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# Effect of LED light spectra on exogenous prolactin-regulated circadian rhythm in goldfish, *Carassius auratus*

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We investigated the effect of light spectra on circadian rhythm by exogenous prolactin (PRL) using light-emitting diodes (LEDs): red, green and purple. We injected PRL into live fish or treated cultured brain cells with PRL. We measured changes in the expressions of *period 2* (*Per2*), *cryptochrome 1* (*Cry1*), melatonin receptor 1 (MT1) mRNAs, and MT1 proteins, and in the plasma PRL, serotonin and melatonin levels. After PRL injection and exposure to green light, MT1 expression and plasma melatonin levels were significantly lower, but the expressions of *Per2* and *Cry1* were significantly higher than the others. Plasma serotonin after PRL injection and exposure to red light was significantly lower than others. These results indicate that injection of high concentration PRL inhibits melatonin, and inhibited melatonin regulates circadian rhythm via clock genes and serotonin. Thus, exogenous PRL regulates the circadian rhythm and light spectra influence the effect of PRL in goldfish.

**Keywords:** circadian rhythm; *cryptochrome 1*; *period 2*; prolactin; melatonin receptor 1; serotonin



## 1. Introduction

All organisms have daily circadian rhythms, called circadian clocks, to regulate physiological, biochemical and behavioural functions (King & Takahashi 2000). Among the many factors that control circadian rhythms, light is the most important one (Jin et al. 2009). Various wavelengths of light, induces/or inhibits the photoreceptors in organisms, and influences not only the physiology but also the reproduction and behaviour of an organism (Pierce et al. 2008).

In addition, circadian rhythm is adjusted by internal factors such as serotonin, a neurotransmitter and melatonin, a hormone secreted by the pineal gland (Falcón et al. 2009; Kim et al. 2012).

Melatonin is a "time-keeping" hormone, and it is altered by arylalkylamine *N*-acetyltransferase-1 (AANAT1) to a neurotransmitter serotonin (5-HT; 5-hydroxytryptamine). Further, secretion of prolactin exogenous prolactin (PRL) is increased during the daytime when secretion of melatonin, the signal of night, is suppressed. Melatonin is reported to regulate the synthesis and secretion of PRL by acting directly on PRL synthesis in cells (Falcón et al. 2003).

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Biological effects of melatonin are initiated by melatonin receptors (MTs) which belong to the G-protein-coupled receptor superfamily. MTs are found in the diencephalon and various regions of the central nervous system (CNS) of vertebrate species, which adjust the levels of melatonin for a specific physiological function in the organisation of diencephalon and CNS (Reppart et al. 1996; Park et al. 2013). Dubocovich et al. (2000) reported three different subtypes of MTs, namely, MT1, MT2 and MT3. MT1 has been identified in the brain of vertebrates, including the hypophyseal pars tuberalis and hypothalamic suprachiasmatic nucleus. MT1 detects seasonal changes and mediates the regulation of circadian rhythms (Reppart et al. 1996).

Serotonin is a precursor of melatonin. It stimulates secretion of cortisol by adjusting the hypothalamic–pituitary–interrenal axis activity to reduce the stress in fish (Lim et al. 2013). In experiments using Nile tilapia *Oreochromis niloticus*, serotonin promotes secretion of PRL by stimulating the synthesis of PRL cells (Grau & Helms 1990; Herndon et al. 1991).

PRL is secreted from the pituitary gland. It is a multi-functional polypeptide hormone present in all vertebrates. PRL is known to exert a wide variety of actions; it is involved in water retention and electrolyte balance, growth, maturity, endocrine metabolism, reproduction and immune regulation (Bole-Feysot et al. 1998; Clapp et al. 2012). PRL in fish is known as the freshwater adaptation hormone as it helps in freshwater osmoregulation by regulating on exchange together with arginine vasotocin production (An et al. 2008; Motohashi et al. 2009). PRL also regulates circadian rhythm (Ribelayga et al. 2002) by stimulating dopamine production (Zilberman-Peled et al. 2006). However, there are no systematic studies on the effects of PRL on circadian rhythms in fish.

Light is one of the most important external factors that regulate circadian rhythms (Pierce et al. 2008; Jin et al. 2009). The lights of various wavelengths, in organisms with photoreceptors induce/or inhibit not only physiological changes but also affect the reproduction, growth and behaviour (Pierce et al. 2008). Shin et al. (2012, 2014) reported the benefits of a narrow bandwidth light that promotes the early growth and reproduction in yellowtail clownfish *Amphiprion clarkii* and sexual maturation in goldfish *Carassius auratus*. Thus, recent research has shown the use of LED wavelengths to study circadian rhythms. In addition, previous studies reported that specific LED wavelengths are able to regulate circadian rhythm by regulating the expression of clock genes *period 2* (*Per2*), *cryptochrome 1* (*Cry1*) and melatonin receptor 1 (MT1) (Shin et al. 2011; Park et al. 2013).

Therefore, we investigated the effect of PRL on the expressions of *Per2*, *Cry1* and MT1, and on the plasma levels of serotonin in goldfish. We also examined the effects of three wavelengths (red, green and mixed purple) on the circadian rhythm according to PRL treatment (*in vivo*; 5 μg/g, *in vitro*; 10 and 100 ng/μL).

