A prolonged photoperiod using pellets containing long-afterglow phosphorescent pigment can stimulate ovarian development in a tropical damselfish

Running title: Ovarian development by LumiNova light

Keywords: Coral reef, *Chrysiptera cyanea*, gonadal development, LumiNova, Photoperiod

* Co-corresponding authors
Abstract

The present study aimed to examine whether light emitted by long-afterglow phosphorescent pigments (LumiNova) would stimulate gonadal development in fish outside of the breeding season. Pellets containing LumiNova powder (LumiNova group) were prepared and placed on the calvaria of specimens of the sapphire devil *Chrysiptera cyanea*, a reef-associated damselfish with a long-day preference for gonadal recrudescence. A pellet without LumiNova powder was placed on the calvaria of the control fish (control group). Fish were reared at 26°C under a light–dark cycle (12 h photophase and 12 h scotophase; LD 12:12) for 4 weeks. No differences in the gonadosomatic index (GSI) and ovarian histology were observed among the control, sham-operation, and LumiNova groups 1 week after the start of the experiment. At 4 weeks, the GSI of the control and sham-operation groups remained at low levels and their ovaries contained immature oocytes at the peri-nucleolus stage. In contrast, the LumiNova group had a significantly higher GSI and developed ovaries with yolk-laden oocytes, demonstrating that long-day conditions are produced by light emitted from the LumiNova pellets and stimulate ovarian development in the damselfish. Therefore, long-afterglow phosphorescent pigments can be used as an alternative to standard light sources for purposes of artificial stimulation of gonadal development in fish.
Introduction

Seasonal changes in day length are critical factors that influence the initial cueing, timing, and subsequent synchronization of gonadal development in various fish species [Bromega et al., 2001; Migaud et al., 2010]. Reproductive strategies utilizing day length are likely to be diverse among species. For example, a steady increase in day length stimulates gonadal development in the black seabass *Centropristis striata* [Howell et al., 2003], the Japanese amberjack *Seriola quinqueradiata* [Hamada et al., 2006], and the Eurasian perch *Perca fluviatilis* [Migaud et al., 2003], whereas a decrease in day length is the trigger for the Atlantic cod *Gadus morhua* [Skjaeraasen et al., 2004] and most salmonid species [Bromega et al., 2001]. It is believed that in both cases of photoperiodicity in fish, changes in day length (i.e., long-day or short-day conditions) are perceived by the photosensory organ(s) and transduced as an internal signal, and that gonadal development is subsequently stimulated by the cascade activation of the hypothalamus–pituitary–gonadal (HPG) axis at an appropriate time of a year. Melatonin secreted by the pineal gland appears to be a potent transducer of photic information to central and peripheral organs because seasonal changes in diurnal melatonin profiles have been reported in some fish species [Randall et al., 1995]. However, how this indoleamine hormone regulates the HPG axis remains unclear [Bromega et al., 2001].

Artificial stimulation or suppression of gonadal development by manipulating photoperiodic conditions has potential industrial advantages for efficiently breeding fish with high commercial value at a desired time of year, and for reducing the excessive use of drugs and hormones for maturational purposes. Past studies have used incandescent, fluorescent, metal–halide, and tungsten–halogen lamps as standard light sources and have successfully controlled the gonadal development of various fish species [Bromega et al., 2001]. From aspects of long-operating life and low-energy consumption, light-emitting diodes (LEDs)
have recently been used as a new light source to stimulate gonadal development [Bapary et al., 2011; Leclercq et al., 2011] and growth [Yamanome et al., 2009]. However, waterproofing and barotolerance issues make the usage of this new light technology in aquaculture problematic.

Recently, long-afterglow phosphorescent pigments (LumiNova) was used as a light source and light emitted by LumiNova sheets has a positive effect on prolonging the reproductive season of the sapphire devil *Chrysiptera cyanea* [Bapary et al., 2012], a reef-associated damselfish with a long-day preference for gonadal recrudescence [Bapary et al., 2009]. Additional advantages for the use of LumiNova are glow-in-the-dark pigments without the necessity of supplying electrical energy, activation by a broad band of wavelengths, a high emission level relative to the amount of radiation, chemical stability and lack of hazardous and radioactive substances, and light- and waterproof pigments (http://www.nemoto.co.jp/en/products/luminova/luminova.html). The goal of the present study was to improve the methodology for LumiNova application and to stimulate gonadal development in the sapphire devil outside of the breeding season.

**Materials and methods**

**Fish and experimental design**

Adult sapphire devils with 3.5 ± 0.4 cm mean body length and 1.3 ± 0.6 g mean body mass were collected from coral reef lagoons in Urasoe, Okinawa, Japan, using a small round haul net during daytime low tide. They were transferred to the Campus of the University of the Ryukyus, Nishihara, Japan, and reared in aquaria equipped with filtering and aeration systems under short-day conditions (10 h photophase and 14 h scotophase; LD 10:14, light on at 08:00 h and off at 18:00 h) and a water temperature of 26°C. A fluorescent bulb (14 W) was
used as a light source, and the light intensity at the water surface was 3.6 W/m². Fish were fed daily at 10:00 h with commercial pellets (EP1; Nisshin-Marubeni, Tokyo, Japan).

