

The stimulatory effect of LED light spectra on genes related to photoreceptors and skin pigmentation in goldfish (*Carassius auratus*)

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Abstract This study aimed to assess differences in genes related to skin color of goldfish (*Carassius auratus*) exposed to light-emitting diodes (LEDs): red, green, and purple. We investigated differences in the expression of mammalian-like melanopsin (Opn4m), rhodopsin (RH), melanin-concentrating hormone (MCH), melanin-concentrating hormone receptor (MCH-R), and proopiomelanocortin (POMC) in goldfish exposed to different LED light spectra. Opn4m, RH, MCH, and MCH-R mRNA levels were significantly higher in the green and purple LED groups than in the white fluorescent bulb (control) and red LED groups. Furthermore, skin cells were isolated to measure the MCH-R mRNA expression levels. The results show that the mRNA expression levels were significantly higher in the green and purple LED groups than in the control and red LED groups. In addition, body weights in the green and purple LED groups were significantly higher than those in the control and red LED groups. However, POMC mRNA expression levels in the green and purple LED groups were significantly lower than those in the control and red LED groups. These results suggest that specific wavelengths regulate fish skin color through neuropeptide hormones and photoreceptors, and POMC,

which is related to stress hormones and melatonin, is associated with stress levels as well as skin color.

Keywords Melanopsin · Melanin-concentrating hormone (MCH) · Proopiomelanocortin (POMC) · Light-emitting diodes · Skin color

Introduction

Ornamental fish production is an important component of the aquaculture industry (Hargrove 1998; Wilson and Vincent 1998). In the United States, ornamental fish production is the fourth largest sector in aquaculture (Tlusty 2002). The skin color of ornamental fish is an especially important aspect of their appearance, and environmental factors such as light wavelength, intensity, salinity and temperature can affect skin color (Wardley 2001).

It is known that the spectral composition of incidental light is different in underwater environments and that rapid attenuation occurs with increasing depth. The short or blue end of the visible spectrum becomes predominant in deeper waters, whereas red light only penetrates in shallow waters (McFarland 1991). The emission of narrow bandwidths by various lighting types could be tuned into the environmental sensitivity of a target species (Lythgoe 1979). Various light wavelengths affect the growth, survival and stress responses of fish (Papoutsoglou et al. 2000;

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Cobcroft and Battaglene 2009). Fish live in environments with varied light intensity; thus, they possess photoreceptor cells adapted to their habitats (Lythgoe 1979).

Vertebrate photoreception relies on opsin- or retinal-based photoreceptors, which work by absorbing light through light-sensitive chromophores and driving changes in the membrane potential through G-protein signaling (Provencio et al. 1998). Melanopsin photoreceptors constitute a branch of the vertebrate opsin family, which is involved in nonvisual photoreception and was initially isolated from the photosensitive dermal melanophores of *Xenopus laevis* (Provencio et al. 1998).

In zebrafish (*Danio rerio*), melanopsin expression was observed in the retina (Bellingham et al. 2002), and the phylogeny of the melanopsin family was clarified by the recognition of two different melanopsins in nonmammalian vertebrates: the “*Xenopus*-like” melanopsins (Opn4x) and the “mammalian-like” melanopsin (Opn4m) (Bellingham et al. 2006). The Opn4m melanopsin is present in all vertebrates (Bellingham et al. 2006).

Also, light also controls the expression of the gene encoding rhodopsin (RH), a dim-light photoreceptor belonging to the G-protein-coupled receptor (GPCR) family (Khorana et al. 2002). RH is located in rod cells and transduces extracellular light signals into the cells (Yokoyama 2000; Terakita 2005). It consists of a protein, opsin, and a retinal chromophore and is the primary photoreceptor responding to external light signals in the retina (Deguchi 1981; Khorana et al. 2002).

These melanopsin and RH photoreceptors convey light information from the retina to the brain (Foster 1998; Shin et al. 2012a, b). The light information received regulates various genes in the brain; in teleost fish, melanin-concentrating hormone (MCH) has been suggested to play the role of a central neurotransmitter or neuromodulator, in addition to being a neurohypophyseal hormone (Batten and Baker 1988). In addition, MCH directly affects the concentration of melanin granules in the skin (Kawauchi et al. 1983; Castrucci et al. 1987; Baker and Bird 2002).