## 2. Materials and methods

### *2.1. Experimental fish and conditions*

For the experiments, common goldfish (length, 6.1 ± 0.5 cm; mass, 12.5 ± 0.4 g) were purchased from the commercial aquarium (Busan, Korea) and were allowed to acclimate for two weeks in three 300-L circulation filter tanks in the laboratory. The goldfish were reared with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) and allowed to acclimatise to the conditions for 24 h.

The fish were divided into experimental groups, each exposed to 22 °C water temperature. Each experimental group was exposed to light-emitting diodes (LEDs), red (peak at 630 nm), green (530 nm) or purple [mixed blue (450 nm) and red (630 nm)] (Daesin LED Co., Kyunggi, Korea); the control group was irradiated with a white fluorescent bulb (Figure 1). The photoperiod was a 12-h light (L):12-h dark (D) cycle (lights on at 07:00 and lights off at 19:00). The LEDs were placed 40 cm above the water surface, and the irradiance at its surface was maintained at approximately 0.9 W/m$^2$. The fish were reared under these conditions with a daily feeding of commercial feed until the day prior to the sampling. Spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD Inc., CO, USA). Fish were anaesthetised with 200 mg/L tricaine methanesulphonate (MS-222; Sigma, USA) to minimise the stress prior to blood collection. Blood was collected rapidly 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 analysis. Fish were killed by spinal transaction (the first sampling at 11:00) at 12-h sampling intervals to collect the hypothalamus and blood samples under dim light using an attenuated white fluorescent bulb.

### *2.2. PRL injection*

To investigate the effects of PRL, the fish were anaesthetised with tricaine methanesulphonate (MS-222; Sigma, USA) prior to injection. PRL (Prolactin; Sigma, USA) was dissolved in 0.9% physiological saline, and each fish was injected with PRL (5 μg/g of body mass [BM]) at a volume of 10 μL/g BM. The sham group was injected with an equal volume of 0.9% physiological saline (10 μL/g BM). After 12 h, hypothalamus samples were removed from the fish at 12-h sampling intervals for one day (the first sampling at 11:00). During the experimental period, water temperature and photoperiod were maintained at 22 °C and 12-h L:12-h D cycle, respectively.

All fish were anaesthetised using tricaine methanesulphonate (Sigma, USA) and decapitated prior to tissue collection. Brain samples from the fish were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis. The plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, 5 min) and stored at −80 °C until analysis.

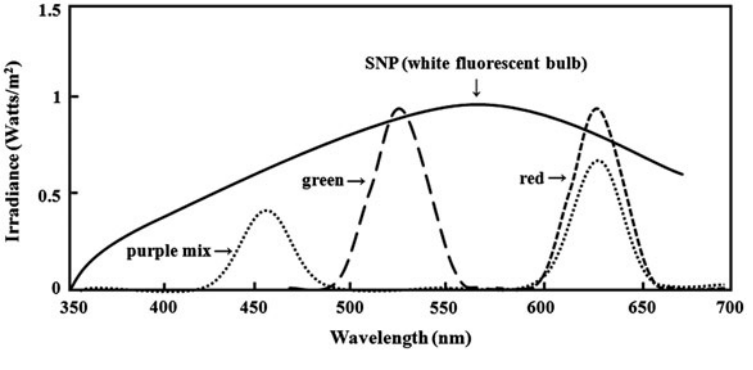


Figure 1. Spectral profiles of red (630 nm), green (530 nm), and purple [mixed blue (450 nm) and red (630 nm)] LEDs used in this study. SNP: simulated natural photoperiod by using a white fluorescent light.

### 2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using TRI Reagent® (Molecular Research Center, Inc., USA) according to the manufacturer's instruction. Concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Two micrograms of total RNA were reverse transcribed in a total volume of 20 μL, using an oligo-d$(T)_{15}$ anchor and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in polymerase chain reaction (PCR) and quantitative PCR.

### 2.4. Quantitative PCR (QPCR)

Quantitative PCR was carried out following 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 using a TRI Reagent® (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, USA) following the manufacturer's instructions. qPCR using cDNA was conducted to determine the relative expression levels of *Cry1* (GenBank accession no. **EF690700**), *Per2* (**EF690697**), MT1 (**AB481372**) and β-actin (**AB039726**) mRNAs using total RNA extracted from the hypothalamus. Primers used in qPCR are shown in Table 1. These primers were designed for each gene using Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed against the BLAST database to ensure the specificity of primers. PCR amplification was conducted using Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions. The qPCR was performed as follows: 95 °C for 5 min, followed by 50 cycles each of 95 °C for 20 s and 55 °C for 20 s. As a control, experiments were duplicated with β-actin and all data were expressed relative to the corresponding β-actin-calculated threshold cycle (ΔCt) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated using $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = 2\text{^} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$] (Livak & Schmittgen 2001).

