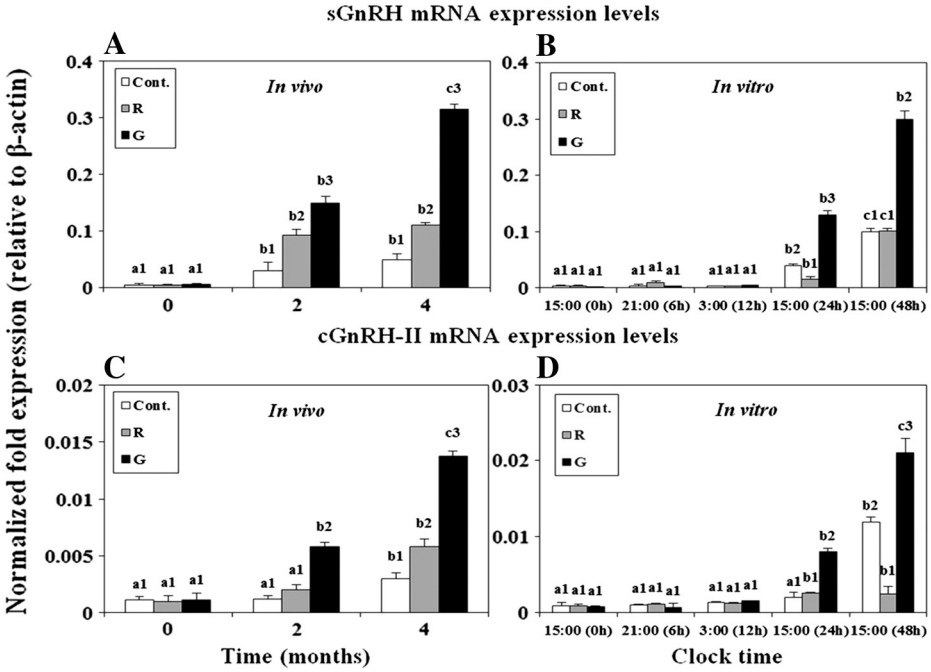

Fig. 1. Changes in the expression levels of sGnRH (A) and cGnRH-II (C) mRNA in the hypothalamus, and sGnRH (B) and cGnRH-II (D) in the hypothalamus cells in goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by quantitative real-time PCR. Total RNA (2.5 μg) from the hypothalamus and hypothalamus cells was reverse-transcribed and amplified. The results are expressed as normalized fold expression levels with respect to the β-actin levels in the same sample. Values with different characters are significantly different at different time points (months or hours) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

2.3. In vitro culture of pituitary cells

After the fish were anesthetized, the pituitary was dissected and placed in ice-cold medium (pH 7.5) composed of 25 mM HEPES, 4 mM $NaHCO_3$, 0.3% bovine serum albumin (BSA), 0.1% collagenase, 0.25 mg/mL fungizone and RPMI medium containing antibiotics (100 U/L penicillin and 100 mg/L streptomycin; Penicillin-Streptomycin, Gibco, USA). The pituitary cut by scalpel into 1–3 mm^3 pieces was weighed, placed in a 24-well culture plate (SPL Life Science, Korea) containing 1 mL of medium and incubated at 20 ± 1 °C in an incubator for 1 day. For the experimental groups, pituitary cells were exposed to red (peak at 630 nm) and green (530 nm) LEDs (Daesin LED Co. Kyunggi, Korea) in addition to a white fluorescent bulb (Cont.). The LEDs were set 50 cm above the surface of the cell culture plate, and the irradiance at the surface of the plate was maintained at approximately 0.9 W/m^2, and a 12-h light (L):12-h dark (D) period (lights on 07:00 h and lights off 19:00 h). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®), and the temperature was maintained using heat prevent system of LEDs.

The cultured pituitary was sampled at 6-, (21:00 h), 12- (03:00 h), 24- (15:00 h) and 48-h (15:00 h) intervals, and each sample was centrifuged (18 °C, 800 *g*, 10 min) to separate the cultured pituitary. The cell was stored at −80 °C until RNA extraction.

