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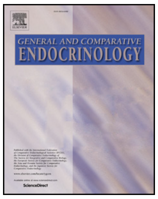

Involvement of melatonin in transducing moon-related signals into the reproductive network of the female honeycomb grouper *Epinephelus merra*

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ABSTRACT

Most groupers (genus *Epinephelus*) inhabiting tropical and subtropical waters exhibit lunar-related reproductive cycles. Their gametes develop synchronously toward and are released around the species-selected moon phase. Periodical changes in cues from the moon are likely used as zeitgeber, and the hypothalamic-pituitary-gonadal (HPG) axis may be activated after cues are perceived by the sensory organ and transduced as internal signals. The objective of this study was to examine weekly changes in mRNA expression profiles of gonadotropin-releasing hormones (*gnrh1* and *gnrh2*) and the β-subunit of gonadotropins (*fshβ* and *lhβ*) during the spawning season (May to June) of the female honeycomb grouper *Epinephelus merra*, which spawns around the full moon period. When mature females were collected based on the lunar cycle, the gonadosomatic index peaked around the full moon. Ovarian histology revealed that oocytes laden with yolk developed toward the full moon and, subsequently, ovulatory follicles appeared around the last quarter moon, confirming lunar-related spawning with a full moon preference. Real-time quantitative polymerase chain reaction analyses revealed high abundances of *fshβ* and *lhβ* toward the first quarter moon, whereas concentrations of *gnrh1* and *gnrh2* increased around the last quarter moon and the first quarter moon, respectively, suggesting that transcription levels of these hormones fluctuate with the lunar cycle. The measurement of melatonin in the eye around the new moon and the full moon revealed that the ocular melatonin content was higher around the new moon than around the full moon, suggesting that the honeycomb grouper can perceive changes in moonlight. In addition, implantation of an osmotic pump containing melatonin into the body cavity of *E. merra* reduced the transcription levels of gonadotropins, suggesting that melatonin negatively affects hormonal synthesis at the HPG axis. We concluded that melatonin plays an essential role in transducing periodical changes in moonlight and that decreases in melatonin levels from the new moon to the full moon activate the HPG axis for entrainment of gonadal development and spawning.

1. Introduction

The reproductive activity of teleost fish functions within suitable ranges of environmental factors including photoperiod and water temperature (Alvarado et al., 2013; Carnevali et al., 2011; Sébert et al., 2008a). Following the perception of changes in environmental factors by sensory organs and their transduction as internal signals, the hypothalamic-pituitary-gonadal (HPG) axis, the endocrine network of reproduction in vertebrates, is activated (Migaud et al., 2010; Pankhurst and Munday, 2011). Subsequently, a secretory cascade of hormones is produced from the HPG axis of female fish; gonadotropin-releasing hormone (GnRH) is synthesized in GnRH neurons in the hypothalamus and released into the portal vein; gonadotropins (GtHs), i.e., follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are synthesized in gonadotrophs in the anterior pituitary and released into the blood; and sex steroids are synthesized in the follicle layers surrounding oocytes in ovaries to stimulate vitellogenic synthesis in the liver and final oocyte maturation (Levavi-Sivan et al., 2010; Patino and Sullivan, 2002; Zohar et al., 2010).

In fish species that exhibit a synchronous pattern in oocyte growth (e.g., anadromous *Oncorhynchus* species and catadromous eels), activation of the HPG axis occurs once during their life span (Wallace and

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Selman, 1981). On the other hand, fish exhibiting a group-synchronous pattern in oocyte growth are likely to repeat the activation and deactivation of the HPG axis according to the periodicity of their gonadal development. Previous studies have reported that the hormonal cascade from the HPG axis of lunar-synchronized spawners may be activated according to a lunar cycle (Rahman et al., 2000, 2001, 2002). For example, in the goldlined spinefoot *Siganus guttatus*, which has a spawning preference around the first quarter moon, vitellogenesis is accelerated with a steady increase in plasma levels of estradiol-17β (E2) toward the first quarter moon, and final oocyte maturation subsequently occurs under the influence of maturation-inducing steroids such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) around this moon phase (Rahman et al., 2000). *In vitro* culture of developing oocytes has revealed that the synthesis of sex steroids in the follicle layers is regulated by GtH, as additions of human chorionic gonadotropin to the medium stimulated the synthesis of steroid hormones according to the lunar cycle (Rahman et al., 2002). It is hypothesized that rhythmicity in gonadal development according to the lunar cycle is regulated at the higher part of the HPG axis or higher regions of the brain (Takemura et al., 2010). However, how these regions are involved in the endogenous regulation of lunar-related reproduction in fish remains unknown.

An influence of moonlight on lunar-related reproduction has been proposed in certain fish species (Falcón et al., 2010; Oliveira et al., 2009, 2010; Saavedra and Pousão-Ferreira, 2006; Takemura et al., 2010). For example, experiments rearing the goldlined spinefoot under constant full moon and new moon conditions resulted in disruption or retardation of their predicted spawnings around the first quarter moon, suggesting that periodical changes in moonlight are necessary for recognition of the spawning lunar phase (Takemura et al., 2004). Such findings suggest that melatonin, a nocturnally-synthesized hormone, may become a key transducer of “brightness at night” (Ikegami et al., 2014), as exposing the goldlined spinefoot to moonlight around the full moon period lowered melatonin levels in the plasma (Takemura et al., 2004) and in ocular regions (Rahman et al., 2004). This insight is provided from evidence that the levels of melatonin influence the reproduction of short-day and long-day spawners (Falcón et al., 2010). In addition, differences in the mRNA abundance of melatonin receptors (MT1 and Mel_{1c}) were observed in the pineal organ between those two moon phases (Park et al., 2014). To date, it remains unclear how melatonin plays a role in regulating the HPG axis of lunar-synchronized spawners, although it has been reported that melatonin acts as a transducer of environmental cues and influences the kisspeptin system and the HPG axis in certain fish species (Alvarado et al., 2015; Carnevali et al., 2011; Sébert et al., 2008b).

