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Effect of long-afterglow phosphorescent pigment on reproductive parameters and ovarian maturation in the yellowtail damselfish, *Chrysiptera parasema*

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ABSTRACT

Photoperiod is considered the most important factor that entrains animal rhythms, including the reproductive cycle. The present study tested differences in sex maturation and sex steroid hormones of yellowtail damselfish (*Chrysiptera parasema*) exposed to a white fluorescent bulb (12L:12D and 14L:10D) or long-afterglow phosphorescent pigment (LumiNova sheet) for 4 months. At the end of the experiment, in the phosphorescent group, mRNA expressions of gonadotropin hormones [(GTHs, including gonadotropin (GTH) α and luteinizing hormone (LH) β)], estrogen receptor (ER), and vitellogenin were significantly higher than in the photoperiod groups (12L:12D and 14L:10D), and these results are consistent with those of Western blotting for protein expression. Furthermore, in the phosphorescent group, plasma FSH, LH, and estradiol-17β (E_2) levels were significantly higher than in the photoperiod groups. However, plasma melatonin levels were significantly lower than in the photoperiod groups. Because LumiNova sheets continue to emit green light (520 nm) for approximately 2 h after sunset, the extended light conditions probably contributed to reproductive ability in the experimental fish. In conclusion, long-afterglow phosphorescent pigment can be used for energy-efficient aquaculture to regulate the reproduction of fish, although its effect needs to be evaluated in other species.

1. Introduction

The timing of sexual maturation and spawning in fish may be affected by artificial endocrine regulation and environmental parameters, such as water temperature, photoperiod, and salinity (i.e., FW and/or SW adaptation) (Hirano et al., 1990; Pankhurst and Thomas, 1998; Duncan et al., 2000). Among these, photoperiod and type of light regulate physiological, behavioral, and biochemical activities in vertebrates through photoreceptors (Tomohiro et al., 2003). Vertebrates perceive their environment (shapes, colors, and irradiance) through rod and cone photo receptors in the retina. In addition, non-visual functions such as the entrainment of the circadian clock, melatonin secretion, gonadal developments, and body color change are regulated by light (Campbell et al., 2001; Falcón, 1999; Oshima, 2001).

Photoperiod is a crucial environmental factor that stimulates reproduction in teleost fish (Pankhurst and Porter, 2003; Migaud et al., 2010). For example, switching to long-day conditions enhances spawning performance in the Nile tilapia, *Oreochromis niloticus*, and the Mexican silverside, *Chirostoma estor estor*, indicating that fish can use artificial light regimes as photoperiod cues for reproduction (Campos-Mendoza et al., 2004; Martinez-Palacios et al., 2007). On the other hand, short-day conditions reduce spawning performance in the spotted snakehead, *Channa punctatus* (Srivastava and Singh, 1992). Long-day conditions stimulate gonadal development in many fish, although short-day conditions also influence reproductive activities in certain types of fish such as salmonids (Amano et al., 2000; Migaud et al., 2010).

Photoperiod plays an important role in the reproductive performance of fish, and various studies on the effect of circadian rhythm on gonad development have been carried out (Pankhurst and Porter, 2003; Bapary et al., 2012). Sexual development and maturation in fish are regulated by various sex hormones in the hypothalamus–pituitary–gonad (HPG) axis, including gonadotropin-releasing hormone (GnRH), gonadotropin (GTH), steroid hormones, and other neurohormones (Lee et al., 2001; Habibi and Andreu-Vieyra, 2007). The pituitary GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are members of the glycoprotein hormone family and consist of a common α subunit and a specific β subunit (Pierce and Parsons, 1981). It is known that GTHs are key regulators of gonadal development and differentiation as well as control of the synthesis of gonadal hormones that regulate reproduction in vertebrates, including fish (Colombo and Chicca, 2003; Kim et al., 2012). In general, FSH regulates estradiol-17β (E_2) for both vitellogenesis and spermatogenesis,

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and LH promotes follicular maturation, ovulation, and the synthesis of steroid hormones in teleosts (Nagahama et al., 1995; Kobayashi et al., 2006).

The steroid hormone estrogen is essential in reproduction and plays important roles in sexual maturation and differentiation, including oogenesis, vitellogenesis, and testicular development. In addition, estrogen influences growth, gonad sex differentiation, the reproductive cycle, and lipid and bone metabolism through mechanisms that are primarily mediated by nuclear estrogen receptors (ERs) (Ryffel, 1978; Ishibashi and Kawashima, 2001). The induction of vitellogenin (VTG), a precursor yolk protein, in response to estrogens by an ER-mediated pathway is well documented in several oviparous (egg-laying) fish species (Ryffel, 1978). In largemouth bass, *Micropterus salmoides*, and Atlantic salmon, *Salmo salar*, ERα is highly correlated with VTG mRNA levels in the liver during spawning (Sabo-Attwood et al., 2004; Meucci and Arukwe, 2006).

Melatonin is produced and secreted by the pineal gland, and the rate of melatonin production is affected by the photoperiod. During the light photoperiod (day), little melatonin is produced; however, melatonin production increases during the dark photoperiod (night) (Falcón et al., 2007). Melatonin inhibits the secretion of gonadotropins (FSH and LH) from the anterior pituitary gland and promotes normal growth (Zeman et al., 1993; Sébert et al., 2008).