The other gene related to MCH that can cause changes in skin color is proopiomelanocortin (POMC), which is a precursor of adrenocorticotrophic hormone (ACTH) and melanin-stimulating hormones

(MSH). The major tissue that produces POMC is the pituitary gland (Takahashi and Kawauchi 2006). POMC secretes melanocortins, which have diverse functions that are involved in body color changes (Ebelre 1988), steroid synthesis (Larmers et al. 1992), and immunomodulation (Harris and Bird 2000).

Light-emitting diodes (LEDs), which represent a new form of lighting technology that is still being developed, can be manufactured to output-specific wavelengths (Migaud et al. 2007). Narrow bandwidth light using such new technologies, in particular, a high-energy, short wavelength, can most likely improve the efficiency of lighting systems compared to those currently used in the fish-farming industry. The potential for the improvement in the existing lighting systems is due to the fact that the emission of narrow bandwidths by the new lighting types can be tuned into the environmental sensitivity of a target species (Villamizar et al. 2009). Therefore, early molecular and physiological studies have analyzed the effects of LED spectra as one of various photic environmental factors using short wavelengths of LED (blue and green) as a light source for aquaculture fish (Shin et al. 2011; Choi et al. 2012).

To investigate the effects of specific LED wavelengths on the photoreceptors and skin pigment factors of fish, we observed the differences in Opn4m, RH, MCH, MCH-R, and POMC mRNA expression levels and MCH-R mRNA expression in skin cells. Therefore, we measured these factors to investigate the correlation with photoreceptors and skin pigment factors affecting color changes by endocrine changes in goldfish reared under fluorescent light or red, green, or purple LED lights for 6 months.

Materials and methods

Experimental fish and conditions

For each experiment, common goldfish ($n = 80$; length, 6.1 ± 0.5 cm; weight, 12.5 ± 0.4 g) were purchased from a commercial aquarium (Choryang, Busan, Korea) and were allowed to acclimate for 2 weeks in four 300-L circulation filter dark opaque tanks in the laboratory. Each tank represented an experimental group and housed 20 fish, and external light in outside of tanks was completely blocked. During the acclimation period, the fish were exposed to light from a white fluorescent bulb

(27 W); in addition, this type of light exposure was used for the control group. The light intensity near the water surface of the tanks was approximately 0.96 W/m^2 . The water temperature was $20 \pm 1 \text{ }^\circ\text{C}$, and the photoperiod consisted of a 12-h light (L):12-h dark (D) cycle (lights on 07:00 h and lights off 19:00 h, respectively). The fish were fed a commercial feed (SE WHA PET FOOD Co., Incheon, Korea) twice daily (09:00 h and 17:00 h) about 1 % of body weight. For the four experimental groups, the fish were exposed to either red (peak at 630 nm), green (530 nm), or purple (mixed 450 nm and 630 nm) LEDs (Daesin LED Co., Kyunggi, Korea), while the control group was exposed to a white fluorescent bulb (Fig. 1). The lights were set 50 cm above the surface of the water, and the irradiance at the surface of the water was maintained at approximately 0.9 W/m^2 . The fish were reared under these conditions until the day before the sampling. The spectral analysis of the lights was performed using a spectroradiometer (FieldSpec[®], ASD, Colorado, USA). The 80 fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) and then euthanized by spinal transection in 2-month sampling intervals (0, 2, 4, and 6 months) to collect retina, pituitary, and skin samples. Samples were collected under white dim light using an attenuated white fluorescent bulb.

Real-time quantitative PCR (RT-qPCR)