Most groupers (genus *Epinephelus*) exhibit protogynous hermaphroditism and undergo sex-reversal from the female phase to the male phase. Because of the high economic value of certain groupers, much attention has been paid to the endocrine mechanisms of gonadal development as well as sex-reversal (Johnson et al., 1998; Kanemaru et al., 2012), but not to endocrine mechanisms affecting lunar-related reproduction; although, the seed production of groupers in aquaculture takes into account the lunar cycle (Teruya et al., 2008). The honeycomb grouper *Epinephelus merra* is a small, common species inhabiting coral reefs of tropical and subtropical waters. Although the honeycomb grouper is not the subject of aquaculture, it has been uses as an ideal species for studying the reproductive physiology of groupers (Bhandari et al., 2003, 2004; Kanemaru et al., 2012; Lee et al., 2010). This species repeats a typical lunar-related reproductive cycle with spawning preference around the full moon period during the spawning season from May to August (Lee et al., 2002). The objective of the present study was to clarify how lunar-related reproduction is endogenously regulated in the female honeycomb grouper. Weekly changes in *gnrhs* (*gnrh1* and *gnrh2*) in the diencephalon and the β-subunit of *gths* (*fshβ* and *lhβ*) in the pituitary were measured using real-time quantitative polymerase-chain reaction (qPCR). In addition, we examined the involvement of melatonin in transducing cues of moonlight as an internal signal.

2. Materials and methods

2.1. Animals

The females of honeycomb grouper (n = 111, total length 193.9 ± 2.8 mm, body weight 102.5 ± 4.0 g) were collected from coral reefs around Sesoko Island (26°38′46″N, 127°51′54″E) or Ou-jima Island (26°07′46″N, 127°46′22″E) using a hook and line. Captured fish were transported to Sesoko Station, Tropical Biosphere Research Center (TBRC), University of the Ryukyus, Okinawa, Japan. Photoperiod and water temperature during the experimental period ranged from 13.09 h to 13.47 h and from 24.5 °C to 30.5 °C, respectively. 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.

For the study on lunar-periodical reproduction (Experiment 1), fish (n = 71, total length 191.9 ± 3.2 mm, body weight 97.6 ± 4.3 g) were collected weekly from coral reefs around Sesoko Island in May and June 2016. Fish were housed in outdoor tanks (2 metric tons capacity) with running seawater and aeration under natural photoperiod at Sesoko Station, TBRC. Tissue collection at midnight around the first quarter moon, full moon, last quarter moon, and new moon was conducted within 2 days under a dim red light, according to the conditions by the previous studies (Bayarri et al., 2002; Oliveira et al., 2007; Takeuchi et al., 2014). After anesthetizing with 2-phenoxyethanol (Kanto Chemicals, Tokyo, Japan) and measuring total length and body weight, fish were sacrificed by decapitation. The brain was removed from the skull and divided into the pituitary, telencephalon, optic tectum, cerebellum, diencephalon, and medulla oblongata, and immediately immersed in RNA*later* (Sigma-Aldrich, St. Louis, MO, USA), frozen in liquid nitrogen, and then stored at −80 °C until RNA extraction. The ovary was also removed from the body cavity, weighed, and cut into small pieces. Some pieces of ovary were fixed in Bouin’s solution for 24 h. The gonadosomatic index (GSI) was calculated using the following formula: GSI = (ovarian mass/body weight) × 100. In addition to these samples, fish (n = 2) were preliminarily collected on 5 May to check ovarian development at that point in the year.

To measure melatonin content in the eye (Experiment 2), fish (n = 24) were collected in June and July around Sesoko Island and kept in polystyrene tanks (300-L capacity) with running seawater and aeration under natural photoperiod at Sesoko Station, TBRC. Fish were housed in two glass aquaria (60-L capacity; six individuals per aquarium) one day before the full moon. Fish were taken from the aquaria at 1200 h and 0000 h of the full moon and anesthetized with 2-phenoxyethanol. After measuring total length and body weight, the eyes were sampled and frozen in liquid nitrogen. Sample collection at 0000 h occurred under a red dim light. Sample collection using an identical protocol was conducted at 1200 h and 0000 h of the new moon.

The effects of melatonin treatment on gene expression in the brain were examined during the spawning season (Experiment 3). Fish used for the experiment (n = 14, total length 206.4 ± 4.7 mm, body weight 125.0 ± 9.4 g) were collected from coral reefs around Ou-jima Island in May 2018. They were transported to Sesoko Station and acclimatized in an outdoor tank with running seawater and aeration for one week under natural photoperiod.

Melatonin was purchased from Sigma-Aldrich, dissolved in ethanol, and diluted to a concentration of 1 mg/ml with saline. At the start of the experiment around the last quarter moon, fish were removed from the outdoor tank and anesthetized with 2-phenoxyethanol. An osmotic pump (Alzet Model 1004, Durect, Cupertino, CA, USA) containing melatonin was peritoneally implanted into fish (n = 7) of the treatment group (MT+). The concentration of melatonin released from the osmotic pump was set at 110 pg/h. An osmotic pump containing the vehicle only was also implanted into the peritoneal cavity of control fish (n = 7; MT−). Fish of the treatment and control groups were separately housed in two tanks (100-L capacity) and kept until sampling around the next full moon (3 weeks after implantation). Fish were fed daily with commercial pellets (Himesakura, Higashimaru, Kagoshima, Japan). After anesthetizing with 2-phenoxyethanol and sacrificing by decapitation, the brain (pituitary and diencephalon) and ovary were collected at 1200 h. The subdivided brain parts were immersed in RNA*later*, frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction. After weighing, small pieces of ovary were preserved in Bouin's solution.