In teleosts, artificial ways of controlling photoperiod are currently used throughout the aquaculture industry to control sex maturation and reproduction; in particular, they are commonly used in aquaculture seed production (Martin-Robichaud and Berlinsky, 2004; van der Meeren and Ivannikov, 2006). A long-afterglow phosphorescent (glow-in-the-dark) pigment was recently developed in LumiNova sheets; in this study, we used the LumiNova light source to improve the energy efficiency of an aquaculture system. This approach is based on strontium aluminate and activated by various light sources that emit green light (520 nm) in the dark (Bapary et al., 2012; Imamura et al., 2014). Recently, a study investigated the utility of green light emitting diode (LED) lights as new photo-environmental factors and found that they increased the maturation of teleosts (Shin et al., 2013). However, studies on the effect of phosphorescent rather than direct light on aspects of molecular endocrine such as physiological response and expression of maturation-related genes are very limited in the fish.

In the present study, we examined the effects of phosphorescent light on sexual maturation and development in the yellowtail damselfish, *Chrysiptera parasema*. This species is a reef-associated damselfish that is widely distributed in shallow waters. It has a commercial value as an ornamental fish and is widely used as an experimental model in science. In this study, we evaluated three groups under different photoperiods and with or without LumiNova sheets for 4 months. The gonadosomatic index (GSI) and ovarian histology were used to compare and evaluate the responses of the experimental and control groups as well as to investigate the changes in the expression of GTHs (GTHα, LHβ), ER, VTG mRNA, and E_2. In addition, we measured the levels of plasma melatonin to confirm the effect based on photoperiod and the presence of phosphorescent light.

2. Materials and methods

2.1. Experimental fish

Immature yellowtail damselfish [$n = 270$, length, 3.6 ± 0.4 cm; mass, 1.1 ± 0.2 g; gonadosomatic index (GSI; gonad weight/body weight × 100) = 0.74 ± 0.07] were purchased from a commercial store and maintained in three 300-L tanks with circular filtration prior to laboratory-based experiments for 2 weeks.

The fish were randomly divided into tanks; each tank was exposed to a specific photoperiod [12-h light (L):12-h dark (D) and 14L:10D] or phosphorescent conditions (12L:12D), with the light photoperiod beginning at 07:00. The light source was a white fluorescent bulb (27 W, 200 lx at the surface of the water), and the lights were set and maintained at 50 cm above the water surface. The water temperature was maintained at 28 ± 1 °C. The fish were provided commercial feed twice daily (09:00 and 17:00 h) and were reared under these conditions for 4 months.

2.2. Phosphorescent conditions

The sides of the tanks for the phosphorescent group were covered with white polystyrene foam boards (5-mm thick), to which LumiNova sheets (M095-200-A; Nemoto Co., Ltd., Tokyo, Japan) were attached. The sides of the other tanks (12L:12D, the control, and 14L:10D) were covered with the same boards without LumiNova sheets. We measured light intensity using a luminometer. Using a luminometer, the measurement range cannot be measured at 30 min after the light is turned off, but brightness could be observed for at least 2 h with the naked eye.

2.3. Sampling

For sampling, fish were reared for 4 months in 2 different photoperiod groups (12L:12D and 14L:10D) and a phosphorescent group (12L:12D + LumiNova sheet), and the fish were sampled every 2 months.

We collected the pituitary, liver, and gonads from fishes in each group (12L:12D, 14L:10D, and phosphorescent) at 14:00 h of 2 and 4 month experimental periods. Immediately after collection, the tissues were frozen in liquid nitrogen and stored at −80 °C until total RNA extraction was performed. In addition, a blood sample was collected from the caudal vasculature using a 1 mL syringe coated with heparin. After centrifugation (4 °C, 10,000 ×*g*, 5 min), the plasma was stored at −80 °C until analysis. Specially, the amount of blood and pituitary were very low for the analysis from a single yellowtail damselfish. So, ten yellowtail damselfishes were used to blood- and pituitary-sampled and the mixed in each aquarium, respectively. Also, for the statistical analysis, three experimental groups [12L:12D, 14L:10D, and a phosphorescent group (12L:12D + LumiNova sheet)] with triplicates were used in this study.

After dissecting and weighing, the gonads were fixed in Bouin's solution and subjected to histological observation. The GSI was calculated for each fish.

2.4. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative mRNA expression levels of GTHs (GTHα, LHβ), ER, and VTG using the total RNA extracted from yellow damselfish tissues. The primers for QPCR are shown in Table 1. These primers were designed for each gene using the Beacon Designer software (Bio-Rad, Hercules, CA, USA). Primer alignments were performed with the BLAST database to ensure the specificity of primers. QPCR amplification was conducted in a manner similar to previous studies using a Bio-Rad CFX96™ Real-Time System (Bio-

Table 1
Primers used for amplification of QPCR.

