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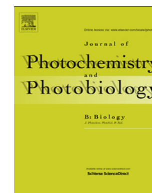

Light-emitting diode spectral sensitivity relationship with reproductive parameters and ovarian maturation in yellowtail damselfish, *Chrysiptera parasema*

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ABSTRACT

The present study investigated the effects of exposure to different light spectra and intensities on ovarian maturation in yellowtail damselfish, *Chrysiptera parasema* over a 4-months period. We used a white fluorescent bulb and three different light-emitting diodes (LEDs: red, peak at 630 nm; green, 530 nm; blue, 450 nm), at three different intensities each (0.3, 0.6, and 0.9 W/m^2). The effects of different illuminations were assessed by measuring the mRNA and protein expressions of vitellogenin (VTG) and estrogen receptor (ER), gonadosomatic index (GSI), and plasma estradiol-17β (E_2) hormone level. For green and blue lights, significantly higher levels of VTG and ER expressions, GSI, and plasma E_2 were obtained, compared to the other light spectra. Histological analysis revealed the presence of vitellogenic oocytes in fish exposed to short wavelengths (green and blue) light. In addition, we observed significantly greater ovarian maturation in fish exposed to low and medium light intensities. The results indicate that exposure to green low intensity lighting accelerates gonadal maturation, and is likely to facilitate development of more energy-efficient aquaculture procedures.

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1. Introduction

The influence of environmental factors on the growth and reproduction of fish has been extensively studied [1]. It is well known that light and temperature are among the most important natural environmental factors that regulate reproduction in fish. Lighting characteristics including wavelength (quality), intensity (quantity), and periodicity (daily cycle) are among factors that regulate seasonally dependent changes in reproductive and growth physiology of fish [1]. The reproductive physiology of fish is closely related with the perception of environmental factors by the sensory systems and the transduction of suitable signals to the hypothalamo–pituitary–gonadal axis [2,3]. The spectral composition (quality) of incident light are key properties affecting the physiological response of teleosts with, among others, effects on growth, reproduction, behavior and stress documented [1].

In various reproductive hormones, estrogen is an essential steroid hormone in reproduction, playing an important role in sexual maturation and differentiation, including oogenesis, vitellogenesis, and testicular development [4]. Estrogen activity is mediated by nuclear estrogen receptors (ERα and ERβ), and ERα is a member of a superfamily of transcription factors that induce target gene expression by binding *cis*-acting enhancer elements located in the promoter region of their responsive genes [5]. Furthermore, the induction of hepatic vitellogenin (VTG), which is a precursor yolk protein, in response to estrogens by an ER-mediated pathway has been well documented in several oviparous fish species [6,7]. Thus, VTG and ER might serve as indicators of reproduction and maturation in fish.

The application of artificial lighting in recirculating aquaculture systems requires appropriate combination of light hours (photoperiod), intensity and spectrum. There are numerous data related to photoperiod and light intensity effects on several farmed fishes and life stages [1]. In most studies fluorescent lamps are used, resulting in what humans perceive as white light, despite the fact that in natural fish habitat, wavelength of light penetrating water varies greatly, fish vision and spectrum perception are strongly adapted to each species natural habitat and living ethology [8], and recent studies indicate that light spectrum affects farmed fish growth performance [9], behavior [10] and physiological status [9].

To date, it has been shown that periodicity is a crucial determinant of reproductive success in fish, with extensive studies on its importance in the initiation and termination of gonadal

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development [11,2,3]. Also, the effects of light-intensity have been well studied over recent years and findings clearly suggest that exposure to threshold intensity levels is required to manipulate physiological functions in various teleosts [12–15]. Hence, it is important to evaluate the impact of different types of lighting on reproduction.