### 2.5. Western blot analysis

Total protein isolated from the brain of goldfish was extracted using T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. A volume of 25 μg of total protein was loaded per lane onto Mini-PROTEAN® TGX™ Gels (Bio-Rad, USA). Protein ladder (Bio-Rad, USA) was

Table 1. Primers used for QPCR amplification.

| Genes (accession no.) | Primer | Sequences |
|---|---|---|
| *Cry1* (EF690700) | Forward | 5′-CGG AGA CCT GTG GAT CAG-3′ |
| | Reverse | 5′-GTG GAA GAA TTG CTG GAA-3′ |
| *Per2* (EF690697) | Forward | 5′-CTG GAG CCG CAA AGT TTC-3′ |
| | Reverse | 5′-CTG GAT GTC TGA GTC TAA-3′ |
| MT1 (AB481372) | Forward | 5′-TTG GCA GTA GCG ATT TCT C-3′ |
| | Reverse | 5′-CTC ACG ACG GAA GTT CTG-3′ |
| β-actin (AB039726) | Forward | 5′-TTC CAG CCA TCC TTC CTA-3′ |
| | Reverse | 5′-TAC CTC CAG ACA GCA CAG-3′ |

loaded as a reference. Samples were electrophoresed at 180 V. The gels were immediately transferred to a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 85 V for 3 min using Trans-Blot® Turbo™ Transfer System (Bio-Rad, USA). Thereafter, the membranes were blocked with 5% skimmed milk in 0.04% Tris-buffered saline with Tween (TTBS) for 45 min and subsequently washed in TTBS. The membranes were incubated with MT1 antibodies (Melatonin Receptor 1A Antibody (NBP1-28912); Novus Biologicals® LLC, USA; dilution, 1:4,000; Approximate 45 kDa), followed by horseradish peroxidase- conjugated anti-rabbit IgG secondary antibodies (Bio-Rad, USA; dilution, 1:4,000) for 60 min. As an internal control, β-tubulin primary antibody (ab6046; Abcam, UK; dilution, 1:4,000) was followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (dilution, 1:4,000; Bio-Rad) for 60 min. Bands were detected using a sensitive electro-chemiluminescence (ECL) system (ECL Advance; GE Healthcare Life Sciences, Uppsala, Sweden) and exposed for 2 min using a Molecular Imager® ChemiDoc™ XRS$^+$ System (Bio-Rad, USA).



### *2.6.* In vitro *PRL-treated brain cells culture*

The culture of goldfish brain was prepared using enzymatic and mechanical procedures. The brain 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; contained 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL fungizone; Gibco-BRL, Rockville, MD, USA). Isolated brain tissues were then transferred to 6 mL of fresh dispersion buffer containing 0.25% trypsin (Type II-S from porcine pancreas; Sigma, USA). Connective tissues and other impurities were removed and brain tissues were chopped into small pieces with scissors. Brain cells and minced brain tissues 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 resuspended in a culture medium (Neurobasal Medium, Gibco-BRL, Life Technologies, USA; without L-glutamine, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 1% foetal bovine serumwith the medium's osmolarity adjusted to match goldfish plasma osmolarity of 353 mOs). The cell suspension was centrifuged at $800 \times g$ for 10 min, and the cells were then resuspended in a fresh culture medium. Each experimental group was treated with PRL (10 and 100 ng/μL).

The brain cells ($1.2 \times 10^6$ cells/800 μL/well) were applied to a 24-well tissue culture plate, and starting from 07:00 they were sampled at ZT4, ZT16, ZT28 and ZT40. Brain cells in each experimental group were exposed to LEDs, red (peak at 630 nm) and green (530 nm), or purple [mixed blue (450 nm) and red (630 nm)] LEDs (Daesin LED Co., Korea); control group was exposed to a white fluorescent bulb. 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 L:12-h D photoperiod (lights on at 07:00 and lights off at 19:00). The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec®, ASD), and the temperature was maintained using the heat-prevention system of LEDs.

### *2.7. Plasma parameter analysis*

#### *2.7.1. Plasma PRL level*

Plasma samples were separated by centrifugation (4 °C, $10{,}000 \times g$, 5 min), and plasma PRL level was analysed using the immunoassay technique with Fish Prolactin/Luteotropic

Hormone (PRL/LTH) ELISA Kit (Cusabio, China). An anti-antibody that was specific to the antibody of PRL was pre-coated onto a microplate, followed by the addition of 50 μL of plasma, 50 μL of HRP-conjugate and 50 μL of antibody to each well. These were mixed well and incubated for 1 h at 37 °C. Following the last wash, any remaining wash buffer was aspirated or decanted off and 50 μL each of the 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 colourless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in the colour 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.

#### *2.7.2. Plasma serotonin level*

Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, 5 min), and plasma serotonin level was analysed using the immunoassay technique with Fish 5-hydroxytryptamine/serotonin (5HT/ST) ELISA kit (MyBioSource Inc., San Diego, California, USA). An anti-antibody that was specific to the antibody of the serotonin was pre-coated onto a microplate, followed by the addition of 50 μL of plasma and 50 μL of HRP-conjugate to each well. These were mixed well and then incubated for 40 min at 37 °C. Following the last wash, any remaining wash buffer was aspirated or decanted off, and 90 μL of TMB substrate were added to each well. These TMB solutions were then incubated for 20 min at 37 °C in the dark, during which time they changed from colourless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in the colour 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.