2.2. Histological observation

Pieces of the ovarian samples were dehydrated in an ethanol series, permutated with xylene, and embedded in histoparaffin (Paraplast Plus, Sigma-Aldrich). Samples were then serially sectioned at 7 µm and stained with hematoxylin-eosin for microscopic observation. Following previous histological observations (Hoque et al., 1998), oocytes in an ovary were classified into five stages: peri-nucleolus (PNS), oil-droplet (ODS), primary yolk (PYS), secondary yolk (SYS), and tertiary yolk (TYS). Post-ovulatory follicles (POFs) and atretic oocytes (AOs) were also observed in the present study.

2.3. Real-time quantitative PCR (qPCR)

Total RNA was extracted from the brain using RNAiso Plus (Takara Bio, Kusatsu, Japan), according to manufacturer's protocol and reverse-transcribed using Prime Script RT reagent kit with gDNA Eraser (Takara Bio) to obtain the first strand cDNA. After RNA quality was checked by agarose electrophoresis, samples with distinct ribosomal bands were selected for the assay. Primers used for measurement of *gnrh1*, *gnrh2*, *fshβ*, *lhβ*, and elongation factor 1 alpha (*ef1α*) of the honeycomb grouper were designed using Primer3Plus software (version: 2.4.2) (Table 1). Efficiency (E) and correlation coefficient (R^2) obtained from the primer check were as follows; E = 100.2% and $R^2 = 0.971$ for *gnrh1*; E = 105.3% and $R^2 = 0.983$ for *gnrh2*; E = 95.0% and $R^2 = 0.994$ for *fshβ*; E = 103.0% and $R^2 = 0.993$ for *lhβ*; E = 101.8% and $R^2 = 0.991$ for *ef1α*. The mRNA abundances of *gnrh1* and *gnrh2* in the diencephalon and *fshβ* and *lhβ* in the pituitary were measured using the CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq (Takara Bio). The PCR cycles were as follows: one cycle of 95 °C for 2 min; 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Expression levels were normalized using the ΔΔCt method with *ef1α* as a housekeeping gene. All qPCR experiments were performed in duplicate.

2.4. Melatonin measurement

Melatonin content in the eye was measured using a time-resolved fluoroimmunoassay as previously described (Takemura et al., 2004). After weighing, the left eye was homogenized in phosphate-buffered saline (PBS) and centrifuged at 10,000 × *g* at 4 °C for 20 min. Supernatant was applied to a Sep-pak C18 cartridge (Waters, Milford, MA, USA), which had been previously equilibrated with 10 ml of methanol followed by 10 ml of distilled water (DW). After a one-time wash with 10 ml of 0.1 M citric buffer (pH 4.0) and a two-time wash with 10 ml of DW, fractions containing melatonin were eluted with 60% methanol and dried using a centrifugal vaporizer (CVE-100, EYELA, Tokyo, Japan). The resultant was reconstituted with assay buffer, which consisted of 0.05 M Tris, 0.9% NaCl, 0.5% bovine serum albumin (BSA), 0.05% NaN_3, 0.01% Tween-40, and 20 mM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (pH 7.75).

Melatonin was conjugated with BSA (melatonin-BSA conjugate) according to the previous method (Yamada et al., 2002). Rabbit antiserum against melatonin (HAC-AA92–03RBP86) was a gift from the Institute for Molecular and Cellular Regulation, Gunma University. Europium (Eu)-labeled immunoglobulin was prepared using an Eu-labeling kit (PerkinElmer, Waltham, MA, USA) according to manufacturer's protocol. A total of 100 µl of melatonin-BSA conjugate (diluted to a final concentration of 5 ng/ml with carbonate buffer, pH 9.6) was immobilized to the surface of a microplate (AGC Techno Glass, Shizuoka, Japan). After incubating the plate at room temperature for 2 h and washing with PBS-Tween, 50 µl of rabbit antiserum against melatonin (1:200,000 in assay buffer) and 50 µl of standards (2000 to 7.81 pg/ml) and reconstituted samples were added to the wells. The reaction was incubated overnight at 4 °C. After washing with PBS-Tween, 100 µl of Eu-labeled immunoglobulin (1:1000 in assay buffer) was added to each well and incubated at room temperature for 1 h. After washing with PBS-Tween, 100 µl of DELFIA enhancement solution (PerkinElmer) was added to each well to measure the intensity of the dissociated EU using a time-resolved fluorometer (PerkinElmer).

Table 1
Primer set sequences used in the present study.

Primer	Sequence
gnrh1-forward[*]	5′-CTCAGACGCACCAGGCAATA-3′
gnrh1-reverse[*]	5′-TGTCGGTGACACTGTCGAGA-3′
gnrh2-forward[**]	5′-AGCTGGACTCTTTTGGCACG-3′
gnrh2-reverse[**]	5′-GGCCAAGGCTTCCAGAATAATGTT-3′
fshβ-forward[***]	5′-TGCCACTCCGACTGTTCATCT-3′
fshβ-reverse[***]	5′-TCTGTTCAGCCCAGTCATCG-3′
lhβ-forward[***]	5′-GGAGAAGGAAGGCTGTCCAA-3′
lhβ-reverse[***]	5′-TGACAGGGTCCTTCGTGATG-3′
ef1α-forward[***]	5′-GGCTGGTGATAGCAAGAATG-3′
ef1α-reverse[***]	5′-GAACTTGCAGGCAATGTGAG-3′

[*] These were designed based on the contig sequences of the honeycomb grouper, which were assembled from the read data by RNA-sequencing.

[**] These were designed based on the cDNA sequence of the orange-spotted grouper *E. coioides* (Genbank Accession Number; **GU143808**).

[***] These were designed and used in the previous report (Kobayashi et al., 2010).