Metal halide bulbs are the present source of underwater artificial lighting used in the industry, but in many aspects they are not suitable for fish farming as they are neither environment nor species specific. They create a bright point source of light, involve high running costs and much of their light energy is wasted in the form of unsuitable wavelengths (i.e. longer wavelength yellow–red light) which are rapidly absorbed in the water column and therefore cannot be detected by fish [15,16]. Light-emitting diodes (LEDs) can output light at specific wavelengths [17]. Furthermore, LEDs have lower power requirements, electrical running costs and a longer life span than standard metal halide bulbs [17]. Narrow bandwidth high-energy short wavelength light may improve the efficiency of lighting systems compared to those currently used in the fish farming industry since it can be tuned more specifically in line with sensitivity of a target species [18]. Furthermore, it is known that the spectral composition of incidental light is differentially affected in underwater environments, and that rapid attenuation occurs with increasing depth [19].

Recently, several studies have investigated the utility of LED lights as photo-environmental factors, using different LED light wavelength light sources for aquaculture. For instance, Shin et al. [20] reported that green and blue light-emitting diodes (LEDs), which have short wavelengths, increased the level of antioxidants in response to oxidative stress in the yellowtail clownfish *Amphiprion clarkii*. In addition, Volpato and Barreto [21] reported that blue spectrum prevents stress in Nile tilapia *Oreochromis niloticus*. Meanwhile, red LED wavelength affects physiological function, and was found to induce oxidative stress in yellowtail clownfish [20]. However, studies on the effect of LED light wavelengths on fish reproduction remain very limited [22,23].

For energy-savings and the way to enhance the gonad development, in the present study, we examined the effects of LEDs on sexual maturation and development in yellowtail damselfish, *Chrysiptera parasema*. This species is a reef-associated damselfish that is widely distributed in shallow waters. It has commercial value as an ornamental fish and is widely used as a scientific experimental model. We investigated the effect of different types of lighting on ovarian maturation in this species. Fish were reared for 4 months under 3 LED wavelengths and three lighting intensities. Changes in the expression of VTG and ER mRNA, as well as expression of VTG and ER proteins were investigated. In addition, ovarian development was evaluated by measuring steroid hormone (estradiol-17β [E_2]) levels, and by determining oocyte development in relation to histological indices of gonadal maturation.

2. Materials and methods

2.1. Experimental fish

The immature yellowtail damselfish ($n = 600$; total length, 2.1 ± 0.4 cm; mass, 1.1 ± 0.2 g) were purchased from a commercial store. Fish length and weight were measured swiftly when the fish were divide to each experimental tanks, and then fish were allowed to acclimate for 1 week in 300-L circulation filter tanks with circular filtration prior to laboratory-based experiments under 12-h light:12-h dark photoperiod (lights on at 07:00 and light off at 19:00) using white fluorescent bulb at 27 °C. The water temperature and photoperiod were 27 ± 1 °C, with a 12L:12D photoperiod, and fed commercial feed twice daily (at 09:00 and 17:00).

The fish were exposed to a white fluorescent bulb (27 W; a simulated natural photoperiod; SNP) was used for the control group. In the experimental groups, fish were exposed to either blue (peak at 450 nm), green (530 nm), or red (630 nm) LEDs (Daesin LED Co. Kyunggi, Korea) for 4 months (Fig. 1). The LEDs were set 50 cm above the water surface, and irradiance at the water surface was maintained at approximately 0.3, 0.6, and 0.9 W/m^2, respectively (Fig. 1).

2.2. Sampling

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

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

2.3. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of VTG (accession No. JQ906787) and ERα (JQ906788) mRNA, using total RNA extracted from the liver of yellowtail damselfish, respectively. Primers for QPCR were designed in reference to known yellowtail damselfish sequences as follows: VTG forward primer (5′-ACC CGT CAG TGC TCA GTA-3′), VTG reverse primer (5′-TCG CTG CTG GTC TTA ATC A-3′), ERα forward primer (5′-TGA CTA GCA TGT CTC CTG AT-3′), ERα reverse primer (5′-ATG GTG ACC TCG GTG TAA-3′), β-actin forward primer (5′-GCA AGA GAG GTA TCC TGA CC-3′), and β-actin reverse primer (5′-CTC AGC TCG TTG TAG AAG G-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad, CA, USA), according to the manufacturer's instructions. QPCR was performed as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s and 55 °C for 20 s [24]. As internal controls, the experiments were duplicated with β-actin calculated threshold cycle (Ct) levels. The calibrated ΔCt value (ΔΔCt) for each sample and internal control (β-actin) was calculated using the formula: [$\Delta\Delta Ct = 2^{\wedge}-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})$] [25].