2.4. Real time quantitative PCR (RT-qPCR)

To carry out this study we have considered the recommendations of the MIQE (Minimum Information for publication of quantitative real-time PCR Experiments) guidelines (Bustin et al., 2009). Total RNA was extracted from the hypothalamus and pituitary using a TRIzol kit (Gibco/BRL, USA) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. RT-

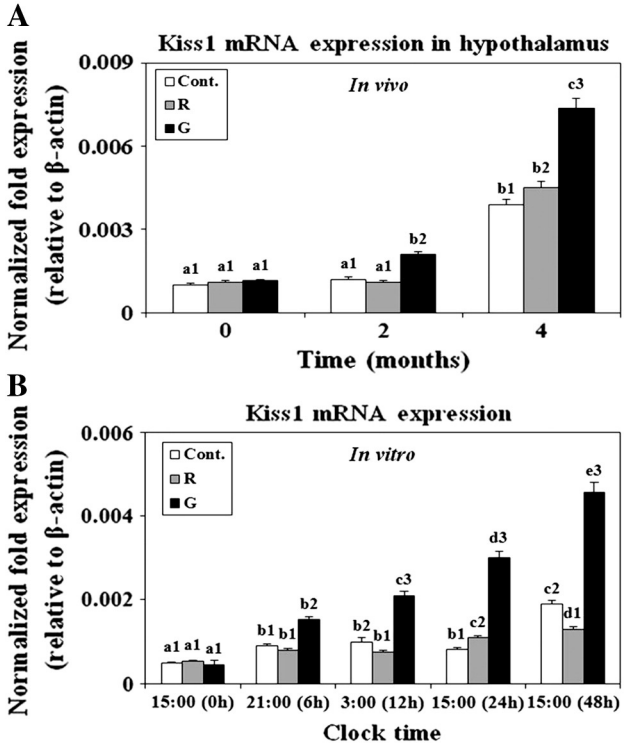

Fig. 2. Changes in the expression levels of Kiss1 mRNA in the hypothalamus (A) and in the hypothalamus cells (B) in goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by quantitative real-time PCR. Values with different characters are significantly different at different time points (months or hours) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative expression levels of sGnRH (GenBank accession no. U30301), cGnRH-II (U40567), Kiss1 (FJ236327), GPR54 (FJ465139), GTHα (D86552), FSH-β (D88023), LH-β2 (D88024) and β-actin (AB039726) mRNA using total RNA extracted from the hypothalamus and pituitary. The primers used for QPCR are shown in Table 2. 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 the specificity of primers. The PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The RT-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 all data were expressed relative to the corresponding β-actin calculated threshold cycle (ΔCt) levels. The efficiencies were found to be 95.0% for β-actin, 94.9% for sGnRH, 96.5% for cGnRH-II, 95.1% for Kiss1, 96.0% for GPR54, 95.4% for GTHα, 95.5% for FSH-β, and 96.2% for LH-β2. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using the $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = 2\hat{}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$] (Livak and Schmittgen, 2001).

2.5. Western blotting

Total protein was extracted from the pituitary of goldfish using a protein extraction buffer (5.6 mM Tris, 0.55 mM ethylenediaminetetraacetic acid (EDTA), 0.55 mM ethylene glycol tetraacetic acid (EGTA), 0.1% sodium dodecyl sulfate (SDS), 0.15 mg/mL phenylmethylsulfonyl fluoride and 0.15 mg/mL leupeptin). It was then sonicated and quantified using the Bradford method (Bio-Rad). Total protein (30 μg per lane) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel, and a protein ladder (Fermentas, Hanover MD, USA) was used for reference. Samples were electrophoresed at 80 V through the stacking gel and at 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 difluoride membrane (Bio-Rad) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min, after which they were washed in TBS. The membranes were incubated with GTHα antibodies (antigoldfish GTHα; a polyclonal rabbit antibody; dilution, 1:2000; courtesy of Kobayashi [Kobayashi et al., 2006]), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:5000; Bio-Rad, 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 antibodies (1:5000; Bio-Rad) for 60 min. Bands were detected using the sensitive electrochemiluminescence (ECL) systems (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDocTM XRS + Systems (Bio-Rad).