#### *2.7.3. Plasma melatonin level*

Plasma samples were separated by centrifugation (4 °C, 10,000 × *g*, 5 min), and plasma melatonin level was analysed using the immunoassay technique with ELISA kit (IBL, Hamburg, Germany). An anti-antibody that was specific to the antibody of the melatonin was pre-coated onto a microplate, followed by the addition of 50 μL of plasma and 50 μL of antiserum. These were mixed well and then incubated for 14 h at 37 °C. Incubation solution was discarded, the plate was washed with 250 μL of wash buffer, and 50 μL of enzyme conjugate was added to each well. These enzyme conjugates were then incubated for 1 h at 25 °C on an orbital shaker. The wash buffer was aspirated or decanted off, and 100 μL of TMB substrate was added to each well. These TMB solutions were then incubated for 20 min at 37 °C in the dark, during which time they changed from colourless or light blue to darker shades of blue. Following incubation, 50 μL of stop solution was added to each well, resulting in the colour 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.

### *2.8. Statistical analysis*

All data were analysed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's *post hoc* test was used to compare differences in the data ($p < 0.05$). The values are expressed as the means ± standard error (SE).

## 3. Results

### 3.1. *Change of* Per2 *and* Cry1 *mRNA expression levels (*in vivo *and* in vitro*)*

This study investigated the expression levels of *Per2* and *Cry1* mRNAs under different LED light spectra with PRL treatment (*in vivo* and *in vitro*).

Both *Per2* and *Cry1* mRNA expression levels followed the same trend (Figures 2 and 3). They were significantly higher in the photophase group with simulated natural photoperiod (SNP) and with all tested LED light spectra than in the scotophase. The levels in groups injected with PRL (*in vivo*) and treated with 100 ng/mL PRL (*in vitro*) were significantly higher than the levels in other groups. Particularly, the levels were significantly higher after the samples were exposed to green LED but significantly lower after exposure to red LED.

### 3.2. *Change in MT1 mRNA and protein expression levels (*in vivo *and* in vitro*)*

To assess the protein expression levels, we performed western blot analysis using protein extracted from goldfish brain. MT1 mRNA and protein expression levels in the scotophase group of SNP and all LED light spectra were significantly higher compared to the photophase group. The levels significantly decreased with PRL treatment. In particular, MT1 protein levels in the group exposed to red LED were high compared to other groups (Figure 4(a)). Additionally, we investigated the expression levels of MT1 mRNA under different LED light spectra with PRL treatment (*in vivo* and *in vitro*). The levels of MT1 mRNA expression in the scotophase of all LED spectra group were significantly higher compared to the photophase. The levels in the group injected with PRL (*in vivo*) and group treated with 100 ng/mL PRL (*in vitro*) were significantly lower than the levels in other groups. Particularly, MT1 mRNA expression levels in groups exposed to red LED were significantly higher compared to other LED spectra, but significantly lower in groups exposed to green LED (Figure 4(b) and (c)).

### 3.3. *Plasma melatonin levels*

The analyses of measured plasma melatonin levels under different LED light spectra with PRL injection revealed that plasma melatonin levels in all light spectra groups significantly decreased with PRL injection. Melatonin levels in the scotophase were significantly higher compared to the photophase. Additionally, plasma melatonin levels in the control groups in photophase were 394.58 ± 19.7 pg/mL, but the levels decreased to 272.0 ± 13.6 pg/mL after the injection of 5 μg/g PRL.

In particular, plasma melatonin levels in the group exposed to green LED and with PRL injection were significantly lower in the photophase (220.2 ± 11.0 pg/mL) and the scotophase (660.4 ± 33.0 pg/mL) compared to other LED groups (Figure 5).

### 3.4. *Plasma prolactin levels*

Plasma prolactin levels in all light spectra groups significantly increased with PRL injection. The prolactin levels in the photophase were significantly higher compared to scotophase. Additionally, plasma prolactin levels in the control groups in photophase were 8.8 ± 0.4 μIU/mL, but the levels increased to 11.3 ± 0.5 μIU/mL after the injection of 5 μg/g PRL. Particularly, plasma prolactin levels in group exposed to green LED and with PRL injection were significantly higher in the photophase (13.05 ± 0.7 μIU/mL) and the scotophase (8.9 ± 0.4 μIU/mL) compared to other LED groups (Figure 6).