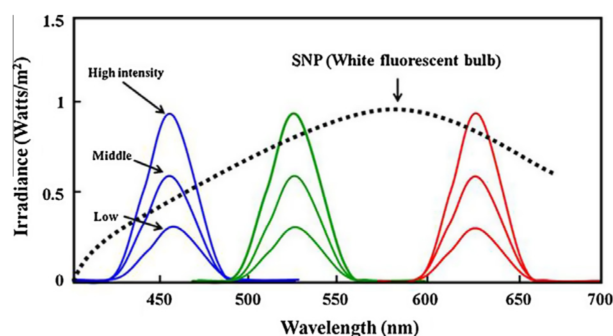

Fig. 1. Spectral profiles of the blue (B), green (G), and red (R) LEDs. Low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) light intensities were used for each type of LED in this study. Square dotted line shows the spectral profile of a white fluorescent light. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Western blot analysis

Total protein isolated from the liver of yellowtail damselfish was extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg/mL phenylmethylsulfonyl fluoride (PMSF) and 0.15 mg/mL leupeptin). It was then sonicated, and quantified using the Bradford method (Bio-Rad, CA, USA). In each lane, total protein (30 μg) was loaded onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. For reference, a protein ladder (Fermentas) was used. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel until the bromophenol blue dye front had run off the gel. The gels were then immediately transferred to a 0.2-μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad, CA, USA) at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5% milk in Tris-buffered saline (TBS) (pH 7.4) for 45 min followed by washing in TBS. The membranes were incubated with VTG (ABIN326357, 1:2000 dilution, Antibodies-online, USA), followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (dilution 1:2000; Bio-Rad, CA, USA). A separate set of membranes were incubated with ERα (E1528 1:2000 dilution; Sigma, USA), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1:2000; Bio-Rad, CA, USA) for 60 min. The internal control was β-tubulin (dilution 1:5000, ab6046; abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000; Bio-Rad, CA, USA) for 60 min. Bands were detected using a standard ECL system, in addition to the more sensitive ECL system (ECL Advance; GE Life Sciences, Sweden), and were exposed to autoradiography-sensitive film for 2 min.

2.5. Gonadosomatic index (GSI) and gonadal histology

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

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

In accordance with oocyte staging of the white-spotted spinefoot, *Siganus canaliculatus*, and sapphire devil, *Chrysiptera cyanea*, oocytes in the ovary of yellowtail damselfish were classified into the following stages: peri-nucleolus (PNS), primary yolk stage (PYS), secondary yolk stage (SYS), and tertiary yolk stages (TYS) [23,26].

2.6. Analysis of plasma parameters

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

2.7. Statistical analysis

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

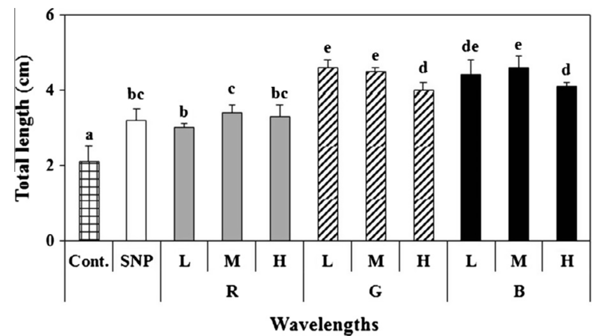

Fig. 2. Changes in the total body length of yellowtail damselfish, which were reared for 4 months under a simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LED lights. Low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) light intensities were used for each different LED light type. Cont. (control) indicates the initial total body length at the start of experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