2.5. Statistical analyses

All data are shown as mean ± standard error of the mean (SEM). The data were analyzed using the Kruskal–Wallis test together with the Steel–Dwass post-hoc test or Scheffe post-hoc test comparisons between each sampling dates. Student's *t*-test was used to test for significant difference between the MT+ and MT− groups as well as the 1200 h and 0000 h groups. Differences were considered significant at $P < 0.05$. Statistical analyses were performed using Statcel3 (Excel Add-in) and R version 3.4.2.

3. Results

3.1. Weekly changes of GSI and oocyte development during the spawning season (Experiment 1)

Lunar-related changes in GSI and ovarian histology were evaluated in the present study. When fish were collected weekly according to the lunar cycle in May and June, GSI was lowest level on 14 May (first quarter moon). Values slightly increased on 22 May (full moon) and remained at the same levels on 29 May (last quarter moon) and 5 June (new moon). A rapid increase in GSI occurred on 12 June (first quarter moon). After the peak on 20 June (full moon), values dropped substantially on 28 June (last quarter moon) (Fig. 1).

Histological observation of ovaries revealed that only immature oocytes at PNS were detected in ovaries on 5 May (data not shown). Similar ovarian features were observed in many ovaries from 14 May to 5 June (Fig. 2a). However, some ovaries on these days had oocytes laden with yolk. Many oocytes laden with yolk (at PYS and SYS) were observed in ovaries on 12 June (Fig. 2b). Subsequently, the proportion of vitellogenic oocytes at TYS increased on 20 June (Fig. 2c). Ovaries on 28 June contained immature oocytes at PNS. POFs were also observed in ovaries on 28 June (Fig. 2d). When we calculated weekly changes in

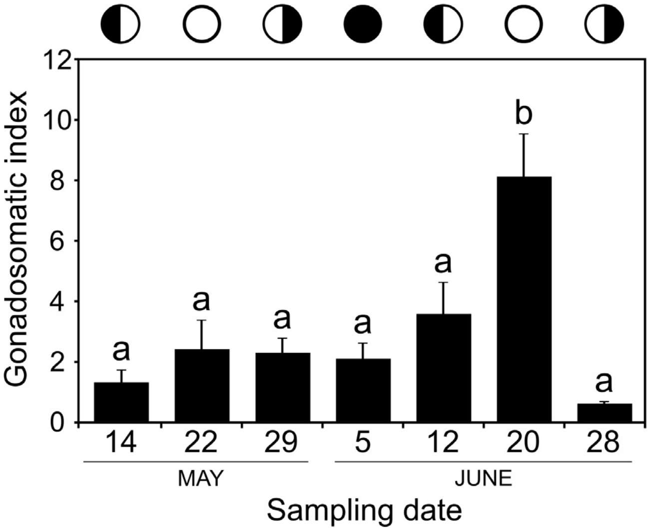

Fig. 1. Weekly change in GSI of the female honeycomb grouper (n = 6–11) during May to June in 2016. Each data is shown as means ± SEM. Lunar phases are indicated as ◐; first quarter moon, ○; full moon, ◑; last quarter moon, ●; new moon. Different letters indicate statistically significant difference at $P < 0.05$ (Non-parametric Kruskal-Wallis ANOVA on ranks, $P = 0.003$; Scheffe post-hoc test, $P < 0.05$).

oocyte stage composition, oocytes at TYS increased in the full moon in June and, subsequently, disappeared around the last quarter moon (Fig. 3).

3.2. *Gene expression profiles in the brain according to the lunar cycle (Experiment 1)*

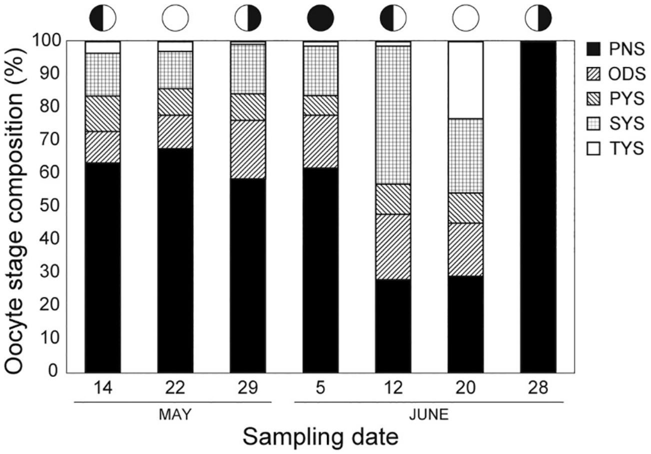

Fig. 3. Weekly change in the oocyte stage composition from May to June in 2016. Lunar phases are indicated as ◐, first quarter moon; ○, full moon; ◑, last quarter moon; ●, new moon. ODS; oil-droplet stage, PNS; peri-nucleolus stage, PYS; primary yolk stage, SYS; secondary yolk stage, TYS; tertiary yolk stage.

Weekly changes in mRNA expression profiles of *gnrh1* and *gnrh2* in the diencephalon and of *fshβ* and *lhβ* in the pituitary were measured using qPCR (Fig. 4). Expression of *fshβ* remained low from 14 May (first quarter moon) to 5 June (new moon) but significantly increased on 12 June (first quarter moon) and 20 June (full moon). Subsequently, expression decreased to the basal level on 28 June (last quarter moon) (Fig. 4a). Stable levels of *lhβ* expression were observed throughout the experimental period, although relatively high expression appeared to occur from 12 June (first quarter moon) to 28 June (last quarter moon) (Fig. 4b). The abundance of *gnrh1* increased around the last quarter moon in May and June, although the increase was only significant on 28 June (Fig. 4c). The abundance of *gnrh2* increased significantly toward the first quarter moon in June and then decreased (Fig. 4d).