Genes	Primer	DNA sequences
GTHα (KM509061)	Forward	5 -AAT GTT CCC GCC AGA GAA-3
	Reverse	5 -AGA GGT TGG AGA AGG CAG-3
LHβ (KJ737373)	Forward	5 -ACC ATC ATC GTG GAG AGA G-3
	Reverse	5 -GAT AGT TCA GGT CCG TTG TTT C-3
ER (JX218093)	Forward	5 -TGA CTA GCA TGT CTC CTG AT-3
	Reverse	5 -ATG GTG ACC TCG GTG TAA-3
VTG (JX218092)	Forward	5 -ACC CGT CAG TGC TCA GTA-3
	Reverse	5 -TCG CTG CTG GTC TTA ATC A-3
β-actin (JF273495)	Forward	5 -GCA AGA GAG GTA TCC TGA CC-3
	Reverse	5 -CTC AGC TCG TTG TAG AAG G-3

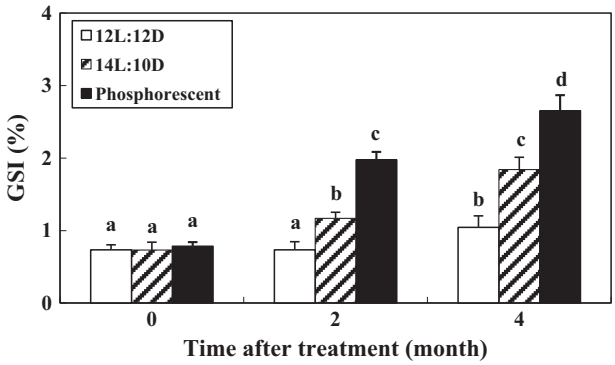

Fig. 1. Changes in the gonadosomatic index (GSI) of yellowtail damselfish under different photoperiods (A; 12L:12D, B; 14L:10D) and phosphorescent (C) conditions. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

Rad). QPCR was performed as follows: 1 cycle of denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 20 s and annealing at 55 °C for 20 s. Each reaction was run in triplicate to confirm consistency. The experiments were duplicated with β-actin as an internal control. The efficiencies of the reactions were determined by performing the QPCR.

2.5. Western blot analysis

Total protein isolated from the pituitary, gonads, and liver 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 PMSF, and 0.15 mg/mL leupeptin), 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 (Bio-Rad) was used for reference. The samples were electrophoresed at 80 V through the stacking gel and at 150 V through the resolving

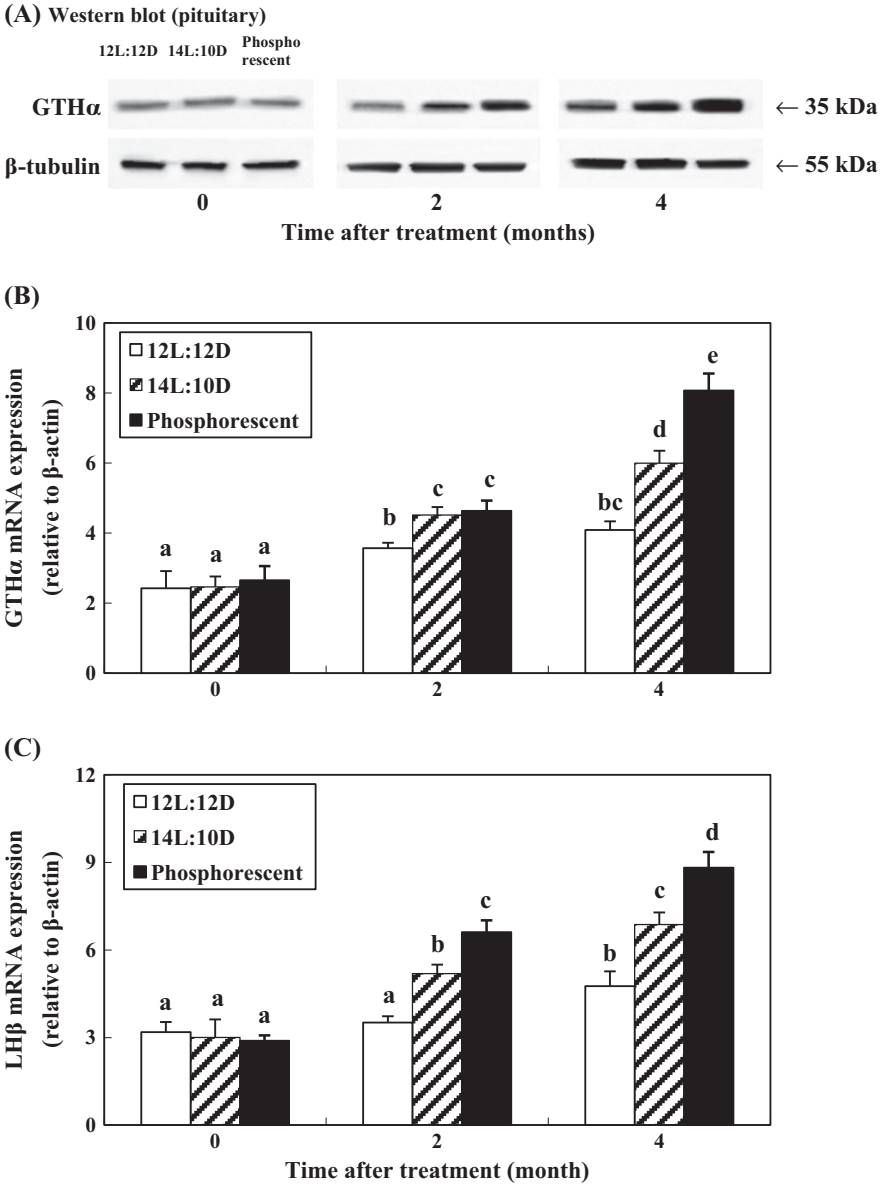

Fig. 2. The expression of GTHs in yellowtail damselfish pituitary after 2 and 4 months under different photoperiods (12L:12D, 14L:10D) and phosphorescent conditions. (A) Western blotting using GTHα (35 kDa) to examine protein expression in the pituitary. The 55 kDa β-tubulin was used as the internal control. GTH (B; GTHα and C; LHβ) mRNA levels relative to β-actin mRNA levels in the pituitary, as measured by quantitative real-time PCR. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