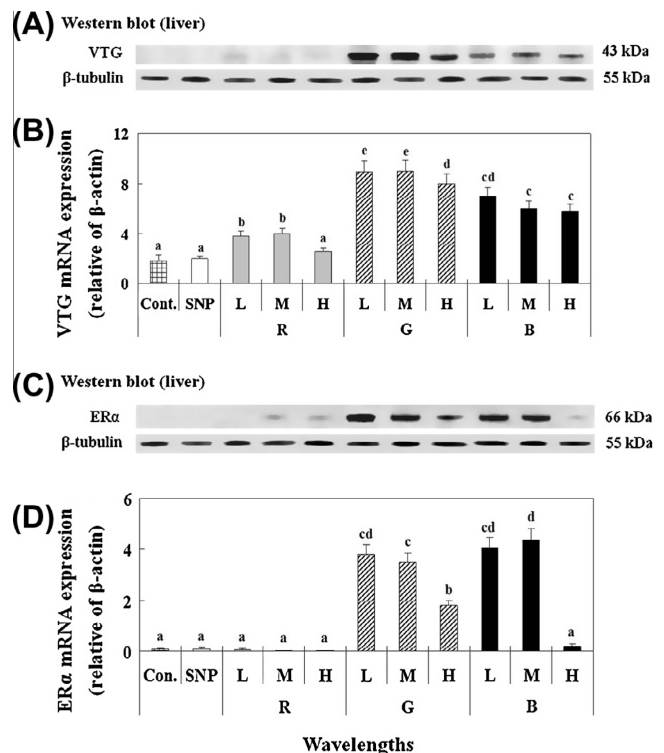

Fig. 3. VTG and ERα mRNA expression levels in the liver of yellowtail damselfish under lighting conditions using a simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LEDs at low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) light intensities. Cont. (control) indicates initial VTG and ERα mRNA levels at the start of the experiment. Western blotting using VTG (43 kDa) (A) and ERα (66 kDa) (C) to examine protein expression in the liver of yellowtail damselfish. The 55 kDa β-tubulin was used as the internal control. VTG (B) and ERα (D) mRNA levels relative to β-actin mRNA levels in the liver and gonads of yellowtail damselfish under lighting conditions using a simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LEDs at low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) lighting intensities, as measured by quantitative real-time PCR. The fish were reared under a light:dark (LD) cycle (12:12). Total liver RNAs (2.5 μg) were 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 letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum ($P < 0.05$). All values are means ± SD ($n = 30$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Total body length

The total body lengths of fish reared under green and blue LED conditions were significantly greater compared to those of fish reared under other light conditions (Fig. 2). The green (4.6 ± 0.2 cm; low) and blue (4.6 ± 0.3 cm; medium) LED groups exhibited the greatest total body lengths, while the red (3.4 ± 0.2 cm; medium) LED and SNP (3.2 ± 0.3 cm) groups exhibited the shortest total body lengths.

3.2. VTG and ER expression in the liver

In the green and blue LED groups, the expression levels of VTG and ERα mRNA were significantly higher than those in fish exposed to other light spectrums (Fig. 3). Especially, the expressions under low and medium intensity were significantly higher than those under high intensity. Western blot analysis identified two protein bands VTG and ERα-immunoreactive proteins corresponding to predicted mass for yellowtail damselfish VTG (43 kDa) and ERα (64 kDa). The expression pattern of the immunoreactive proteins resembled that of VTG and ERα transcript levels in the yellowtail damselfish liver (Fig. 3A and C).

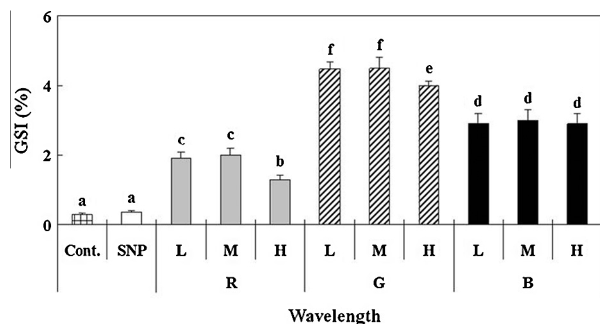

Fig. 4. Changes in the gonadosomatic index (GSI) of yellowtail damselfish under lighting conditions using a simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LEDs at low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) light intensities. Cont. (control) indicates initial total fish body length at the start of the experiment. Values with letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum ($P < 0.05$). All values are means ± SD ($n = 30$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