Powdered LumiNova (G-300M) was purchased from C-task (Tokyo, Japan). Epoxy resin (craft resin) and its hardener were bought from Nisshin Resin KK (Yokohama, Japan). LumiNova, epoxy resin, and hardener were mixed at a ratio (w/v) of 2:1:1. The mixture was poured into a silicone tube (internal diameter = 2 mm, external diameter = 3 mm) and left for 6 h at room temperature. After drying and hardening, the silicone tube containing the LumiNova was cut into approximately 2 mm lengths (Fig. 1a, b) and kept at room temperature until use. The control pellets were prepared by mixing only the epoxy-resin and its hardener at a ratio of 2:1.

The experiment was carried out for one months starting in December, when fish are out of the breeding season and their ovaries are exclusively occupied by immature oocytes [11]. After fish (n = 60, male : female = 1 : 5) were anesthetized with ethyl-3-amino-benzoatemethanesulfonic acid (MS-222, Sigma-Aldrich, St. Louis, MO, USA), a pellet was sewn on the head of each individual (Fig. 1c). Prepared fish were separated into three groups: the LumiNova pellet group (3 males and 15 females), control pellet group (3 males and 15 females), and sham-operation group (4 males and 20 females). Each group was housed in an aquarium equipped with filtering and aeration systems under LD 12:12 conditions (lights on at 06:00 and off at 18:00) and a water temperature of 26°C, and were fed daily with EP1. Then 80- to 100-mm-diameter pipes were set as a spawning nest on the bottom of each aquarium. Fish (5 females per aquarium) were sampled at 1, and 4 weeks after the start of the experiment. Ovaries from 5 females were also collected from the sham-operation group just before the onset of the experiment. The ratio of male to female was kept by taking males at each sampling time. Fish were anesthetized with 2-phenoxyethanol (Kanto Chemical Co., Tokyo, Japan) and decapitated. The body mass and body length of each individual were recorded. The ovaries were removed from the body
cavity, weighed, and subsequently fixed in Bouin’s solution. The gonadosomatic index (GSI) was calculated using the following formula: GSI = (ovarian mass/body mass) × 100.

All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the University of the Ryukyus and the regulations for the care and use of laboratory animals in Japan.

Histological procedures

A piece of the fixed ovary was dehydrated in a series of ethanol and permuted with xylene, and then embedded in paraffin (melting point between 62°C and 64°C; Nacalai Tesque Inc., Osaka, Japan). Serial sections at 7 µm were prepared and stained with Mayer’s hematoxylin–eosin for microscopic observations. Oocyte development classified into six stages: the peri-nucleolus stage, oil droplet stage, yolk vesicle stage, primary yolk stage, secondary yolk stage, and tertiary yolk stage [Bapary et al., 2009].

Statistical analysis

The results of the GSI were expressed as the mean ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) followed by a Tukey–Kramer test was used for comparing the mean GSI among the fish groups (P < 0.05 for a statistically significant difference).

Results

No fish died during the experimental period. The GSI of the initial control was 0.64 ± 0.12. The GSI of the LumiNova pellet group increased to 0.70 ± 0.10 after 1 weeks and 1.88 ± 0.37
after 4 weeks. The GSIs of the control pellet group and sham-operation group did not change, and were 0.98 ± 0.11 and 0.92 ± 0.07 after 4 weeks, respectively. At 4 weeks, the GSI of the LumiNova pellet group was significantly higher than that of the other groups (Fig. 2).

Ovaries of the initial control contained immature oocytes at the peri-nucleolus stage. Similar ovarian features were confirmed in the control pellet and sham-operation groups at 1 week after the start of the experiment (Fig. 3a). On the other hand, ovaries of the LumiNova pellet group at 1 week had oocytes at peri-nucleolus and oil droplet stages (Fig. 3b). At 4 weeks, ovaries of the control pellet and sham-operation groups were occupied only by oocytes at the peri-nucleolus stage (Fig. 3c), whereas those of the LumiNova pellet group contained yolk-laden oocytes at the tertiary yolk stages (Fig. 3d).

Discussion

The present study clearly shows that light emitted by long-afterglow phosphorescent pigments (LumiNova) stimulates ovarian development in the sapphire devil outside of the breeding season. No ovarian development was induced during the experimental period in the control and sham-operation fish. A similar result was previously obtained using the same species, whereby fish in aquaria covered with the LumiNova sheets continued active oocyte growth and repeated spawnings even after the reproductive season of the naturally reared fish had terminated [Bapary et al., 2012]. LumiNova thus emits wavelengths of light that can be perceived and utilized by the fish.