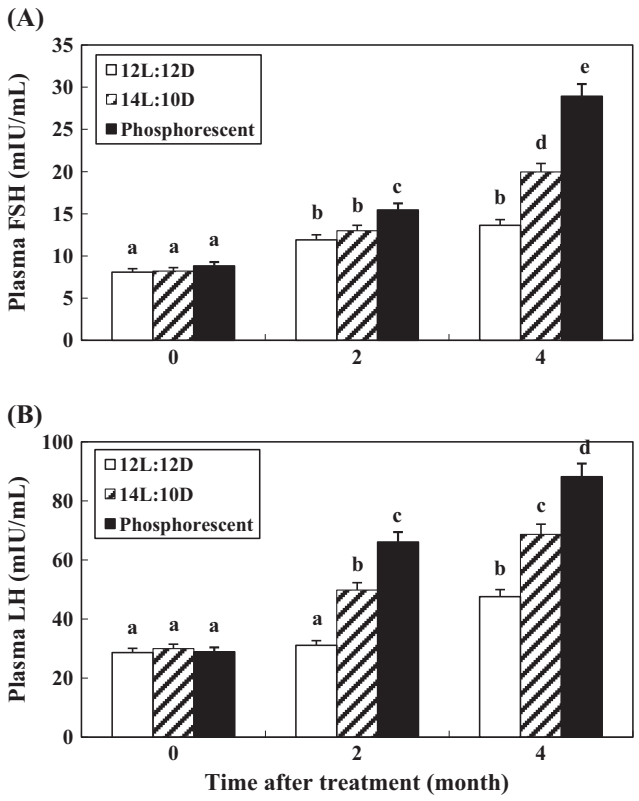

Fig. 3. The activities of FSH and LH in yellowtail damselfish after 2 and 4 months under different photoperiods (12L:12D, 14L:10D) and phosphorescent conditions were analyzed using a plate reader. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

gel until the bromophenol blue dye front had run off of 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 and then washed in TBS. The membranes were incubated with VTG antibodies (dilution 1:4000, ABIN326357, Antibodies-online, USA), followed by a horseradish peroxidase conjugated anti-mouse IgG secondary antibody (dilution 1:5000, Bio-Rad) for 60 min. Additionally, the membranes were incubated with a polyclonal rabbit antibody to GTHα [anti-goldfish GTHα; a polyclonal rabbit antibody; dilution, 1:4000, courtesy of M. Kobayashi (Kobayashi et al., 2006)] and ERα (dilution 1:1000, E1528, Sigma, St Louis, MO, USA), followed by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (dilution 1:5000, Bio-Rad) for 60 min. The internal control was a β-tubulin antibody (dilution, 1:5000, AB6046, Abcam, Cambridge, UK), followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5000, Bio-Rad) for 60 min. The bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, USA) with a 30-s exposure and a Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad). The membrane images were scanned with a high-resolution scanner, and the band density was estimated using a computer program (Image Lab™ Software, version 3.0, Bio-Rad). The ratios of the internal control (β-tubulin) to the genes (GTHα, ERα, and VTG) for each concentration were calculated and plotted against the concentration of the internal control.

2.6. Histological analysis

The gonadal tissues of each experimental group (12L:12D, 14L:10D, and phosphorescent; 0, 2, and 4 months) were fixed in a Bouin's solution to analyze the gonads during sexual maturation. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene, and embedded in paraffin. Sections (5-μm thick) were selected and stained with hematoxylin–eosin for observation under a light microscope (DM 100; Leica, Wetzlar, Germany). The images were captured using a digital camera (DFC 290; Leica).

2.7. Plasma parameter analysis

The plasma FSH, LH, and E_2 levels were analyzed using the immunoassay technique with an ELISA kit [FSH (Catalog no., E15790Fh), LH (Catalog no., E15791Fh), and E_2 (Catalog no., E1317Fh); Cusabio Biotech, Hubei, China].

An anti-antibody specific to the antibodies of hormones (FSH, LH, and E_2) was pre-coated onto a microplate. There was 50 μL of plasma per well; subsequently, 50 μL of HRP-conjugate and 50 μL of an antibody were added to each well. The samples were mixed well and then incubated for 2 h at 37 °C. After the final wash, any remaining wash buffer was removed by aspirating or decanting. Then, 50 μL each of substrate A and substrate B was added to each well, and the samples were incubated for 15 min at 37 °C in the dark. After incubation, 50 μL of the stop solution was added to each well. Finally, the optical density of each well was determined within 10 min using a microplate reader set to 450 nm.