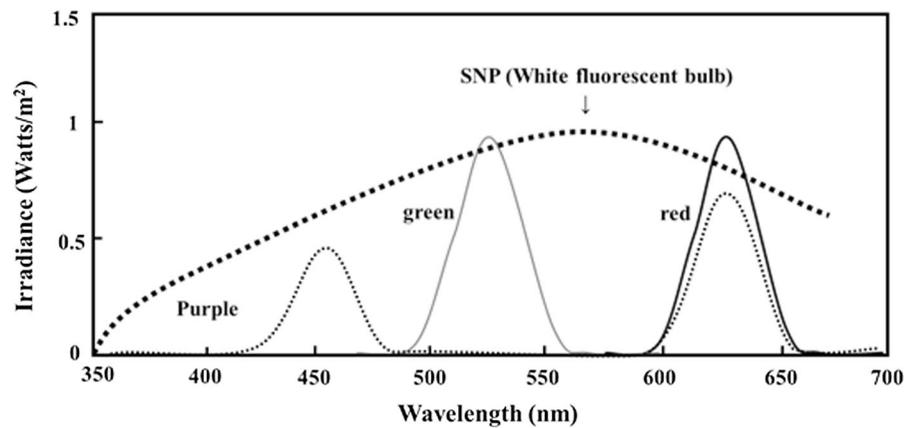
Total RNA (treated by DNase for removing genomic DNA) was extracted from the retina, pituitary, and skin using a TRIzol reagent (Gibco/BRL, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Two and a half 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 (Bioneer, Seoul, Korea) according to the manufacturer's instructions. RT-qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative expression levels of Opn4m (retina; GenBank accession no. KF040055), RH (retina; L11863), MCH (hypothalamus; AM403731), MCH-R (skin and skin cells; AB472590), POMC (pituitary; AJ431209), and β -actin (AB039726) mRNA using total RNA extracted from the retina, pituitary, skin, and skin cells. The following RT-qPCR primers were designed with reference to the known sequences of the goldfish:

Opn4m forward (5'-GCA TAC ACC ATC CTC CTC T-3') and reverse primers (5'-CCA CAG TCC AAC TCT CGT A-3'); RH forward (5'- ATG AAC GGT ACA GAG GGA GAT A-3') and reverse primers (5'-CAT TTC CGA CCT CTT CAT GGT-3'); MCH forward (5'-TAC TGT CCT CAT CTC TGT TGC-3') and reverse primers (5'-ATC TGG CTC CGT GTC TTC-3'); MCH-R forward (5'-AGA TCG TGA GCA CCT ACA-3') and reverse primers (5'-TAT GTG GTT GAA GCG GAT G-3'); POMC forward (5'-GGA GTG AGG ATG TTG TGT C-3') and reverse primers (5'-GAT GTT CTC GTC AGT GGT G-3'); and β -actin forward (5'-TTC CAG CCA TCC TTC CTA-3') and reverse primers (5'-TAC CTC CAG ACA GCA CAG-3'). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA), an iQTM SYBR Green Supermix (Bio-Rad, CA, USA), synthesized cDNA, primers, and distilled water according to the manufacturer's instructions. The RT-qPCR was performed as follows: One cycle of 95 $^\circ\text{C}$ for 5 min, followed by 35 cycles of 95 $^\circ\text{C}$ for 20 s and 55 $^\circ\text{C}$ for 20 s. Experiments were performed in duplicate using β -actin as an internal control, and all data were expressed relative to the corresponding β -actin calculated threshold cycle (ΔCt) levels. The efficiencies were found to be as follows: β -actin = 95.0 %, Opn4m = 94.1 %, RH = 95.4 %, MCH = 95.3 %, MCH-R = 96 %, and POMC = 95.5 %. The calibrated $\Delta\Delta\text{Ct}$ values ($\Delta\Delta\text{Ct}$) for each sample and for internal controls (β -actin) were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, [$\Delta\Delta\text{Ct} = 2^{-(\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{internalcontrol}})}$] (Livak and Schmittgen 2001).

Skin cell dispersion and RT-qPCR

Skin cell dispersion was performed according to the method described by Kobayashi et al. (2009). Skin on the dorsal fins was peeled from the fish and rinsed in Hanks' balanced salt solution (HBSS) (Biological Industries, Kibbutz Beit Haemek, Israel). The fin skin samples were allowed to stand for 20 min at room temperature in the dissociation medium [DM: 1 mg/mL collagenase type III (Worthington, Freehold, NJ, USA), 10^{-4} M epinephrine (Sigma-Aldrich), 2 mg/mL bovine serum albumin (Sigma-Aldrich), 0.1 mg/mL soybean trypsin inhibitor (Roche, Indianapolis, USA), and 5 U/mL DNase I (Takara)]. The samples were then gently agitated for 10 min in the same solution. The

Fig. 1 Spectral profiles of red (630 nm), green (530 nm), and purple [mixed blue (450 nm) and red (630 nm)] light-emitting diodes (LEDs) used in this study. The *filled square-dotted line* spectral profile of white fluorescent light (SNP; simulated natural photoperiod). Reprinted from ref. (Shin et al. 2011), with permission from comparative biochemistry and physiology, part-A



DM was removed, and the fin skin samples were rinsed three times with HBSS. Finally, during gentle agitation in the DM, dispersed skin cells (chromatophores) were isolated under a microscope using glass capillaries. Total chromatophores RNA was extracted 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-qPCR was performed using cDNA. RT-qPCR was conducted to determine the relative expression levels of MCH-R mRNA using total RNA extracted from the isolated chromatophores.