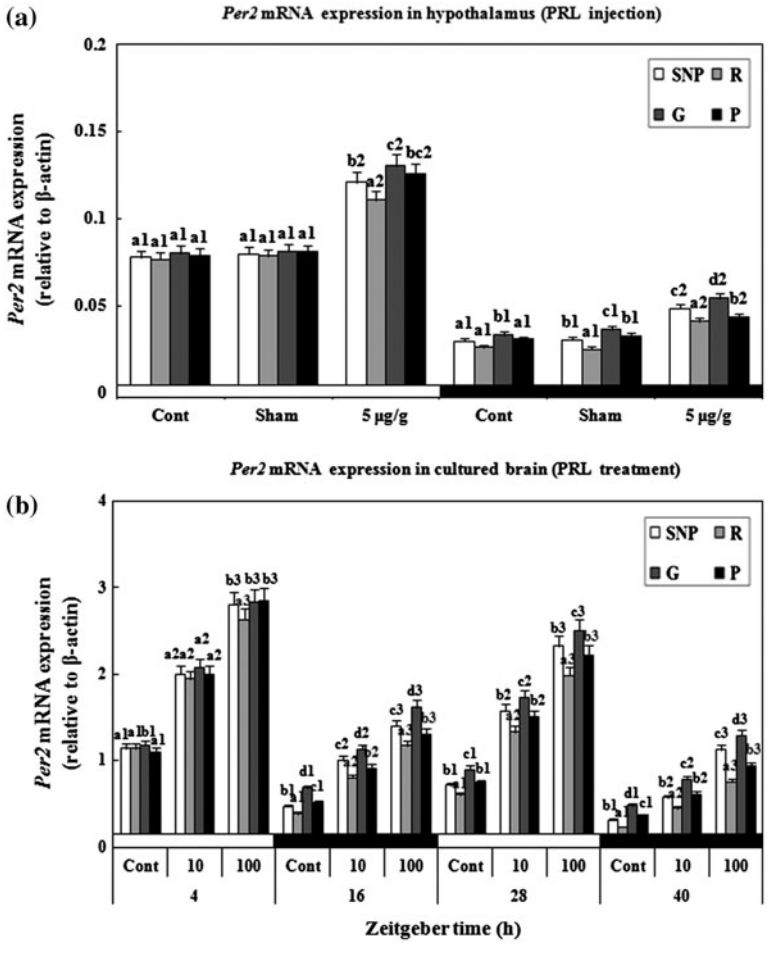


Figure 2. Changes in the expression levels of *Per2* mRNA in the hypothalamus after PRL injection (5 μg/g of body mass) (a) (*in vivo*); and in the brain after PRL (10 and 100 ng/μL) treated cell culture (b) (*in vitro*); in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Total hypothalamus RNA (2.0 μg) and brain RNA (2.0 μg) were reverse-transcribed and amplified. Horizontal white bar represents the photophase (at 11:00), and black bar represents the scotophase (at 23:00). Results are expressed as normalised fold expression levels with respect to β-actin levels in the same sample. Different letters indicate significant difference among treatments exposed to different LEDs spectra but the same PRL injection and treatment levels ($p < 0.05$). Different numbers indicate significant differences between PRL injection and cultured levels within the same LEDs spectra and time ($p < 0.05$). All values are means ± SE ($n = 5$). Cont = control group; Sham = control group injected with saline; 5 μg/g = fish injected with PRL.

### *3.5. Plasma serotonin levels*

The analysis of plasma serotonin levels under different LED light spectra with PRL injection revealed that the plasma serotonin levels in all light spectra groups significantly decreased with PRL injection. The serotonin levels in the scotophase were significantly higher than in the photophase. Additionally, plasma serotonin levels in the control groups in photophase decreased from 2.1 ± 0.1 to 1.9 ± 0.1 ng/mL after the