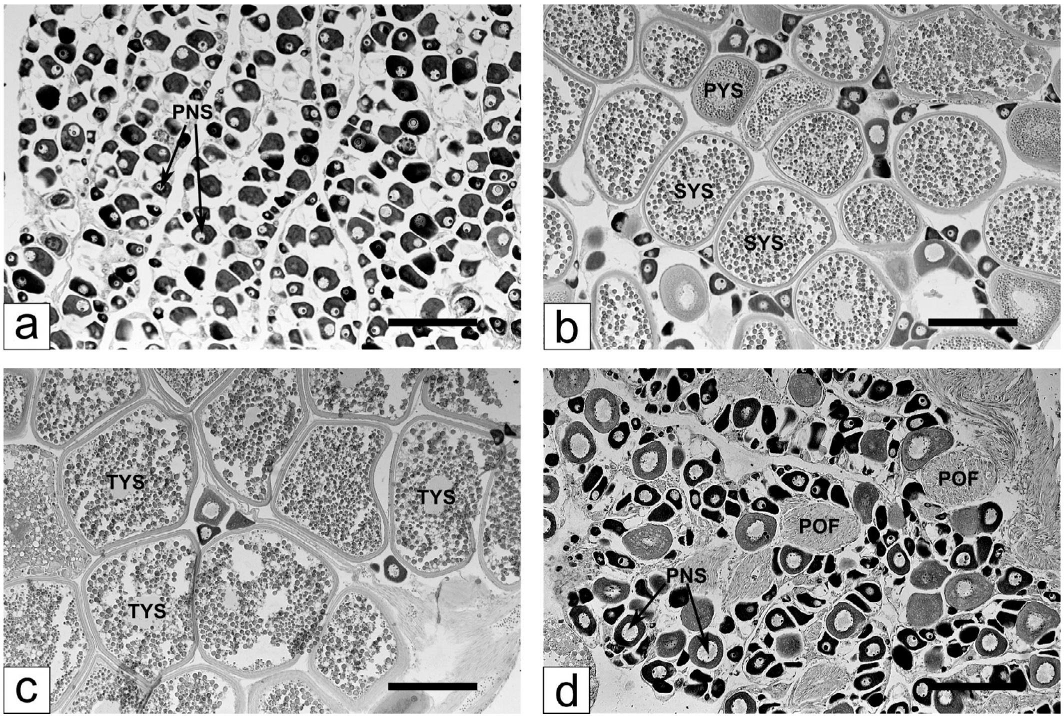

Fig. 2. Ovarian histology in the honeycomb grouper during June in 2016. (a) Cross section (CS) on 5 June (new moon), (b) CS on 12 June (first quarter moon), (c) CS on 20 June (full moon), (d) CS on 28 June (last quarter moon). PNS; peri-nucleolus stage, PYS; primary yolk stage, SYS; secondary yolk stage, TYS; tertiary yolk stage, POF; post-ovulatory follicle. Scale bar = 200 µm.

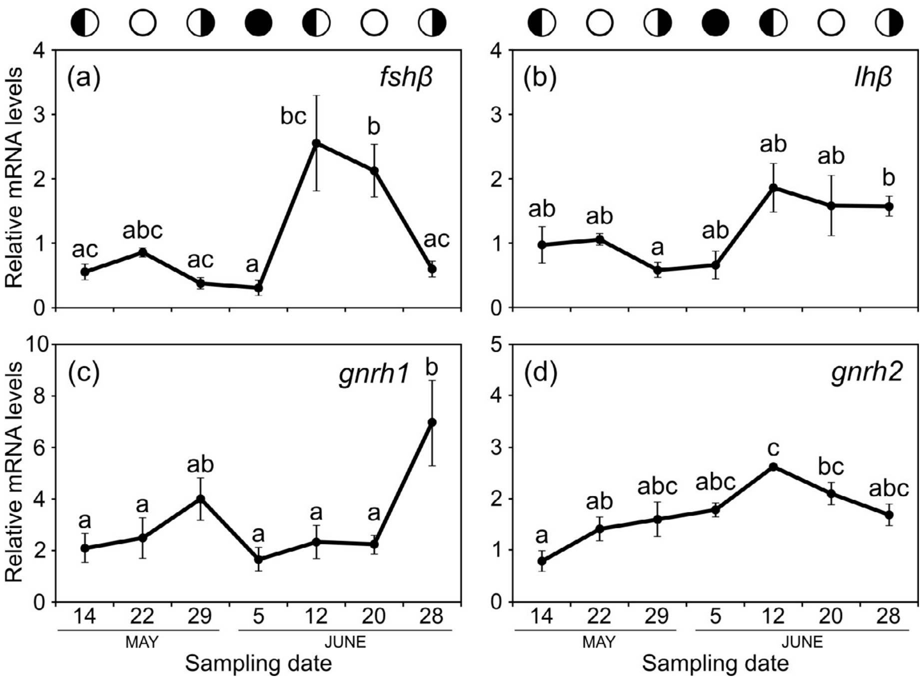

Fig. 4. Weekly changes in the expressions of *fshβ* (a) and *lhβ* (b) in the pituitary and *gnrh1* (c) and *gnrh2* (d) in the diencephalon of female honeycomb grouper (n = 4–9). Each data is shown as means ± SEM. Lunar phases are indicated as ◐; first quarter moon, ○; full moon, ◑; last quarter moon, ●; new moon. Different letters indicate statistically significant difference (Non-parametric Kruskal-Wallis ANOVA on ranks, $P = 3.8E\text{-}05$ for *fshβ*, $P = 0.0058$ for *lhβ*, $P = 0.0505$ for *gnrh1*, $P = 0.0015$ for *gnrh2*; Steel-Dwass post-hoc test, $P < 0.05$).