Statistical analysis

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

Results

Changes in body weights

The body weights of the fish under the green and purple LED conditions were significantly higher compared to those under other light conditions (Fig. 2). At the end of the experiment (end point at 6 months), the green and purple LED groups had the greatest body weight (25.2 ± 1.1 and 24.1 ± 1.0 g,

respectively), whereas the red LED and control groups had the lowest body weight (15.6 ± 0.5 and 18.8 ± 1.2 g, respectively).

Expression of Opn4m and RH mRNA in the retina

We examined the effects of the different light spectra on the expression of Opn4m and RH mRNA in the retina (Fig. 3). Opn4m mRNA expression levels in the green and purple LED groups increased significantly until 6 months, whereas the control and red LED groups had significantly lower values than the green and purple LED (Fig. 3a).

RH mRNA expression levels in the green and purple LED groups increased significantly at 2 months and then maintained, whereas the control and red LED groups had no significant differences between months and significantly lower values than the green and purple LED (Fig. 3b).

Expression of MCH mRNA in the hypothalamus

MCH mRNA expression levels in all spectra groups significantly increased until 6 months, but the levels in the green and purple LED groups were significantly higher than those in the groups exposed to the control and the red LED (Fig. 4).

Expression of MCH-R mRNA in the skin and in the chromatophores

We observed the effects of different light spectra on the expression of MCH-R mRNA in the skin and in the chromatophores (Fig. 5). MCH-R mRNA expression

Fig. 2 Changes in the body weight of goldfish reared under a white fluorescent bulb or under red, green, or purple light-emitting diode lights. Values with different characters are significantly different at different times (months) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same months ($P < 0.05$). All values are the mean \pm SD ($n = 5$)