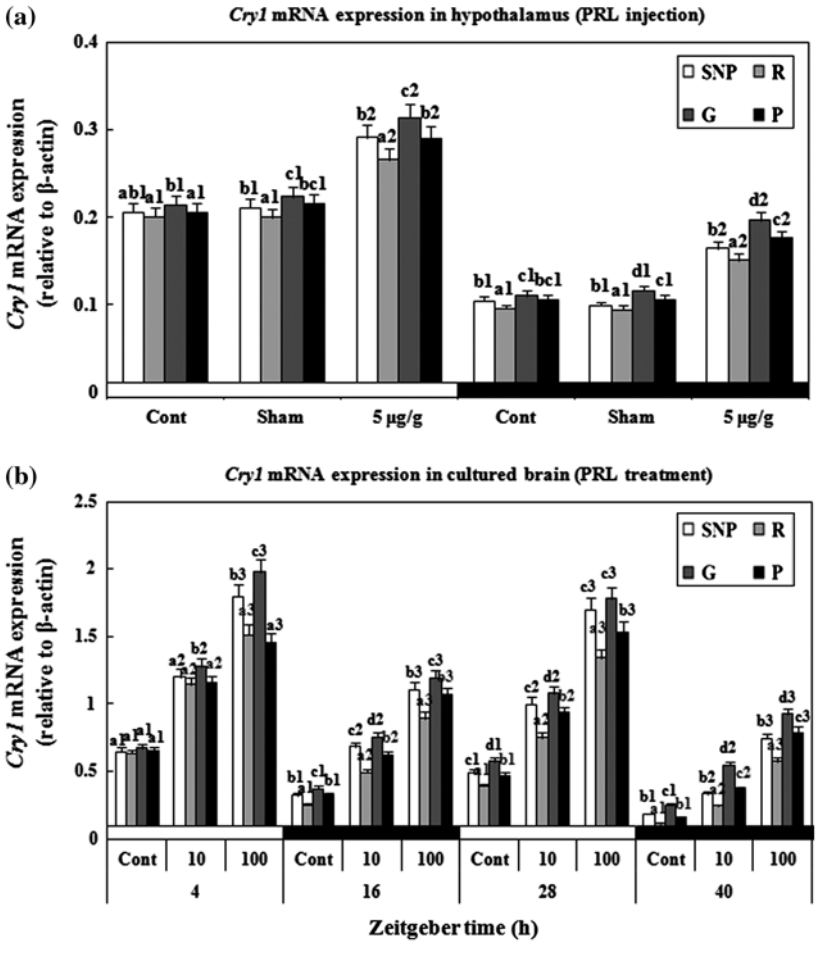


Figure 3. Changes in the expression levels of *Cry1* mRNA in the hypothalamus after PRL injection (5 μg/g of body mass) (a) (*in vivo*); and in the brain after PRL (10 and 100 ng/μL) treated cell culture (b) (*in vitro*); in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Total hypothalamus RNA (2.0 μg) and brain RNA (2.0 μg) were reverse-transcribed and amplified. Horizontal white bar represents the photophase (at 11:00), and black bar represents the scotophase (at 23:00). Results are expressed as normalised fold expression levels with respect to β-actin levels in the same sample. Different letters, different numbers, Cont, Sham, and 5 μg/g are defined in Figure 2.

injection of 5 μg/g PRL. The highest plasma serotonin levels in photophase and scotophase were observed in the group exposed to green LED with PRL injection (2.7 ± 0.1 and 1.2 ± 0.1 ng/mL, respectively) (Figure 7).

## 4. Discussion

To investigate the effects of exogenous PRL and LED spectra on circadian rhythm of goldfish, as produced by different LED spectra (red, green and mixed purple), we examined the mRNA and protein expression levels of clock genes (*Per2, Cry1* and MT1) and plasma PRL, serotonin and melatonin levels.