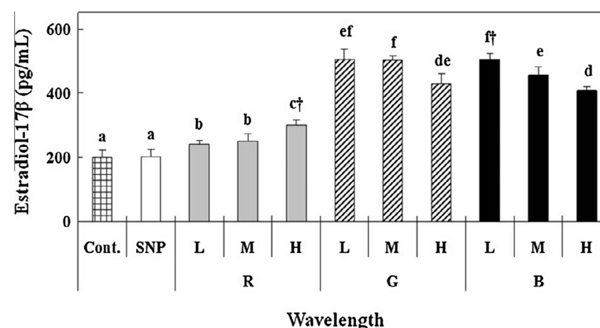

Fig. 6. Plasma E_2 hormone levels of yellowtail damselfish under lighting conditions using a simulated natural photoperiod (SNP), as well as red (R), green (G), and blue (B) LED lights at low (*L*, 0.3 W/m^2), medium (*M*, 0.6 W/m^2), and high (*H*, 0.9 W/m^2) light intensities. Cont. (control) indicates initial estradiol-17β (E_2) level at the start of the experiment. Values with letters indicate significant differences among lights of different wavelengths. The cross (†) indicates significant differences in light intensity within the same spectrum ($P < 0.05$). All values are means ± SD ($n = 30$).

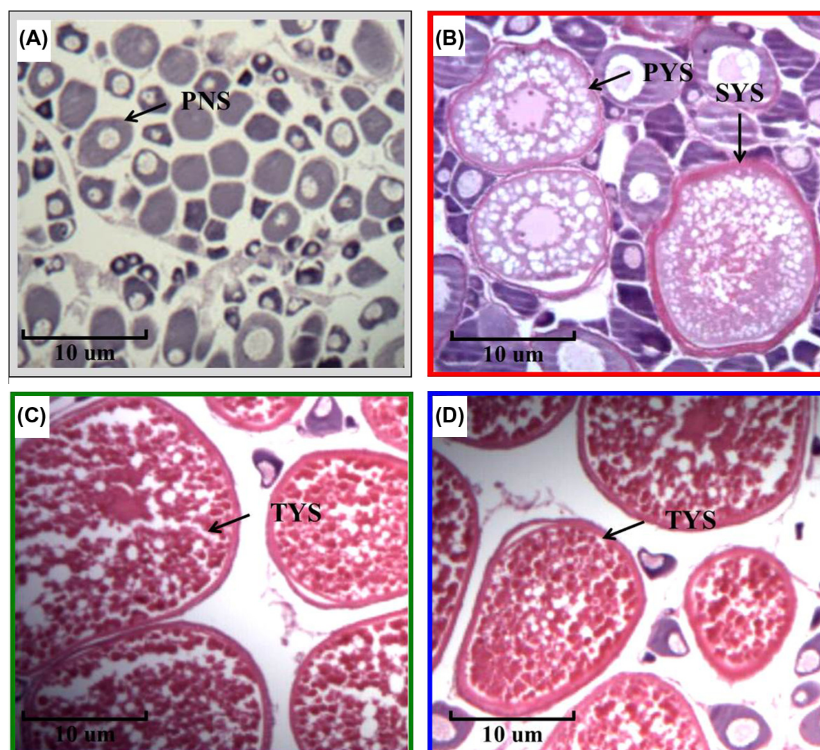

Fig. 5. Changes in cross section of the ovary histology of yellowtail damselfish under different lighting conditions using a simulated natural photoperiod (SNP) (A), as well as red (B), green (C), and blue (D) LED lights at low (*L*, 0.3 W/m^2) light intensity. Scale bar = 10 μm. PNS; peri-nucleolus stage, PYS: primary yolk stage, SYS: secondary yolk stage, TYS; tertiary yolk stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. GSI and histological observation

The GSI values of fish exposed to green (4.5 ± 0.3; low) and blue (3.1 ± 0.3; low) LED lights were significantly higher than that of the SNP group (0.38 ± 0.03). Furthermore, the GSI values at low intensity were significantly higher than those observed at medium or high intensity lights in the LED groups (Fig. 4). To investigate gonadal morphology, we performed histological studies of gonadal samples as shown in Fig. 5. The gonads of all fish in the SNP group contained only immature oocytes at peri-nucleolus stage (Fig. 5A), and red LED group contained oocytes at primary yolk and secondary yolk stages (Fig. 5B). In contrast, well-developed vitellogenic oocytes and most of them were at tertiary yolk stage in the green and blue LED groups (Fig. 5C and D).