3.3. Effect of brightness at night on melatonin levels in the eyes (Experiment 2)

Melatonin content in the eye was measured at 1200 h and 0000 h during the full moon and the new moon. A day-low and night-high difference in melatonin content ($P < 0.05$) was observe around the new moon. Its content around the full moon at midnight (0000 h) did not increase and was not significantly different from that at noon (1200 h) (Fig. 5). The comparison of melatonin content at midnight between the new moon and the full moon showed that its content around the new moon was significantly higher than around the full moon ($P < 0.05$, Fig. 5).

3.4. Effect of melatonin treatment on gonadal condition and expression of fshβ and lhβ, gnrh1 (Experiment 3)

The effect of melatonin treatment on gonadal development and the abundances of *fshβ* and *lhβ* in the pituitary and *gnrh1* in the

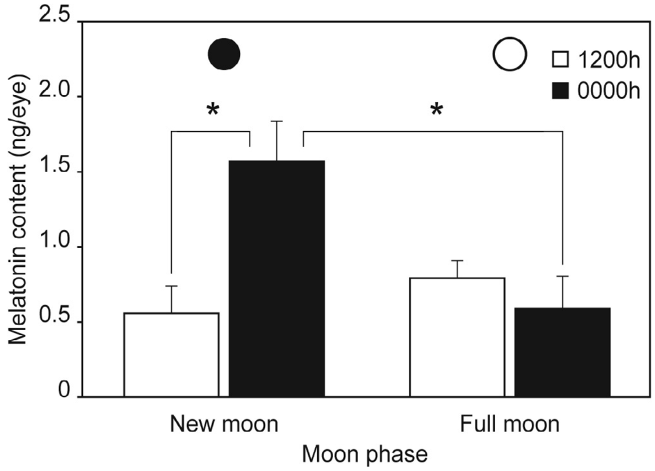

Fig. 5. Melatonin content in the eye at noon (1200 h) and midnight (0000 h) around the new moon and the full moon. Each data is shown as means ± SEM. Asterisk indicates a significant difference (Student's *t*-test, $P < 0.05$).

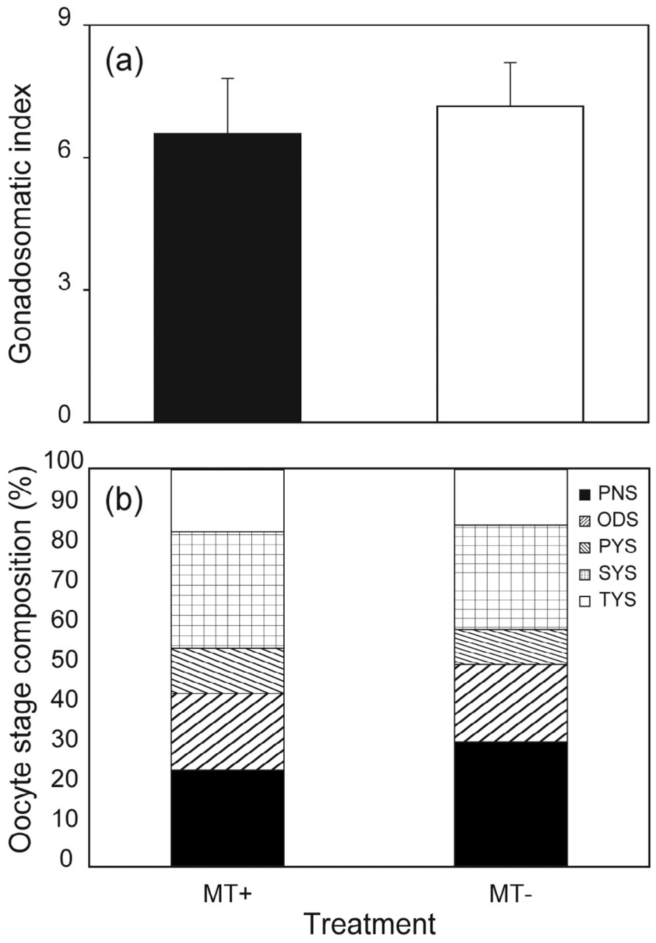

Fig. 6. Effect of melatonin treatment on gonadosomatic index (a) and oocyte stage composition (b) of the female honeycomb grouper. GSI is shown as means ± SEM. ODS; oil-droplet stage, PNS; peri-nucleolus stage, PYS; primary yolk stage, SYS; secondary yolk stage, TYS; tertiary yolk stage.