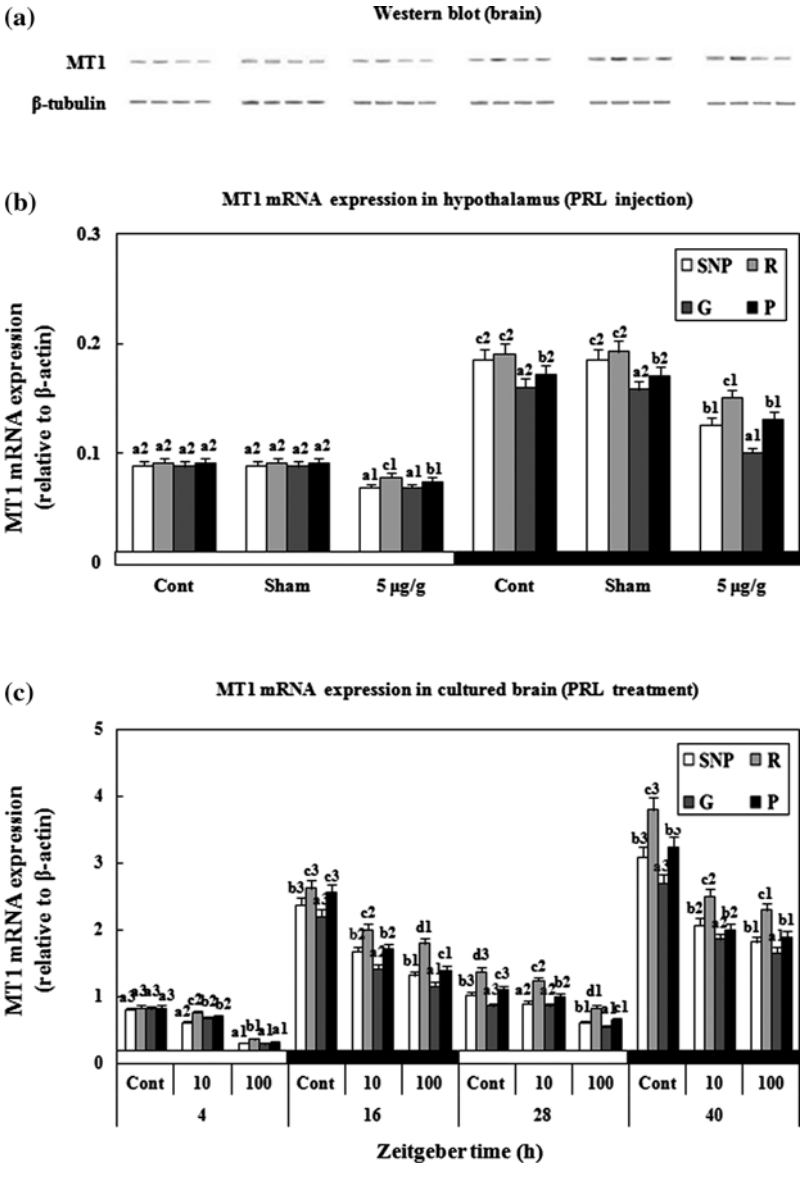


Figure 4. Changes in the expression levels of MT protein (anti-goldfish MT; a polyclonal rabbit antibody; 45 kDa) in the brain (a), and MT1 mRNA in hypothalamus after PRL injection (5 μg/g of body mass) (b) (*in vivo*); and in the brain after PRL (10 and 100 ng/μL) treated cell culture (c) (*in vitro*) in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by quantitative real-time PCR. Total hypothalamus RNA (2.0 μg) and brain RNA (2.0 μg) were reverse-transcribed and amplified. Horizontal white bar represents the photophase (at 11:00) and black bar represents the scotophase (at 23:00). Results are expressed as normalised fold expression levels with respect to β-actin levels in the same sample. Different letters, different numbers, Cont, Sham, and 5 μg/g are defined in Figure 2.

First, the goldfish was irradiated by SNP, red, green and purple LED after the injection of 5 μg/g PRL. The expressions of *Per2* and *Cry1* mRNA increased after the

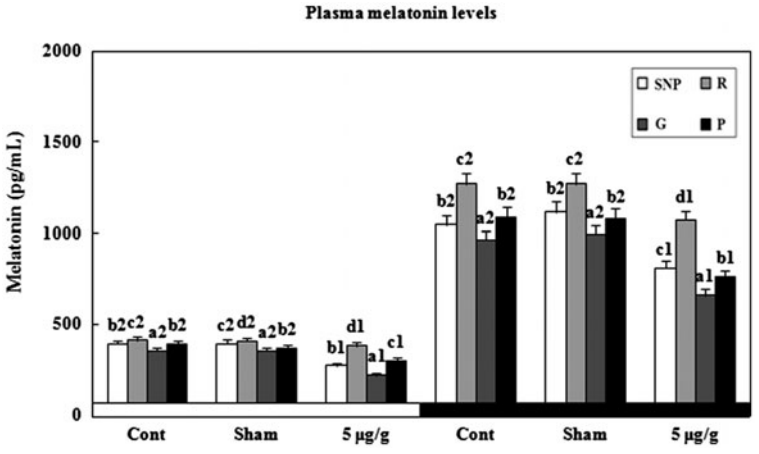


Figure 5. Changes in the levels of plasma melatonin after PRL injection (5 μg/g of body mass) in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by plate reader. Horizontal white bar represents the photophase (at 11:00) and black bar represents the scotophase (at 23:00). Different letters, different numbers, Cont, Sham, and 5 μg/g are defined in Figure 2.

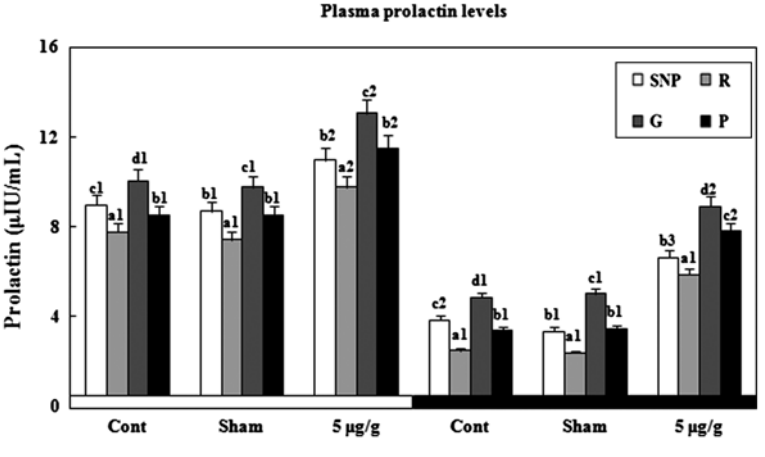


Figure 6. Changes in the levels of plasma prolactin after PRL injection (5 μg/g of body mass) in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by plate reader. Horizontal white bar represents the photophase (at 11:00) and black bar represents the scotophase (at 23:00). Different letters, different numbers, Cont, Sham and 5 μg/g are defined in Figure 2.

injection, while the expression of MT1 protein and mRNA decreased (Figures 2(a), 3(a), 4(a) and (b)). Grattan and Kokay (2008) reported that PRL stimulates gene expression of tyrosine hydroxylase (TH) and also modulates the phosphorylation of TH, resulting in increased dopamine synthesis. Several studies reported that as dopamine increases, plasma melatonin levels decrease and then photosensitivity increases by the activation of cones, photoreceptor cells in retina (Ribelayga et al. 2003; Zilberman-Peled et al. 2006).