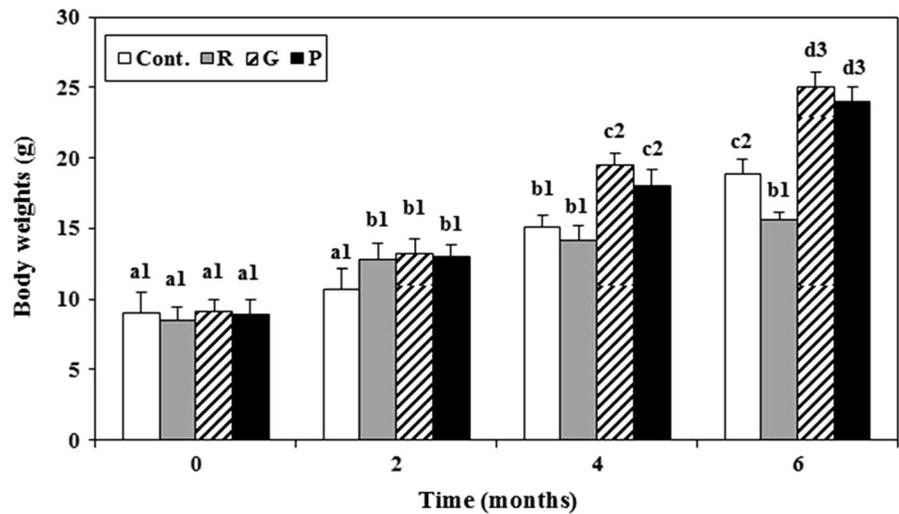
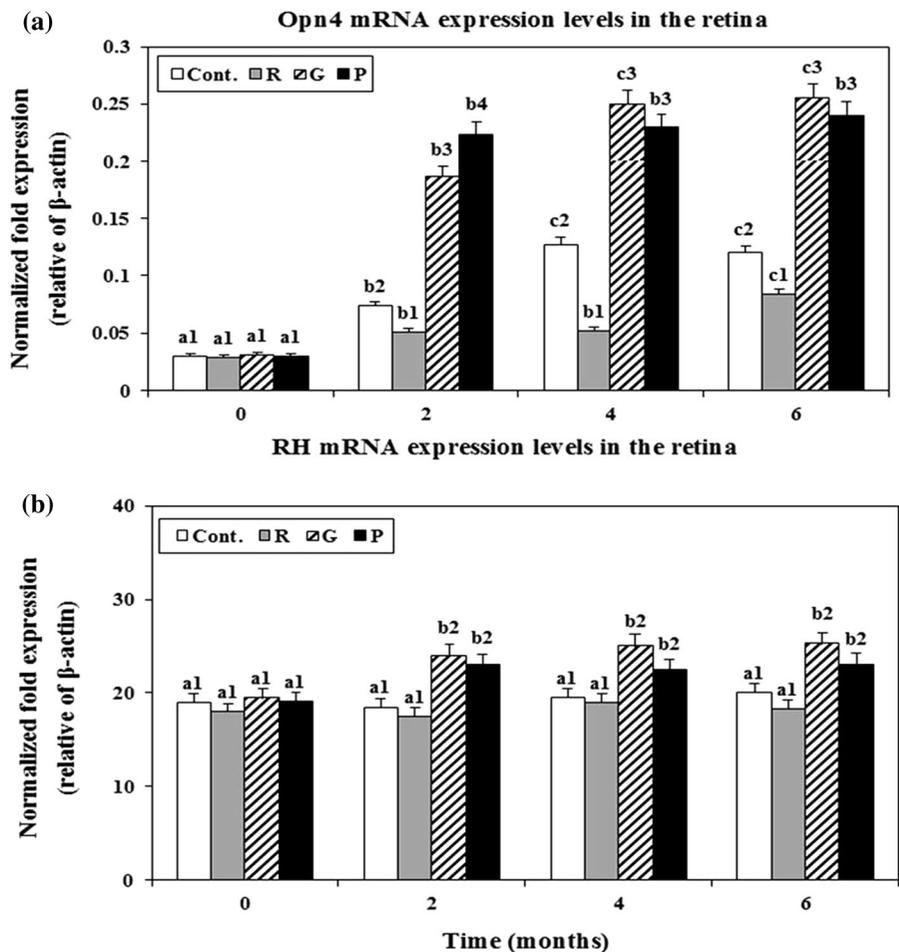


Fig. 3 Changes in the expression levels of Opn4m (a) and RH (b) mRNA in the retinas of goldfish under lighting conditions using red (R), green (G), and purple (P) LEDs and a white fluorescent bulb (cont.), as measured by quantitative real-time PCR. The results were expressed as fold expression levels normalized with respect to the β -actin levels in the same sample. Values with different characters are significantly different at different times (months) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same months ($P < 0.05$). All values are mean \pm SD ($n = 5$)



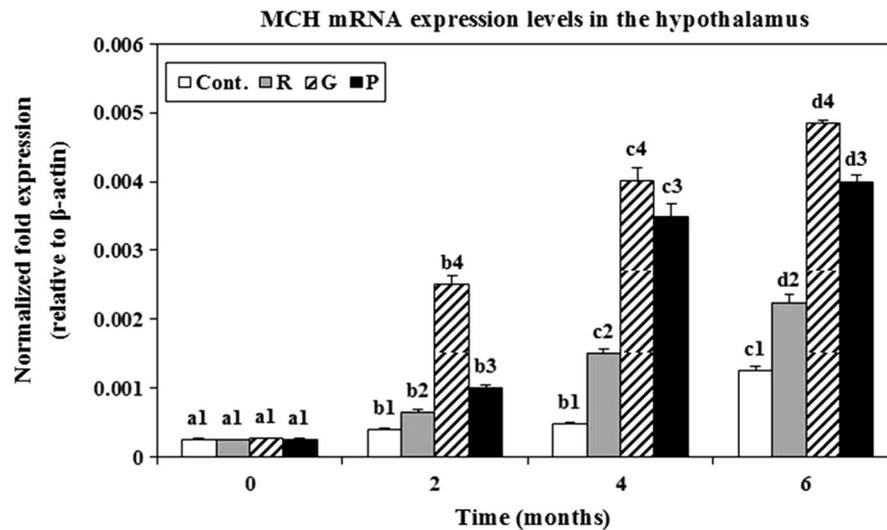


Fig. 4 Changes in the expression levels of MCH mRNA in the hypothalamus of goldfish under lighting conditions using red (R), green (G), or purple (P) LEDs or a white fluorescent bulb (cont.), as measured by quantitative real-time PCR. The results are expressed as fold expression levels normalized with respect to the β -actin levels in the same sample. Values with different

characters are significantly different at different times (months) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same months ($P < 0.05$). All values are the mean \pm SD ($n = 5$)

levels in the green and purple LED groups were significantly higher than those in the groups exposed to the control and the red LED (Fig. 5a).