2.6. Plasma parameter analysis

Plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min), and plasma LH and 17α-hydroxypregnenolone levels were analyzed using the immunoassay technique with the ELISA kits E0830f, E0441f (EIAab Science, Wuhan, China) and CSB-EQ027292FI (Cusabio Biotech, Hubei, China), respectively. An anti-antibody that was specific to the antibody of the LH was pre-coated onto a microplate, following which 50 μL of plasma, 50 μL of HRP-conjugate, and 50 μL of the antibody were added to each well. These were mixed well and then incubated for 2 h at 37 °C. Following the last wash, any remaining Wash Buffer was aspirated or decanted off, and 50 μL each of substrates A and B were added to each well. These substrate solutions were then incubated for 15 min at 37 °C in the dark, during which they changed from colorless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in the color changing from blue to yellow. The optical density of the solution in each well was then determined within 10 min, using a microplate reader set to 450 nm. The following standard curve concentrations were used for the ELISA: LH — 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mIU/mL.

2.7. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA) (McCullough, 1999). Two-way ANOVA followed by Tukey's *post hoc* test was used to assess statistically significant differences among different time points and different light spectra. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of sGnRH and cGnRH-II mRNA in the hypothalamus (in vivo) and hypothalamus cells (in vitro)

We initially investigated the effects of different light spectra on the sGnRH and cGnRH-II mRNA levels in the hypothalamus and hypothalamus cells (Fig. 1). Hypothalamic sGnRH and cGnRH-II mRNA levels in the hypothalamus (*in vivo*) were significantly increased in a time-related manner up to 4 months exposure to red and green LEDs compared to white fluorescent bulb (Cont.), differences were particularly significant after 4 month exposure *in vivo* (Fig. 1A, C).

In the cultured cells, exposure to green LED significantly increased sGnRH and cGnRHA-II mRNA levels in hypothalamus cells, *in vitro* after 24 and 48 h compared to control indicating direct action of neuronal cells (Fig. 1B, D). In comparison exposure to red LED was less effective.

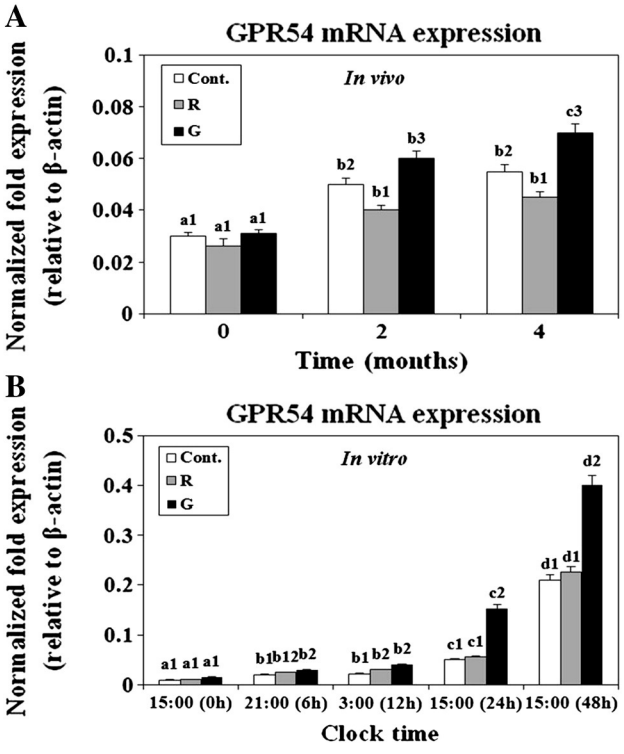

Fig. 3. Changes in the expression levels of GPR54 mRNA in the hypothalamus (A) and in hypothalamus cells (B) in goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by quantitative real-time PCR. Values with different characters are significantly different at different time points (months or hours) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