2.8. Melatonin determination by ELISA

The melatonin concentration in the plasma was determined using the enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). The absorbance was read at 450 nm.

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). A two-way analysis of variance followed by Tukey's post-hoc test was used to test for significant differences in the data ($P < 0.05$). The values are expressed as the means ± standard error (SE). We have checked the normality and homogeneity of the data.

3. Results

3.1. GSI

The GSI was 0.74 ± 0.08 at the beginning of the experiment. The GSI values of the 14L:10D (1.84 ± 0.18) and phosphorescent (2.65 ± 0.22) groups were significantly higher than that of the 12L:12D group (0.85 ± 0.13), and the greatest effect was observed at the end of the experiment (4 months).

3.2. Expression and activity of GTHs under different photoperiod conditions

The effects of different photoperiod and phosphorescent conditions on the expression and activity of GTHs in the pituitary were investigated (Figs. 2 and 3). In the 14L:10D and phosphorescent groups, the mRNA expression levels of the GTHs were significantly higher than those in the 12L:12D group (Fig. 2). In particular, the expression levels in the phosphorescent group increased approximately 2.0-fold and 1.3-fold (GTHα; Fig. 2B), and were 1.8-fold and 1.2-fold (LHβ; Fig. 2C) higher at 4 months compared with those reared in the photoperiod groups (12L:12D and 14L:10D, respectively). Western blot analysis revealed 2 protein bands of GTHα-immunoreactive proteins that corresponded to the predicted mass for yellowtail damselfish GTHα (35 kDa). The expression pattern of the immunoreactive proteins resembled that of the GTH transcript levels in the yellowtail damselfish pituitary (Fig. 2A).

Additionally, under different photoperiod and phosphorescent conditions, the plasma FSH level was 8.08 ± 0.42 mIU/mL at the start of the experiment. In particular, the levels of FSH increased to

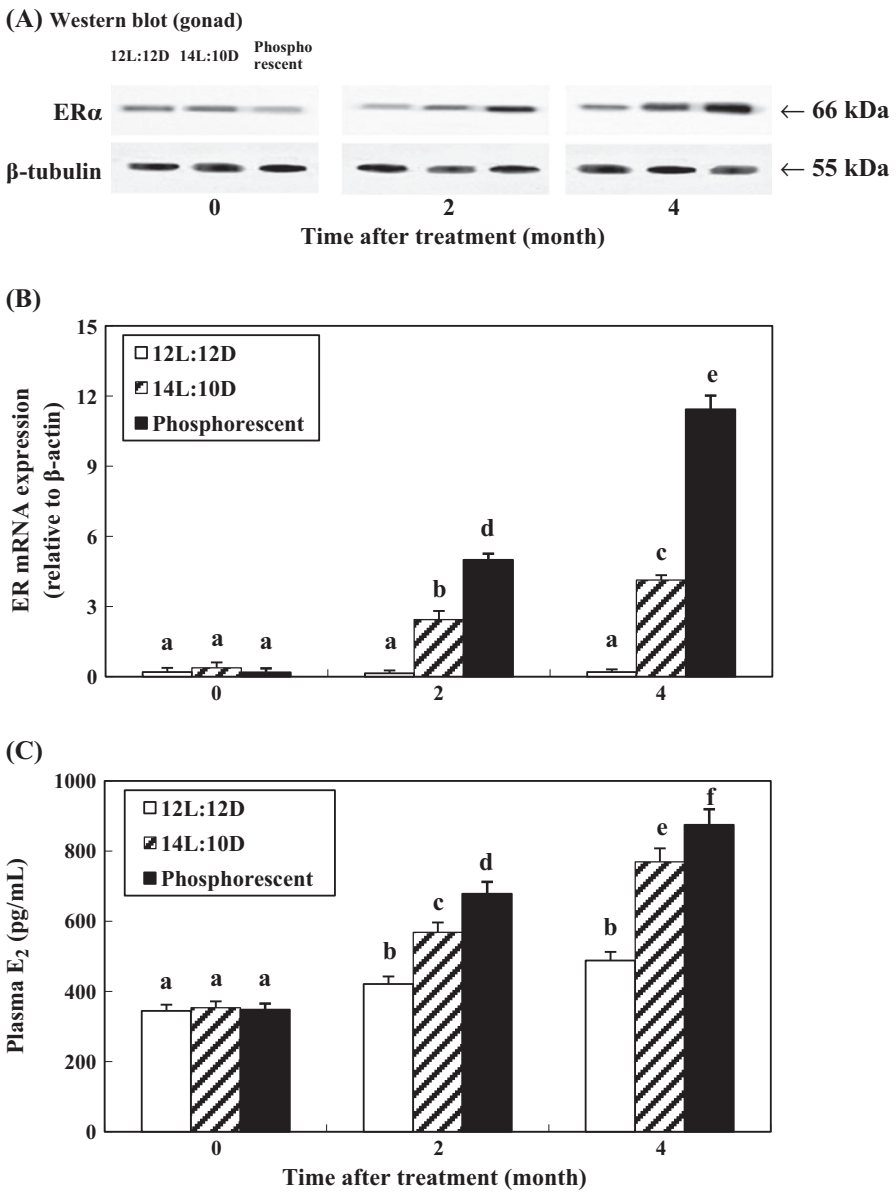

Fig. 4. The expression and activity of ER in yellowtail damselfish gonads after 2 and 4 months under different photoperiods (12L:12D, 14L:10D) and phosphorescent conditions. (A) Western blotting using ERα (66 kDa) to examine protein expression in the gonads. The 55 kDa β-tubulin was used as the internal control. (B) ER mRNA levels relative to the β-actin mRNA levels in the pituitary, as measured by quantitative real-time PCR. (C) Plasma E_2 activity was analyzed using a plate reader. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