MCH-R mRNA expression levels in all spectra groups significantly increased until 6 months, but these mRNA levels in the green and purple LED groups were significantly higher than those in the groups exposed to the control and the red LED (Fig. 5b).

Expression of POMC mRNA in the pituitary

We observed the effects of different light spectra on the expression of POMC mRNA in the pituitary (Fig. 6). POMC mRNA expression levels in all spectra groups increased significantly until 6 months, but the levels in the green and purple LED groups were significantly higher than those in the groups exposed to the control and the red LED.

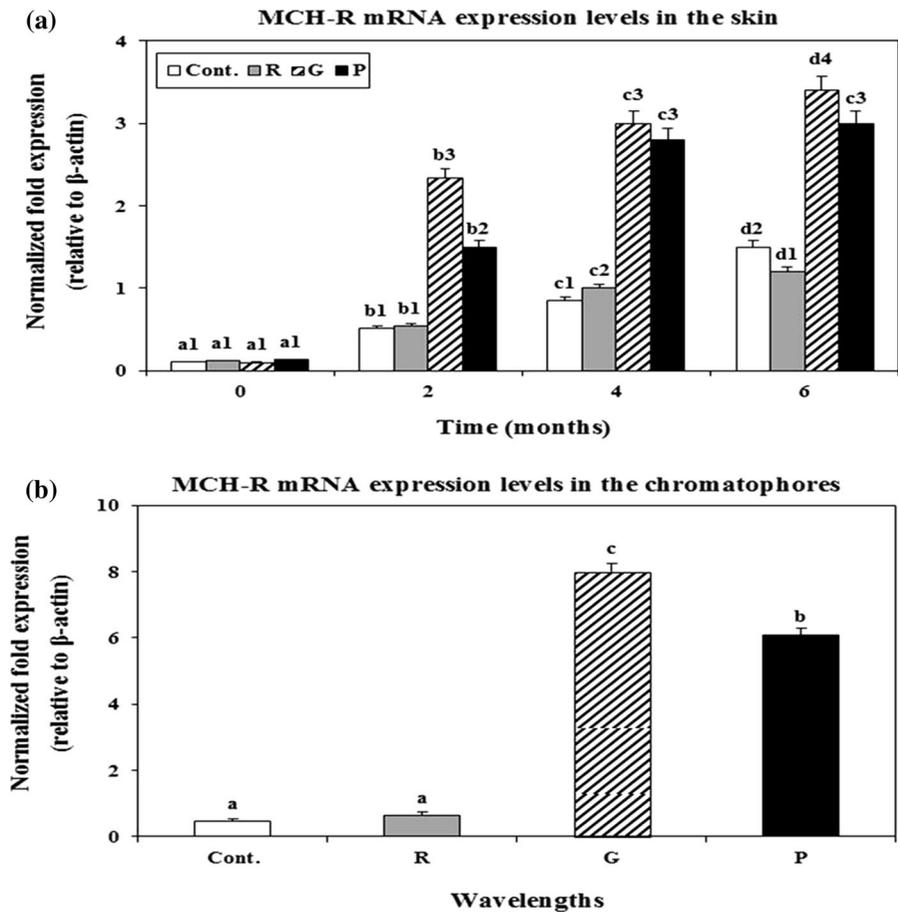
Discussion

To investigate the effect of specific LED wavelengths on hormones that are secreted by the brain and the skin and are associated with changes in the body color of

goldfish, we examined the mRNA levels of Opn4m and RH in the retina, MCH in the hypothalamus, POMC in the pituitary, and MCH-R in the skin and chromatophores by exposing goldfish to a white fluorescent bulb or to red, green or purple LEDs for 6 months.