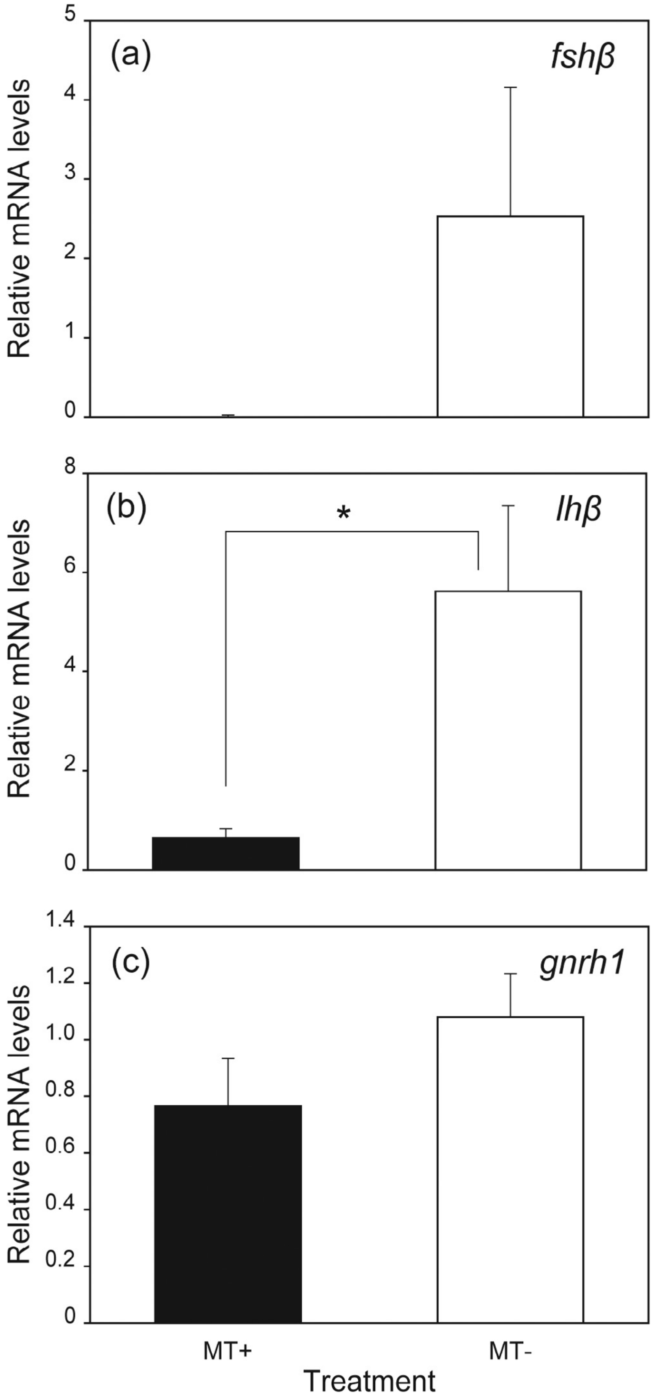

Fig. 7. Effect of melatonin treatment on the expressions of *fshβ* (a) and *lhβ* (b) in the pituitary and *gnrh1* (c) in the diencephalon. Each data is shown as means ± SEM. Asterisk indicates a significant difference (Student's *t*-test, $P < 0.05$).

diencephalon were examined in the present study. Melatonin treatment for three weeks did not vary GSI as well as oocyte stage composition (Fig. 6). In contrast, when the osmotic pump containing melatonin was implanted into the body cavity around the last quarter moon and left there for 3 weeks (until the full moon), the abundance of *fshβ* in the pituitary decreased, although values did not significantly differ between the melatonin-treated and control groups (Fig. 7a). Furthermore, the abundance of *lhβ* in the pituitary significantly decreased in the melatonin-treated group (Fig. 7b). A preliminary experiment revealed that melatonin implantation lowered the abundance of *fshβ* and *lhβ* in the pituitary (Fig. S1). On the other hand, melatonin treatment had little effect on the abundance of *gnrh1* in the diencephalon, although the value of the melatonin-treated group tended to decrease (Fig. 7c).

4. Discussion

The present study confirmed that the honeycomb grouper is a lunar-synchronized spawner with a preference toward the full moon period: weekly changes in GSI increased toward the full moon in June and subsequently decreased around the last quarter moon, at which point POFs could be observed histologically in most ovaries. A similar lunar-spawning pattern has previously been reported in the honeycomb grouper in Okinawan waters, but ovarian development and spawning occurred at least three times around the full moon from May to July, when vitellogenic oocytes and POF were observed in the same ovaries after the full moon period (Lee et al., 2002). In this regard, POFs were not observed in the ovaries collected in May in the present study, although vitellogenic oocytes at TYS were seen in some ovaries during this month. Therefore, fish collected in the present study were not expected to spawn around the full moon period in May.

The transcription levels of *fshβ* exhibited lunar-related variation with a significant increase from the first quarter moon to the full moon period in June, when yolk accumulation into developing oocytes actively occurred in all fish, suggesting that FSH plays a role in regulation of vitellogenesis through activation of E2 synthesis in ovarian follicles. This finding is consistent with results for the European sea bass *Dicentrarchus labrax* (Alvarado et al., 2013), the Japanese flounder *Paralichthys olivaceus* (Kajimura et al., 2001), and the greasy grouper *E. coioides* (Li et al., 2005). On the other hand, the transcription levels of *lhβ* did not show typical lunar-related variation, although relatively high levels of its gene expression were maintained from the first quarter moon to the last quarter moon in June. This transcription pattern may imply a relatively minor role in ovarian development, although LH is likely to be involved in final oocyte maturation and ovulation of certain fish, including the rainbow trout *Oncorhynchus mykiss* (Prat et al., 1996) and the marble eel *Anguilla marmorata* (Huang et al., 2009). Rapid increases in the mRNA abundances of *lhβ* as well as *fshβ* were observed in the pituitary of the red seabream *Pagrus major* during the spawning season (Gen et al., 2003). The present results appear to differ from these previous reports. Notably, when a luteinizing hormone-releasing hormone analog was implanted in the sevenband grouper *E. septemfasciatus* with vitellogenic oocytes at TYS (Shein et al., 2004), the development processes from vitellogenesis to final oocyte maturation were rapidly completed within 42 h. Importance of a LH surge in inducing final oocyte maturation was reported in certain fish (Karigo et al., 2012). These previous findings suggest the possibility that the processes of final oocyte maturation and ovulation under the influence of LH occur during spawning migration in nature, because most groupers, including the honeycomb grouper, aggregate at the spawning ground when the species-selected moon phase for spawning is approaching (Bijoux et al., 2013; Nanami et al., 2017; Tuz-Sulub and Brulé, 2015). Therefore, it is probable that we failed to detect an LH peak between the full moon and the last quarter moon. This hypothesis is further supported by our histological observation that most ovaries contained oocytes at TYS around the full moon period in June and POFs around the last quarter moon, suggesting that endocrine processes from final oocyte maturation to ovulation occurred between these moon phases.