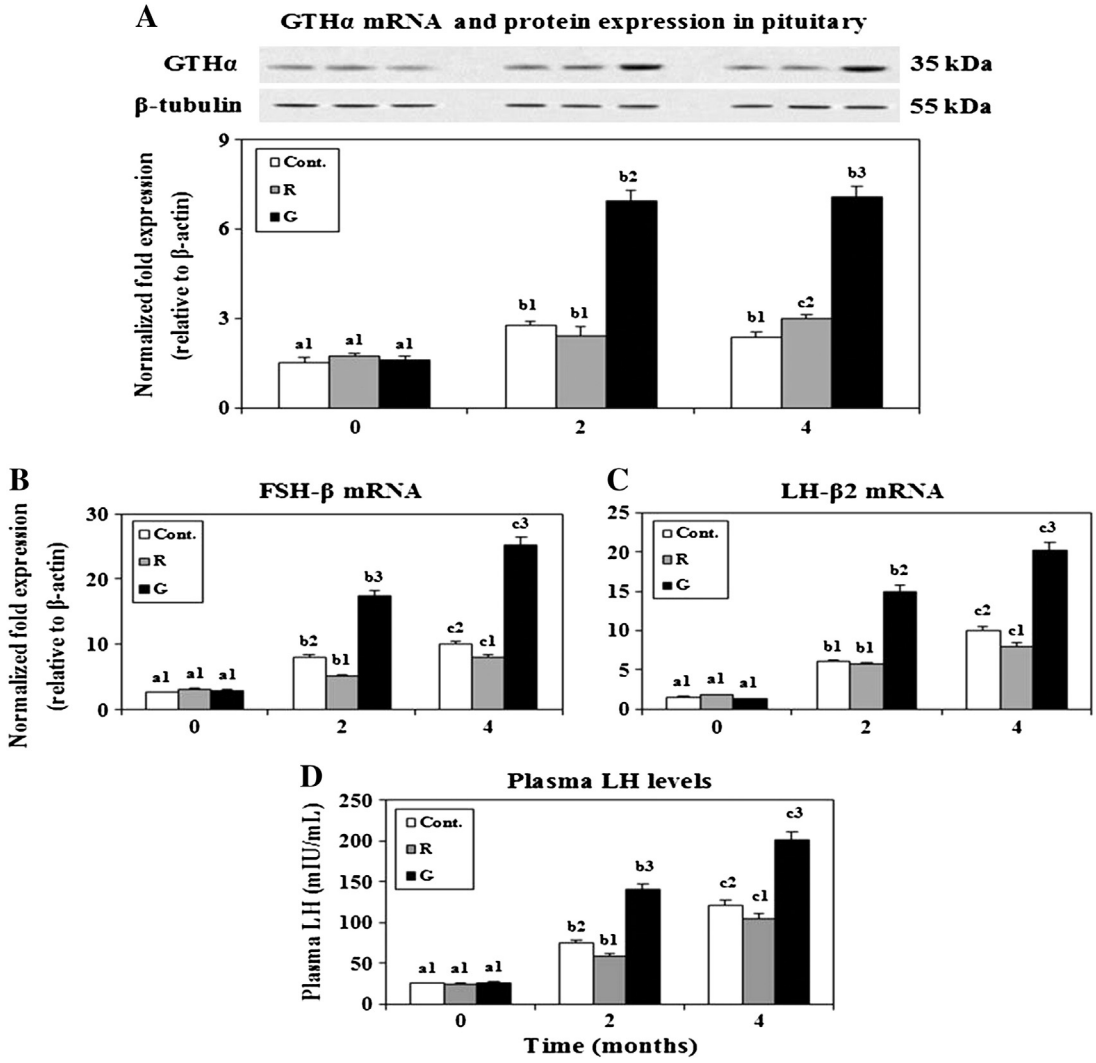

Fig. 4. Changes in the expression levels of GTHα protein and mRNA (A), FSH-β mRNA (B), and LH-β2 mRNA (C) and plasma LH (D) levels in the pituitary and plasma of goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by quantitative real-time PCR and a microplate reader. Values with different characters are significantly different at different time points (months or hours) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

3.2. Expression of Kiss1 mRNA in the hypothalamus (in vivo) and hypothalamus cells (in vitro)

In the same study, we investigated the effects of different light spectra on Kiss1 mRNA levels. Hypothalamic Kiss1 mRNA was significantly increased after 4 month exposure to greed LED, and the levels in the group exposed to green LEDs were higher than those in the other groups (Fig. 2A).

Exposure of cultured neuronal cells to green LED significantly Kiss1 mRNA expression levels in the hypothalamus cells (*in vitro*) at all time points tested (Fig. 2B). No significant changes were observed in the groups exposed to red LED.

3.3. Expression of GPR54 mRNA in the hypothalamus (in vivo) and hypothalamus cells (in vitro)

Exposure of fish to green LED, *in vivo*, resulted in small but statistically significant changes in Kiss-1 receptor (GPR54) mRNA level, *in vivo*. Exposure to green LED lights resulted in small but statistically significant increase in GPR54 mRNA level in the goldfish hypothalamus. The hypothalamic GPR54 mRNA level, however, was lower in the group exposed to red LED for 2 and 4 months (Fig. 3A).