3.4. Plasma E_2 levels

Positive correlations were observed between circulating E_2 levels and gonadal development in the yellowtail damselfish. The plasma concentration of E_2 in fish exposed to green (503 ± 35 pg/mL; low) and blue (504 ± 16.7 pg/mL; low) LED lights were significantly higher than that of the SNP group (202 ± 20.2 pg/mL) (Fig. 6).

4. Discussion

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

The results demonstrate that total yellowtail damselfish body length can be increased significantly when exposed to green and blue wavelengths for 4-months. Our results are consistent with a report demonstrating increased growth in barfin flounder, *Verasper moseri*, exposed to green and blue lights (fluorescent lamp) [27]. In addition, Shin et al. [28] reported significantly higher levels of growth hormone and total body length in yellowtail clownfish, *A. clarkii*, reared under green and blue LED light compared to fluorescent light and red LED.

In the present study, we observed significantly higher expressions of VTG and ERα in yellowtail damselfish under green and blue LED groups compared to the other groups. This is consistent with observed higher circulating level of E_2, GSI and histological characteristics in the same groups. The present results provide a strong support for the hypothesis that exposure of fish to short green and blue wavelengths enhance ovarian maturation. This hypothesis is further supported by the observed mature oocytes (tertiary yolk stage) in the green and blue LED groups. The present results are in accordance with previous report by Volpato [29], the study demonstrated that enhancing reproductive performance of hormone-induced Matrinxa fish, *Brycon cephalus*, and increasing spawning rate in female fish reared under green light. These results collectively demonstrate that exposure to short wavelength lights increase reproductive capacity in cultured fish.

However, little information is available on the mechanisms underlying short wavelength light-induced enhancement of ovarian maturation. It is possible that a single pigment with wide spectral sensitivity, or several photopigments, may be involved in the transduction of exogenous photic stimuli in fish. Furthermore, specific photoreceptors may be involved in this process. It is interesting to note that Urasaki [30] and Garg [31] reported that the extent of gonadal development is lower in blind and pinealectomized fish, and suggest possible mediation of eyes and extra-retinal photoreceptors.

We suggest that a physiological response is involved in the mechanisms linking specific light wavelengths with fish gonadal maturation. It should also be noted that light is closely connected with stress response in fish. Studies by Shin et al. [20] demonstrated lower lipid peroxidation (LPO) and H_2O_2 levels in fish exposed to green and blue lights compared to fish exposed to other spectra. It was suggested that short green and blue LED wavelengths may inhibit oxidative stress in fish compared to those exposed to other light spectra. Furthermore, Volpato and Barreto [21] observed lower cortisol levels in fish exposed to blue spectra. In stressed Nile tilapia, *O. niloticus*, lower level of cortisol was observed in fish exposed to blue light, for 48 h, compared to fish exposed to other light spectra. The findings suggest that certain light spectra may affect the activity of pituitary–adrenal axis, and related physiological parameters.

In conclusion, exposure of fish to short wavelength light lower stress response, enhance the immune function, and enhance gonadal development. In the present study, we demonstrated that low intensity LED lighting significantly increased the expressions of VTG and ERα, increase plasma levels of E_2, and enhance body growth and oocyte maturation. Our findings support the hypothesis that the use of green and blue wavelengths LEDs would be valuable by improving immunity and reproductive ability in cultured fish. Further studies will be required to understand the mechanisms regulating the reproductive response in fish by analyzing photoreceptors connecting reproduction to different light spectra and intensities.

Acknowledgements

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