28.92 ± 1.44 mIU/mL at 4 months under phosphorescent conditions (Fig. 3A). Additionally, under phosphorescent conditions, the plasma LH levels significantly increased and reached levels that were approximately 3.2-fold (88.20 ± 4.44 mIU/mL) higher at 4 months compared with the control group (Fig. 3B).

3.3. Expression and activity of ER under different photoperiod conditions

The effects of different photoperiod and phosphorescent conditions on the expression and activity of ER in the gonads were investigated (Fig. 4). In the 14L:10D and phosphorescent groups, the expression levels of ER mRNA were significantly higher than that in the 12L:12D group (Fig. 4B). In particular, the expression level of the phosphorescent group increased to approximately 57.7-fold and 2.7-fold higher at 4 months than those in the photoperiod groups (12L:12D and 14L:10D, respectively). Western blot analysis revealed 2 protein bands of ER-immunoreactive proteins that corresponded to the predicted mass for yellowtail damselfish ER (66 kDa). The expression pattern of the immunoreactive proteins resembled that of the ER transcript levels in the yellowtail damselfish gonads (Fig. 4A). Additionally, under different photoperiods and phosphorescent conditions, the plasma E_2 level was 344.88 ± 17.22 pg/mL at the start of the experiment. In particular, the levels of E_2 increased to 875.92 ± 43.44 mIU/mL under phosphorescent conditions for 4 months, which was higher than those observed in the12L:12D (488.22 ± 43.44 mIU/mL) and 14L:10D (769.22 ± 23.44 mIU/mL) conditions (Fig. 4C).

3.4. Expression of VTG under different photoperiod conditions

The effects of different photoperiod and phosphorescent conditions on the expression and activity of VTG in the liver were investigated (Fig. 5). In the 14L:10D and phosphorescent groups, the expression levels of VTG mRNA were significantly higher than those in the 12L:12D group (Fig. 5B). In particular, at 4 months, the expression in the phosphorescent group increased to approximately 3.5-fold and 1.8-fold higher than those in the photoperiod groups (12L:12D and

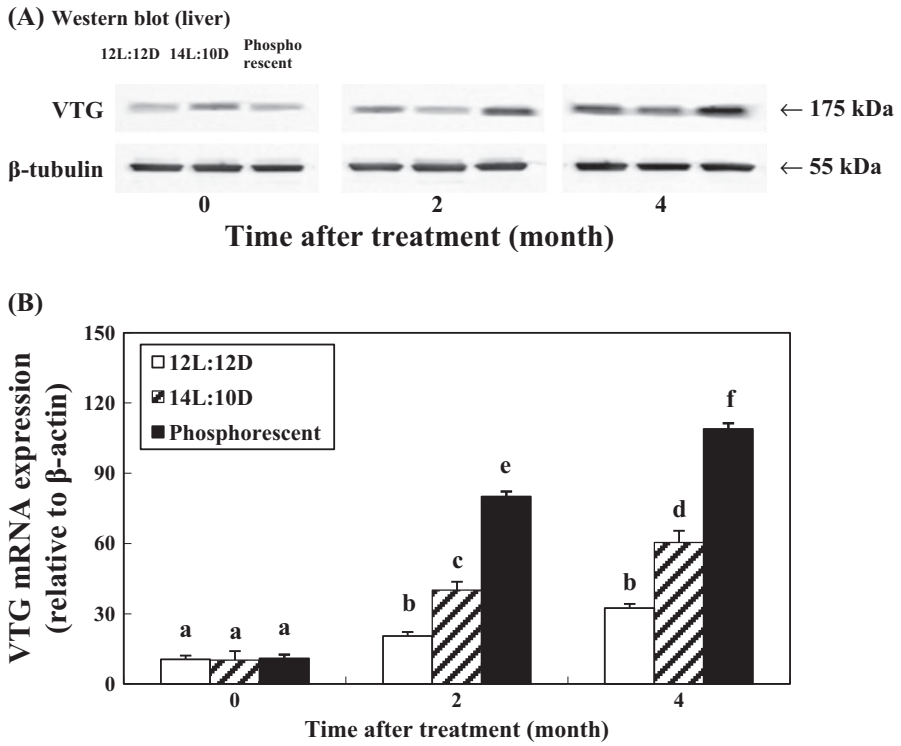

Fig. 5. The expression of VTG in yellowtail damselfish liver after 2 and 4 months under different photoperiods (12L:12D, 14L:10D) and phosphorescent conditions. (A) Western blotting using VTG (178 kDa) to examine protein expression in the liver. The 55 kDa β-tubulin was used as the internal control. (B) VTG mRNA levels relative to the β-actin mRNA levels in the pituitary, as measured by quantitative real-time PCR. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

14L:10D, respectively). Western blot analysis revealed 2 protein bands of VTG-immunoreactive proteins that corresponded to the predicted mass for yellowtail damselfish VTG (178 kDa). The expression pattern of the immunoreactive proteins resembled that of the VTG transcript levels in the yellowtail damselfish liver (Fig. 5A).