We observed that the mRNA expression levels of melanopsin, Opn4m, and RH in the retina increased significantly in all experimental groups: The levels in the green and purple LED groups in particular were significantly higher than those of the other groups (Fig. 3). Bellingham et al. (2002) reported that melanopsin was expressed mainly in the retinal ganglion cells (RGC) of zebrafish; it was expressed by the retinohypothalamic tract (RHT), which conveys external light information to the brain (Hattar et al. 2006). Furthermore, RGC comprising the RHT express Opn4 mRNA, and these cells are photosensitive (Berson 2003). Opn4 knockout studies indicate altered circadian photoentrainment and decreased pupillary constriction in response to pulses of light (Hattar et al. 2003). Third, biochemical data suggest that Opn4 is capable of forming a functional photopigment and of activating transducin (Newman et al. 2003). Also, Davies et al. (2011) reported that when Opn4m photopigment can convert all-trans retinal to a cis-

Fig. 5 Changes in the expression levels of MCH-R mRNA in the skin (a) and chromatophores (b) of goldfish under lighting conditions using red (R), green (G), or purple (P) LEDs or a white fluorescent bulb (cont.), as measured by quantitative real-time PCR. The results are expressed as fold expression levels normalized with respect to the β -actin levels in the same sample. Values with different characters represent significant differences between different light spectra ($P < 0.05$). All values are the mean \pm SD ($n = 5$)



isomer that is, in turn, used by co-expressed melanopins to generate a more pronounced light-dependent inward current.

Studies of green photoreceptors in *Apis mellifera* (*Am*) lacking screening pigments indicate an RH with maximal sensitivity at about 526 nm in these cells (Gribakin 1988). The putative *Am* RH cDNA sequence shows the greatest identity to the green mantid RH, which is thought to have wavelength sensitivities in the green (Towner and Gartner 1994). Chang et al. (1995) reported that comparative analysis of RH sequences has proven useful in identifying residues involved in determining RH absorption spectra.

Based on this theory, we hypothesized that the reason the *Opn4m* and RH gene expression levels detected in the retina were significantly higher in the green and blue LED groups was that different LED wavelength lights are detected differently by fish. The short wavelength, or blue, end of the visible spectrum is predominant in deeper waters, whereas red light

only penetrates shallow waters (McFarland 1991). Together, these data support the hypothesis that *Opn4m* may be involved in detection of amount of light according to specific photic environments. Also, 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. Therefore, fish detected the large amount of light in short wavelengths, the green and purple lights, because they penetrate deeper waters than the long wavelengths, such as red light; therefore, we hypothesized that *Opn4m* expression levels would be significantly higher in the green and purple LED groups.

Opn4m in the retina detects light and conveys the detected light information to the brain (Foster 1998). Therefore, when the increased of *Opn4m* expression levels in green and purple LED groups, we hypothesized that it is regulated by hormones and genes related to the circadian rhythm and the processing of light information well in green and purple LED groups.

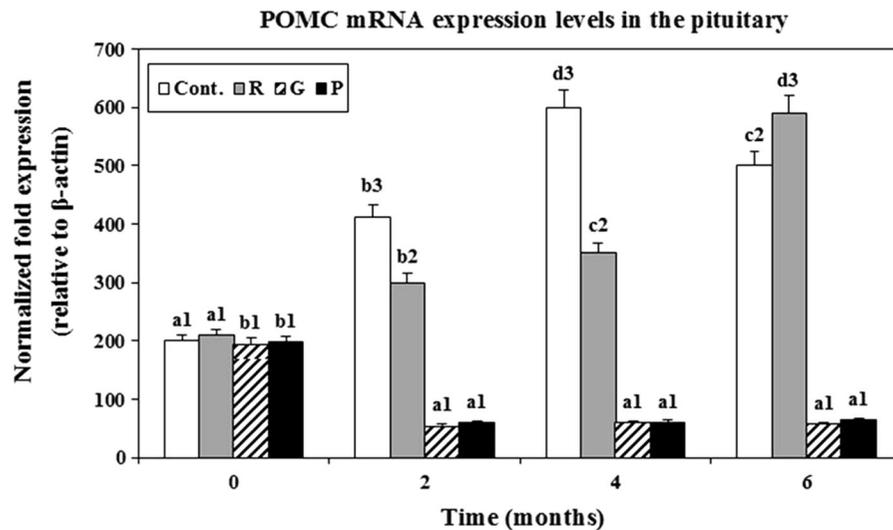


Fig. 6 Changes in the expression levels of POMC mRNA in the pituitary of goldfish under lighting conditions using red (R), green (G), or purple (P) LEDs or a white fluorescent bulb (cont.), as measured by quantitative real-time PCR. The results are expressed as fold expression levels normalized with respect to the β -actin levels in the same sample. Values with different