It was observed histologically that ovarian development of the sapphire devil in Okinawan water initiates in March and peaked in May, when photoperiod and water temperature are increasing [Bapary et al., 2009]. When females were reared at 25°C under experimental conditions of LD 10:14, LD 12:12, and LD 14:10 using a fluorescent bulb as a light source, ovaries with yolk-laden oocytes were observed only under long-day conditions.
[Bapary et al., 2009]. Also, exposing the female sapphire devil to long-day conditions (LD 14:10) with red (627 nm) and green (530 nm) LED lights resulted in the induction of ovarian development, but exposure to blue (455 nm) and white LED lights did not [Bapary et al., 2010]. These previous reports clearly suggest that the sapphire devil perceives long-day conditions from LED lights and starts ovarian development outside of the natural breeding season. In addition, mid- to long-wavelength lights are preferable for initiating ovarian development in this species. Because LumiNova emits green light (530 nm) in the dark after energy absorption and emits light for several hours afterward (http://www.nemoto.co.jp/en/products/luminova/luminova.html), fish are likely exposed to the desired wavelengths of light after turning lights off. Since photoperiodic conditions of LD 12:12 was used in the present study, additional emission of light by LumiNova produces resultant long-day conditions to stimulate gonadal development.

Because the pellet was placed on the calvaria of each individual, the majority of light emitted with LumiNova may stimulate the extraretinal photoreceptors. In this regard, it was reported that following pinealectomy and ophthalmectomy, gonadal development can be induced under short-day conditions in the ayu sweetfish Plecoglossus altivelis altivelis [Masuda et al., 2005]. In addition, Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses revealed that rhodopsin is expressed in the brain of the ayu sweetfish [Masuda et al., 2003]. When the ophthalmectomized sapphire devils were reared under long-day conditions (LD 14:10), vitellogenic oocytes appeared in an ovary [Bapary et al., 2011]. These previous findings indicate that extraretinal photoreceptor(s) play a role in inducing gonadal development. In situ hybridization using the sapphire devil brain revealed that long-wavelength sensitive cone opsins (LWS) mRNA is expressed in the third ventricle periventricular area in the anterior hypothalamus and that its strong signals are predominantly observed in the ventromedial thalamic nucleus (VM), anterior periventricular nucleus (NAPv), and suprachiasmatic nucleus (NSC) [Takeuchi et al., 2011]. In addition, the
expressions of both middle-wavelength sensitive cone (MWS) and rhodopsin mRNA were detected in the brain of this species by RT-PCR [Takeuchi 2012]. Taken together, these opsins are likely to be involved in the perception of green light emitted by LumiNova. However, the possibility that the eyes play a role in transducing light signals from LumiNova cannot be presently excluded because light from the pellet on the head of one individual may be perceived through the eyes of other individuals.

The series of our trials using the tropical damselfish is the first report showing the effectiveness of long-afterglow phosphorescent pigments on the artificial control of gonadal activity. The previous approach was to irradiate the whole aquarium by light emitted from LumiNova [Bapary et al., 2012], while the pinpointed irradiation onto the calvaria of fish was given in the present study. Although there is technical difference in our application of LumiNova, it can become a useful tool for controlling gonadal activity in fish and may provide energy-free and environmentally safe advantages for aquaculture. Further studies would be needed to examine whether LumiNova is widely applicable to other important fisheries species.

Acknowledgement

References


Bapary MAJ, Takemura A (2010) Effect of temperature and photoperiod on the reproductive condition and performance of a tropical damselfish *Chrysiptera cyanea* during different phases of the reproductive season. Fish Sci 76:769–776


Bapary MAJ (2011) Studies on environmental control of the reproductive activities in a tropical damselfish *Chrysiptera cyanea*. PhD dissertation, University of the Ryukyus, Okinawa


Figure legends

Figure 1. Pellets containing long-afterglow phosphorescent pigments (LumiNova pellets). a; LumiNova pellets (taken under a fluorescent bulb), b; Luminova pellets (taken under darkness after exposing to light from a fluorescent bulb for 5 min), c; fish with a LumiNova pellet on the calvaria.

Figure 2. Changes in gonadosomatic index of female sapphire devil with a pellet on the calvaria. Ovaries were collected at 1 and 4 weeks after the onset of the experiment. White, shaded, and black columns indicate sham-operation fish, fish with a control pellet, and fish with a pellet containing LumiNova, respectively. Each value (n = 5) represents mean ± SEM. Different letters indicate significant difference at $P < 0.05$.

Figure 3. Changes in ovarian histology of the sapphire devil with a pellet on the calvaria. Ovaries were collected at 1 and 4 weeks after the onset of the experiment. A cross section of an ovary (CSO) of (a) the control pellet group at 1 week, (b) CSO of the LumiNova pellet group at 1 week, (c) CSO of the control pellet group at 4 weeks, and (d) CSO of the LumiNova pellet group at 4 weeks. ODS; oil droplet stage, PNS; peri-nucleolus stage, TYS; tertiary yolk stage. Scale bar = 200 µm.
Gonadosomatic index

Weeks after treatment

- Sham-operation fish
- Fish with a control pellet
- Fish with a LumiNova pellet

Bar chart showing the gonadosomatic index for different groups of fish at 1 and 4 weeks after treatment.