3.5. Activity of melatonin during different photoperiod conditions

Under different photoperiod and phosphorescent conditions, the plasma melatonin level was 7.92 ± 0.72 pg/mL at the start of the experiment. In particular, the levels of melatonin decreased to 4.85 ± 0.35 mIU/mL after 4 months under phosphorescent conditions, which was lower than those exposed to 12L:12D (7.68 ± 0.34 mIU/mL) and 14L:10D (5.81 ± 0.29 mIU/mL) (Fig. 6).

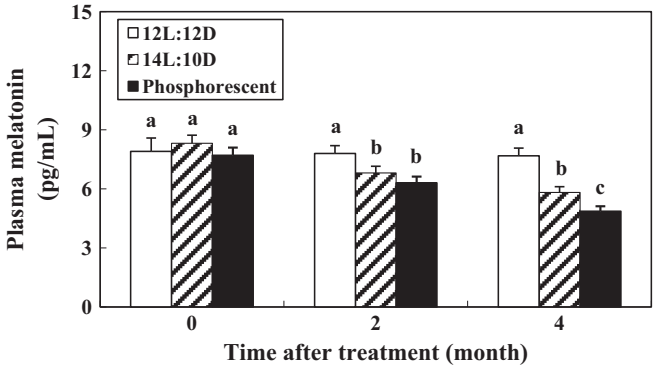

Fig. 6. Plasma melatonin levels in yellowtail damselfish after 2 and 4 months under different photoperiods (12L:12D, 14L:10D) and phosphorescent conditions were analyzed using a plate reader. Values with letters indicate significant differences among lights of different photoperiods ($P < 0.05$). All values are means ± SE.

3.6. Histological observation

To investigate gonadal morphology, we performed histological studies of gonadal samples as shown in Fig. 7. The gonads of all fish in the control and experimental groups were found to contain a smaller number of primary oocytes (Fig. 7A, D, G). After 4 months, in the 12L:12D and 14L:10D groups, ovaries developed vitellogenic oocytes at the secondary yolk stage (SYS) (Fig. 7C, F). In contrast, well-developed vitellogenic oocytes, such as those at the tertiary yolk stage (TYS), were found to be abundant after 4 months in the phosphorescent group (Fig. 7I).

4. Discussion

The results demonstrate that total yellowtail damselfish GSI was significantly increased in the phosphorescent group compared with the 12L:12D and 14L:10D groups after 4 months (Fig. 1). Bapary et al. (2012) reported that the GSI increased in damselfish reared for 2 months under phosphorescent pigment, indicating that the additional light (long-afterglow) emitted from the LumiNova sheets had a stimulatory effect to prolong reproduction. In this study, we determined that the effect of afterglow (long-day conditions) induced gonadal growth. This result is consistent with previous studies that found that longer day lengths promote gonadal development in many species (Koya and Kamiya, 2000; Migaud et al., 2010).

Additionally, to investigate the active pituitary–gonad axis relative to the afterglow effects of the photoperiod and phosphorescent pigment, mRNA and protein expression of the sex maturation-related hormones GTHα and LHβ in the pituitary were evaluated. The expressions of these hormones increased the most after 4 months, and levels of FSH and LH increased with longer light photoperiod and produced a longer breeder period (Figs. 2 and 3). Long-day conditions increased the secretion of sex hormones and ovary growth with phosphorescent afterglow by activating the pituitary–gonad axis. Previous studies