characters are significantly different at different times (months) in fish exposed to the same light spectrum ($P < 0.05$). The numbers indicate significant differences between different light spectra within the same months ($P < 0.05$). All values are the mean \pm SD ($n = 5$)

In addition, we examined the mRNA expression levels of MCH and MCH-R in the hypothalamus and the skin, respectively. The levels found in the green and purple LED groups were significantly higher than those found in the other groups (Figs. 4 and 5a). Furthermore, MCH-R mRNA expression levels in the chromatophores showed patterns similar to the levels in the skin (Fig. 5b).

Amano and Takahashi (2009) reported that MCH is closely involved with background color. The body color of teleost fish is paler against a white background than against a black background (Cerdá-Reverter et al. 2006). In addition, in tilapia (*Oreochromis mossambicus*), rainbow trout (*Oncorhynchus mykiss*), barfin flounder (*Verasper moseri*), and goldfish, MCH expression levels in the brain were higher in fish reared in environments with white backgrounds than those reared in environments with black backgrounds (Gröneveld et al. 1995; Suzuki et al. 1995; Takahashi et al. 2004; Cerdá-Reverter et al. 2006). We found that fish detected a large amount of light in short wavelengths, such as green and purple lights, because these wavelengths penetrate into deeper waters than long wavelengths, such as red light. The results of this study agree with studies of fish reared in tanks with white backgrounds.

Over the 6 months of the study, we observed the changes in body weights. The body weights increased significantly in the green and purple LED groups (Fig. 2). Villamizar et al. (2009) observed that the growth rate and feeding performance of fish larvae were significantly increased under a blue light treatment compared to those resulting from red or white light treatments. These results suggested that blue wavelengths stimulate the visual system sufficiently for the larvae to feed well and that short wavelengths, such as green and blue, positively affect adult fish growth as well as larval growth. Therefore, light of particular wavelengths can stimulate light perception systems and subsequently modulate the neuroendocrine network involved in the feeding behavior or somatic growth of fish (Yamanome et al. 2009). Therefore, MCH expression levels were changed in response to specific wavelengths, which can contribute to body color changes via the aggregation of melanin granules. Thus, given that the production of MCH may be changed by a specific wavelength of light, other hypothalamic peptides related to food intake may also be influenced.

In this study, we found that the expression levels of POMC mRNA that are related to body color were significantly increased in both the control and red LED

groups (Fig. 6). The POMC mRNA levels of barfin flounder adapted to a black background showed the highest pituitary content (Kobayashi et al. 2009). In addition, POMC is a precursor of ACTH and is very closely linked with stress responses. Also, POMC is the common precursor for melanocyte-stimulating hormone (MSH) (Takahashi and Kawauchi 2006), and the release of MSH is modified by the change in background color (Ebelre 1988). According to this theory, we hypothesized that POMC and MSH are suggested to be involved in the regulation of skin color by various photic environments. The POMC mRNA expression levels in the control and red LED groups were significantly higher than the levels found in the green and purple LED groups, indicating that the stress levels in fish exposed to the control and the red LED were significantly higher than the stress levels of fish in green and purple LED groups.

Shin et al. (2011) reported that the oxidative stress values in yellowtail clownfish (*Amphiprion clarkii*) illuminated under a white fluorescent bulb or a red LED were significantly greater than those of other groups. Therefore, POMC secreted melanocortin, which caused changes in body color and is closely related with stress response.

In conclusion, we hypothesized the following: (1) short wavelengths such as those emitted by green and purple LEDs stimulate the *Opn4m* and *RH* gene in the retina, thus conveying light information of various wavelengths to the brain and enhancing MCH-R in the skin by regulating MCH and POMC genes related to body color. (2) Green and purple LED lights regulate stress, enhance growth and body color, and affect the endocrine changes in fish by regulating the expression of genes related to body color.

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