In the cultured neuronal cells, exposure to red light for the most part was without a significant effect. Cells exposed to red LED for 24 and 48 h, however, contained higher GPR54 mRNA levels compared to control (Fig. 3B).

3.4. Gonadotropin levels in the pituitary and plasma (in vivo)

We examined the effects of the different light spectra on the expression of gonadotropin subunit mRNA and GTHα protein levels in the pituitary, as well as plasma LH levels. Pituitary GTHα mRNA and protein expression levels were significantly increased in goldfish exposed to green LED for 2 and 4 months (Fig. 4A). The observed increase in GTHα mRNA was correlated with significant increase in GTHα protein level in the hypothalamus of goldfish exposed to green LED (Fig. 4B). Similarly we observed an increase in LH-β2 and FSH-β mRNA levels in the hypothalamus of goldfish exposed to green LED (Fig. 4). Furthermore, we observed significant increase in circulating LH concentration following exposure to green LED for 2 and 4 months (Fig. 4).

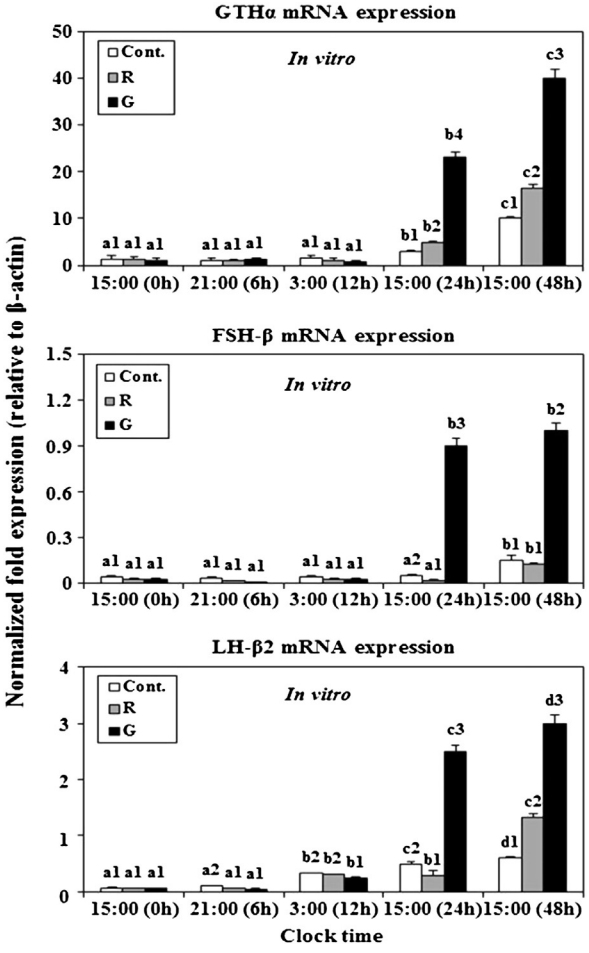

Fig. 5. Changes in the expression levels of GTHα (A), FSH-β (B), and LH-β2 (C) mRNA in the pituitary cells of goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by quantitative real-time PCR. Values with different characters are significantly different at different time points (months or hours) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

We also investigated the effects of different light spectra on gonadotropin subunit mRNA levels in cultured pituitary cells, *in vitro*. Exposure to greed LED consistently increased GTHα, LH-β2 and FSH-β mRNA levels in cultured neuronal cells after 24 and 48 h (Fig. 5). Exposure to red LED caused a small but significant increase in GTHα and LH-β2 mRNA levels, but was without effect on FSH-β mRNA expression following 48 h of incubation (Fig. 5).

3.5. Gonad histology

Histological studies revealed no significant changes in the ovarian structure in goldfish exposed to red LED for 4 months. As in the control group, the ovary of goldfish exposed to red LED was found to be at the peri-nucleolus stage (PNS) (Fig. 6). In the fish exposed to greed LED, however, oil droplets were found in larger ovarian follicles (Fig. 6C).

3.6. Plasma 17α-hydroxypregnenolone level

We investigated the effects of the different light spectra on plasma 17α-hydroxypregnenolone levels in goldfish (Fig. 7). Plasma 17α-hydroxypregnenolone level was found to be significantly increased following exposure to greed LED after 2 and 4 months (Fig. 7). Exposure to red LED was without effect on Plasma 17α-hydroxypregnenolone concentration.