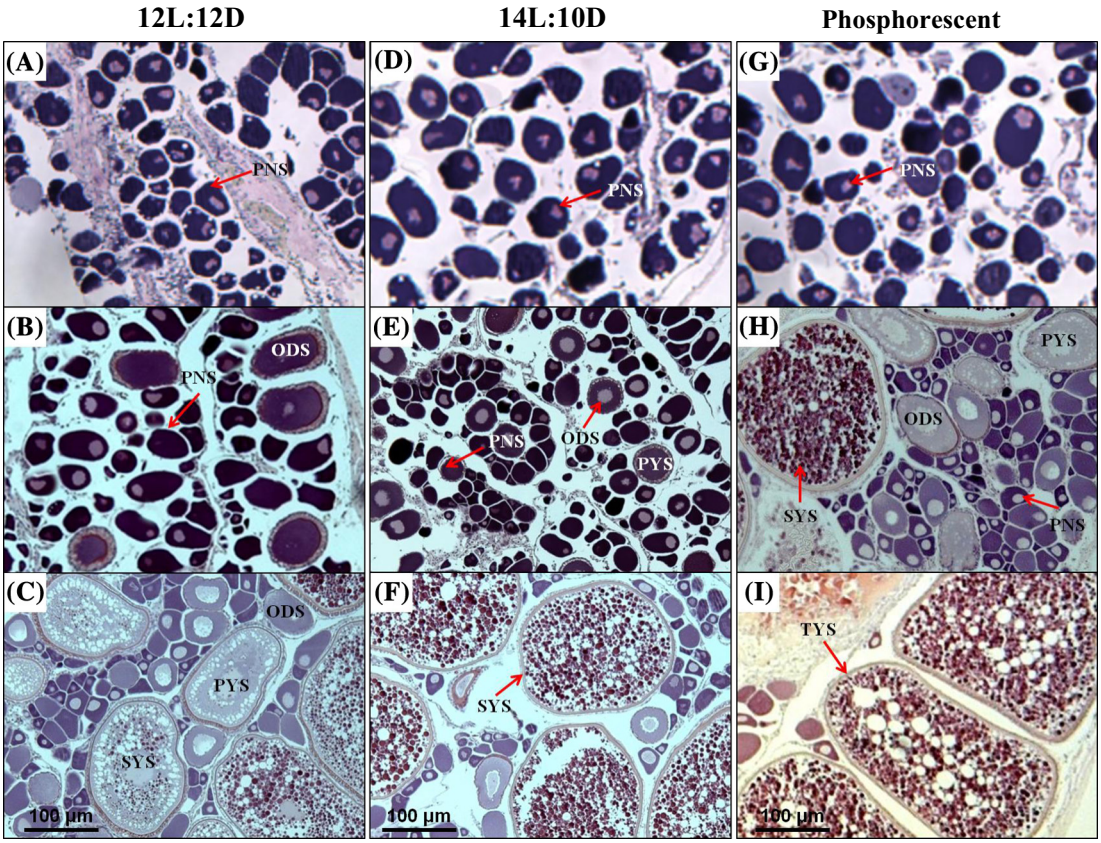

Fig. 7. A photomicrograph of cross-sections of yellowtail damselfish under different photoperiods (A; 12L:12D, B; 14L:10D) and phosphorescent conditions (C). Cross-section of fish ovaries after 2 (B, E, H) and 4 (C, F, I) months under different photoperiod conditions. ODS: oil droplet stage; PNS: peri-nucleolus stage; PYS: primary yolk stage; SYS: secondary yolk stage; TYS: tertiary yolk stage. Scale bar = 100 μm.

determined that long-day conditions directly regulated the sex hormones and were related to maturation of ovarian, secreted axis of the brain–pituitary–gonad axis (Campos-Mendoza et al., 2004; Migaud et al., 2010). Moreover, expressions of ERα and VTG mRNA and protein also increased over the rearing period in long-day conditions (Figs. 4 and 5). These results revealed that high expression levels were significantly observed in the phosphorescence experiment.

The long-afterglow phosphorescent pigment of the LumiNova sheet was activated by various light sources that emit green light (520 nm) in the dark (Bapary et al., 2012). Shin et al. (2013) reported that the green LED wavelength induced sex maturation of yellowtail damselfish and observed a large amount of mature oocyte cells, which indicated that green light induced ovary maturation. In the present study, sexual maturity-related genes and protein levels were higher in the phosphorescence group compared with the 14L:10D group; in addition, there was a lasting effect when phosphorescent pigment was used to stimulate long-day conditions, because the phosphorescence has the same effect as the green wavelength on sexual maturation.

Levels of melatonin were higher in the 12L:12D group, which represented a common photoperiod, and lower in the phosphorescence group (Fig. 6). The sustainment time of melatonin levels was proportional to the length of the night, which affects complex biochemical and physiological processes as well as the day cycle in vertebrates (Arendt, 1995). The action of melatonin occurs via a melatonin receptor that belongs to the G-protein-coupled receptor superfamily (Iigo et al., 1994a; Reppert et al., 1996). Sébert et al. (2008) reported that a 12L:12D photoperiod generally increased the effect of melatonin on dopaminergic systems under melatonin treatment, suppressed the activity of the hypothalamus–pituitary–gonad axis, and reduced the levels of sexual maturation-related hormones. Additionally, Chaube and Joy (2002) reported that the activity of thyroid hormone (TH), the hormone that affects reproductive activity, and GSI are decreased in the telencephalon and hypothalamus of melatonin-treated catfish, *Heteropneustes fossilis*. In this study, we confirmed that the secretion inhibition of melatonin leads to accelerated secretion of sex-related hormones and sex maturation, and this is the result of the sustained effect of Luminova sheets during longer photoperiods.

Moreover, we confirmed by histological observation that the ovaries have a large amount of mature oocytes in the phosphorescence group compared with the other 14L:10D group in this study (Fig. 7). In this study, the afterglow effects of fluorescence induced long-day condition (14L:10D). Previous study reported that green color light induced maturation of fish (Shin et al., 2013), similarly this study of authors has suggested that the expressions of VTG and ERα mRNA and protein and levels of plasma E_2 showed higher at phosphorescence group by the green light of phosphorescence. Then, they increased the maturation-related hormones and genes effectively promoted the maturation of the yellowtail damselfish.

We determined that fish maturation is associated with light wavelength in terms of both mechanism and physiological response. Our findings support the hypothesis that the use of phosphorescent pigment via a LumiNova sheet by the emission of green light induces the effect of long-day conditions and would be valuable for inducing ovary maturation and improving the reproductive ability of fish.

However, the studies on the effect of phosphorescence type and wavelength intensity on fish maturation and growth were very

limited. So, further studies will be required to understand the effects of reproduction, growth, and reproduction of second generation in various phosphorescence and wavelength.

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