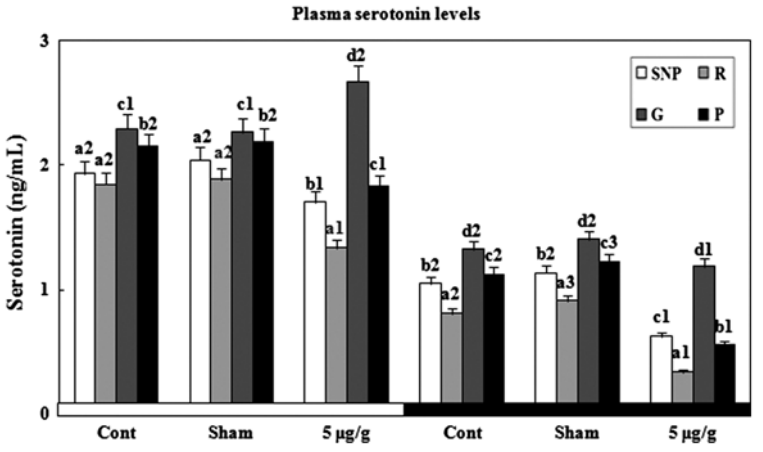


Figure 7. Changes in the levels of plasma serotonin after PRL injection (5 μg/g of body mass) in goldfish exposed to red (R), green (G) and purple (P) LEDs and a simulated natural photoperiod (SNP), as measured by plate reader. Horizontal white bar represents the photophase (at 11:00) and black bar represents the scotophase (at 23:00). Different letters, different numbers, Cont, Sham and 5 μg/g are defined in Figure 2.

In this study, we observed that dopamine synthesis increased with increasing PRL after the PRL treatment. This resulted in decreased melatonin production, suggesting that circadian rhythm is regulated by *Per2* and *Cry1*, as the activation of clock genes by light significantly increased (Figures 2(a), 3(a) and 4(b)). These results were consistent with a previous study, which reported that expression levels of *Per2* and *Cry1* mRNA increased with increasing the activation of photoreceptors by light (Besharse et al. 2004; Kim et al. 2012).

We investigated the change in *Per2*, *Cry1*, MT1 mRNA and MT1 protein expression levels by exposing the brain tissue to LED spectra. *Per2* and *Cry1* mRNA expression levels under green LED were significantly higher in both the PRL treatment group and non-PRL treatment group, while MT1 mRNA and MT1 protein expression levels were significantly higher under red LED (Figures 2(a), 3(a), 4(a) and (b)), thus being in agreement with a previous report by Park et al. (2013). 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. Therefore, a fish can detect better short wavelengths such as green light. In this study, after increasing the photosensitivity by PRL treatment, the expression levels of *Per2* and *Cry1* mRNA, which are regulated by light, were significantly higher in groups exposed to green LED than to red LED, whereas the opposite was detected for MT1 mRNA and protein expression levels (Figures 2(a), 3(a), 4(a) and (b)).

To test whether the wavelength of the LED has the same effect in the *in vitro* culture environment, we examined the expression levels of the clock genes in cultured brain cells treated with 10 and 100 ng/mL of PRL. The increase in PRL concentration resulted in increased expression levels of *Per2* and *Cry1* mRNA, but decreased MT1 level (Figures 2(b), 3(b) and 4(c)). The results of this study were similar to the results obtained in the *in vivo* experiments. Ekström and Meissl (2003) reported that neuroendocrinal receptors in the brain increased the sensitivity to light using the same basic phototransduction pathways. In this study, we found that increased PRL concentration

increases the sensitivity to light by increasing the expression levels of *Per2* and *Cry1* mRNA and decreasing the expression of MT1 mRNA. Further, plasma melatonin exhibited the lowest concentration at a wavelength of green LED and the highest concentration at a wavelength of red LED. The level of plasma melatonin was lower in experimental groups treated with PRL compared to the experimental groups, which were not treated with PRL (Figure 5). As mentioned earlier, previous studies (Zilberman-Peled et al. 2006) reported decreased plasma melatonin levels with increasing dopamine levels. In other words, a synergistic effect occurred during the simultaneous processing of PRL and a specific wavelength of LED in the regulation of biological rhythms.

In this study, we investigated the plasma serotonin levels of the PRL-injected goldfish, which were exposed to LED with various wavelengths to assess the effect of PRL and the wavelengths on the change in the biorhythm. As a result, the lowest concentration of serotonin was observed under the red LED, and it significantly increased when exposed to green LED (Figure 7). Karakatsouli et al. (2007), in their study of rainbow trout reared under LED light, reported that the level of albumin was lower when exposed to the long wavelengths, such as red LED, and higher when exposed to short wavelengths, such as blue LED. Since albumin is a transport protein carrying the serotonin precursor tryptophan (Sasaki et al. 1999), its higher levels in blood trigger the synthesis of serotonin by supplying tryptophan to the brain (Fenerty & Lindup 1989). In this study, when using red LED, increased photoreceptor through PRL treatment may be intended to decrease in the concentration of plasma albumin. There was decrease in the concentration of plasma serotonin in blood, as a result of reduced absorption of tryptophan in the brain. Because of increased sensitivity of the photoreceptors, plasma levels of serotonin are high when exposed to green LED wavelength (Figure 7).

In conclusion, we propose that the circadian rhythms are regulated by increased expression of clock genes, such as *Per2* and *Cry1*, due to an increase in the light sensitivity of photoreceptor by the PRL treatment. Consequently, in this study, we indicated that the hormones involved in circadian rhythm, such as melatonin and serotonin, could be regulated by PRL and wavelength. In particular, the green LED wavelength is able to regulate effectively the circadian rhythm by increasing the effect of PRL.

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