4. Discussion

To investigate the effects of specific LED wavelengths on the sexual maturation of goldfish, we exposed goldfish to white fluorescent bulb as a control as well as to red and green LEDs for 4 months. We investigated sGnRH, cGnRH-II, Kiss1 and Kiss1-receptor (GPR54) in the hypothalamus of intact fish and in cultured hypothalamus cells. We also investigated gonadotropin production by measuring GTHα, LH-β2 and FSH-β mRNA levels as well as circulating LH and 17α-hydroxypregnenolone concentrations and pituitary LH protein content in the pituitary of intact goldfish and in cultured pituitary cells, *in vitro*. We also provide ovarian histology in control fish and those exposed to red and green LEDs. The results clearly demonstrate stimulatory effects of exposure to green LED, *in vivo* and *in vitro* on various hormones involved in the control of reproduction in fish.

The observed stimulatory effects of exposure to green LED provide a strong evidence that exposure to specific wavelengths has direct actions at the level of brain, hypothalamus and pituitary cells. Exposure to green LED significantly increased sGnRH and cGnRH-II mRNA levels and pituitary gonadotropin production provides a strong evidence that green LED stimulates gonadal development by effecting both brain and pituitary levels. Furthermore, greater expression and Kiss1 and Kiss1 receptor, GPR54, provide evidence that green LED increases stimulatory actions of gonadal estrogens of brain GnRH production. In this context, Irwig et al. (2004) demonstrated that Kiss1 neurons are located in close association with the GnRH neuron in the hypothalamus. It has also been reported that Kiss1 is a regulator of GnRH production, and Kiss1 is involved in a feedback mechanism of gonadotropin production (Colledge, 2009). There is evidence that Kiss1 stimulates GnRH neuron through its receptor, GPR54, and this action induces the up-regulation of the hypothalamus–pituitary–gonad axis (Tsutsui et al., 2010). Additionally, Kiss and GPR54 are integral components of the physiological regulation of reproduction and gonadal maturation. Overall, they participate in the regulation of reproductive function and fertilization through regulation of the secretion of gonadotropin hormones, feedback actions of sex steroid hormones, and environmental signally through acceleration of the GnRH neuron in time to puberty (Parhar et al., 2004; Tena-Sempere et al., 2012).

The observed lack of stimulatory action of red LED provides strong evidence for specificity of cells for light wavelengths. However, the exact mechanism underlying the relationship between short-wavelength green light and the enhancement of the secretion of sexual maturation hormones remains unclear. It is known that the short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light can only penetrate short distance and is likely to be effective in shallow waters (McFarland, 1991). This theory suggests that fish could detect green wavelengths because green light penetrates more deeply than red light and affect fish in deeper sections. Thus, it is possible that the amount of light detected by the fish may be an important contributing factor. Furthermore, the observed direct actions on cultured cells indicate that green LED may effect gene expression by affecting receptor–ligand interaction or enzyme substrate kinetics.

The present results are in accordance with a previous report by Volpato (2000), which demonstrated enhanced reproductive performance of hormone-induced Matrinxa fish, *Brycon cephalus* and an increased spawning rate in female fish reared under green light. These results collectively demonstrate that short-wavelength lights affect reproductive behavior and performance, and provide a strong support for the hypothesis that short-wavelength green light has a positive effect on the reproductive capacity of fish.

The mechanism underlying the relationship between short-wavelength green light and the enhancement of the secretion of sexual

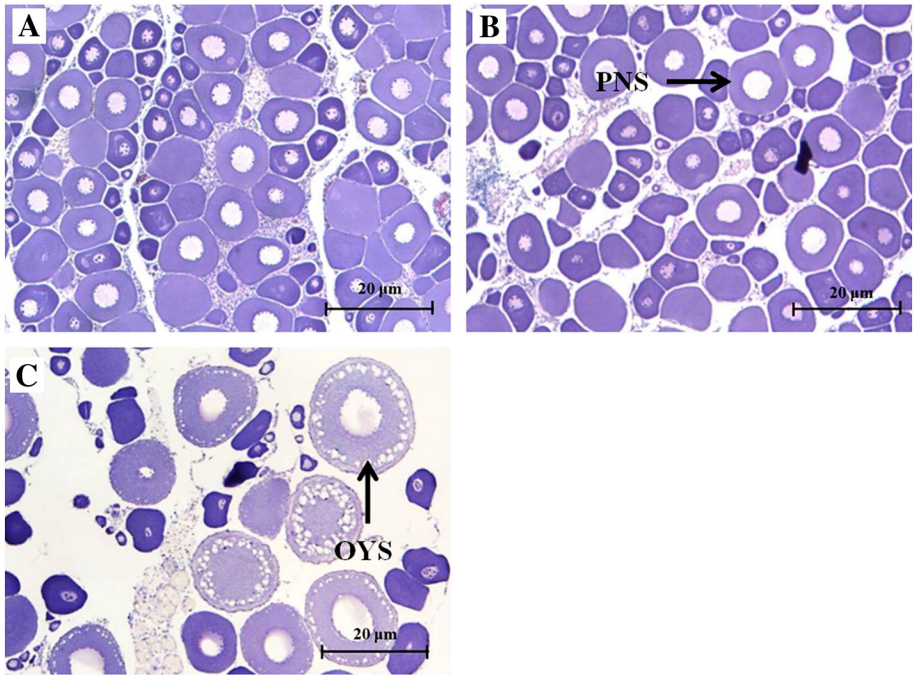

Fig. 6. Changes in gonad histology of goldfish under different lighting conditions using a white fluorescent bulb (Cont.) (A), as well as red (B) and green (C) LEDs. PNS: Peri-nucleolus stage, OYS: Oocytes in the yolk stage, scale bar = 20 μm.

maturation hormones remains unclear. However, the reason that genes are expressed differently according to wavelengths could be due to the characteristics of light wavelengths in water. The short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates in shallow waters (McFarland, 1991). This theory suggests that fish could detect green wavelengths because green light penetrates more deeply than red light and does reach fish beyond these depths. Thus, we hypothesized that these results would be closely related with the amount of light detected by the fish.

To provide further evidence of gonadal development, we examined the gonad tissues from goldfish reared for 6 months in different wavelengths. More mature oocytes at the vitellogenic stage were found in the group exposed to green LEDs compared to control group exposed to white fluorescent bulb and those exposed to red LEDs, indicating that green light more effectively stimulates the maturation of oocytes than other lights. Furthermore, the observed increase in plasma 17α-hydroxypregnenolone levels in the fish exposed to green LEDs indicates greater level of steroidogenesis in the ovary (Hu et al., 2001).

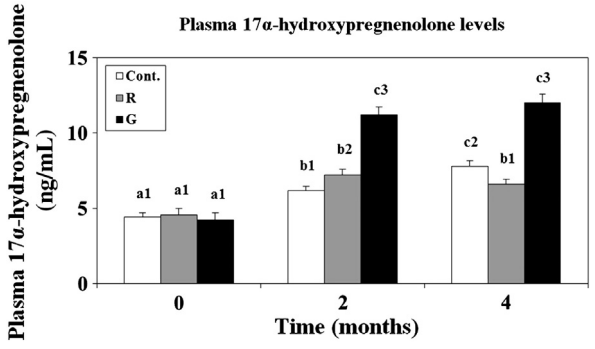

Fig. 7. Changes in the levels of plasma 17α-hydroxypregnenolone in goldfish under lighting conditions using red (R) and green (G) LEDs and a white fluorescent bulb (Cont.), as measured by a microplate reader. Values with different characters are significantly different at different time points (months) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same time point ($P < 0.05$). All values are means ± SD ($n = 5$).

The present results are in accord with those reported by Shin et al. (2012) that exposure of yellowtail clownfish, *Amphiprion clarkii* to green LED resulted in higher levels of growth hormone and faster growth rate compared to fish exposed to other types of light. This result is also in agreement with research showing that short-wavelength green light positively affects growth (Yamanome et al., 2009). Increase in growth hormone may be a contributing factor in stimulating gonadal maturation. In this context, growth hormone was shown to accelerate steroid-enhancing effects of gonadotropin hormone in the goldfish ovary (Van der Kraak et al., 1990).

In summary, the present findings provide a strong support for the hypothesis that short-wavelength green light promotes sexual maturation in goldfish by directly acting on the hypothalamus and pituitary cells.

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