Among three GnRH paralogues (GnRH1, GnRH2, and GnRH3) in the brain of perciformes (Zohar et al., 2010), GnRH1 likely plays a role in gonadal maturation. This thought is supported by immunohistochemical observations that GnRH1 cell bodies located in the preoptic area and its fibers projected into the pituitary of the red seabream (Okuzawa et al., 1997) and the barfin flounder *Verasper moseri* (Amano et al., 2002). Moreover, GnRH1 levels peaked in the brain and pituitary of the red seabream around the spawning season (Senthilkumaran et al., 1999). The present study clearly documented a lunar-related change in *gnrh1* in the hypothalamus of the honeycomb grouper; the transcription levels of *gnrh1* increased toward the last quarter moon in May (minor) and June (major) (Fig. 4c). This profile seemed not to correspond to weekly changes in the transcript levels of *fshß* and *lhß* in the pituitary. In this regard, it may be worth mentioning that day-night variations of *gnrh1* occurs in the brain of *E. coioides* and are different with ovarian development (Chai et al., 2013). Therefore, our weekly sampling is unlike to detect a GnRH1 peak occurring around the full moon, although GnRH1 is related to ovarian development toward this moon phase through synthesis and release of FSH and/or LH. In the medaka *Oryzias latipes*, a well-known daily spawner, GnRH released from neurons in the evening regulates a subsequent LH surge, suggesting that the GnRH1-LH cascade in the brain coordinates a daily ovulatory cycle of this species (Karigo et al., 2012). A similar hormonal cascade may occur in the brain of the honeycomb grouper on a weekly scale. On the other hand, the abundance of *gnrh2* mRNA did not exhibit lunar-related variation. Projection of GnRH2 immunoreactive fibers into the pituitary of the European sea bass was not observed (González-Martínez et al., 2001); thus, GnRH2 is unlikely to play a role in controlling FSH/LH synthesis in this species. Although little is known about the physiological roles of GnRH2 in coordinating reproductive behavior (White et al., 1995), one noteworthy finding is its action as a melatonin-releasing factor in the pineal organ of the European sea bass, in which GnRH2 immunoreactive fibers entered the pineal organ through the pineal stalk and its receptor was expressed in the pineal organ (Servili et al., 2010). Moreover, intraperitoneal injection of GnRH2 enhanced a nocturnal increase in plasma melatonin levels, suggesting that GnRH2 acts as a mediator of photic information perceived by the pineal organ (Servili et al., 2010). Because the pineal organ of the goldlined spinefoot can perceive changes in moonlight (Takemura et al., 2006), future research should focus on the physiological roles of GnRH2 in lunar-synchronized spawners including the honeycomb grouper. In the present study, the mRNA abundance of *gnrh2* increased from the first quarter moon in May to the same moon phase in June; thus, its transcriptional regulation may be not controlled by lunar-related factors.

The measurement of melatonin in the eye of the honeycomb grouper around the new moon and the full moon was performed in the present study. The effect of moonlight on melatonin fluctuation was evaluated using the eyes but not the pineal and plasma samples. The present study demonstrated that ocular melatonin content was higher around the new moon period compared to the full moon period, suggesting that the honeycomb grouper can sense changes in moonlight. These results are consistent with those observed in other lunar spawners, including spinefoots, in which, in addition to a day-low and night-high fluctuation, ocular melatonin content fluctuated with an increase around the new moon period and a decrease during the full moon period (Rahman et al., 2004). In this fish group and the Senegal sole *Solea senegalensis*, changes in moonlight altered the synthesis of melatonin in the pineal organ and/or its levels in the blood (Oliveira et al., 2010; Takemura et al., 2004, 2006), suggesting that melatonin is a possible transducer of changes in moonlight at night. Although we did not confirm the effect of moonlight on melatonin synthesis in the pineal organ of the honeycomb grouper, information regarding moonlight likely reaches the higher part of the HPG axis through the action of melatonin. This hypothesis may be partially reinforced by the evidence showing a day-low and night-high profile of melatonin receptor (*MT1*) as well as opposite profiles of kisspeptin (*kiss1* and *kiss2*) and GnRH (*gnrh1 and gnrh2*) in the brain of the female orange-spotted grouper, suggesting that melatonin plays a key role in regulating light-related expressions of key peptides (Chai et al., 2013).

Implanting an osmotic pump containing melatonin into the body cavity for 3 weeks from the first quarter moon did not alter gonadal conditions. However, this treatment reduced the transcription levels of *lhβ* in the pituitary. Effectiveness was seen on a decrease in the transcript levels of *fshβ* in the pituitary, although statistical difference was not shown between the melatonin-treated and control groups for *fshβ*. Similar inhibitory effects were noted in melatonin-implanted European eel *A. anguilla* females (Sébert et al., 2008a) and European sea bass males (Alvarado et al., 2015), in which the pituitary levels of *fshβ* and *lhβ* (5-month implantation) and plasma levels of FSH and LH (60- and 30-day implantation), respectively, decreased. The opposite effect was observed in females of the zebrafish *Danio rerio*; when fish were immersed in water containing melatonin for 10 days, the expression of *lhβ* in the pituitary was upregulated with concomitant increases in *vtg* and *erα* in the liver (Carnevali et al., 2011). These stimulatory or inhibitory effects of melatonin on fish reproduction may be partially due to difference in species and treatment (timing, dose, and method) used in each experiment, and diversity of reproductive strategies of each species. Melatonin is unlikely to act at the level of the hypothalamus to regulate GtH synthesis and release in the pituitary, because melatonin treatment induced little change in the transcript levels of *gnrh1* in the diencephalon of the honeycomb grouper (Fig. 7c). Correspondingly, melatonin implantation reduced the mRNA expression levels of *gnrh1* in the dorsal brain but not in the hypothalamus of male European sea bass (Alvarado et al., 2015). Melatonin treatment also inhibited dopamine release from perfused hypothalamic cells of carp *Cyprinus carpio* during the reproductive season (Popek et al., 2006) and dopaminergic activity in the brain of the tropical damselfish *Chrysiptera cyanea* (Badruzzaman et al., 2013). Because dopamine acts as an inhibitory factor of gonadotropin release in certain fish species (Aizen et al., 2005; Nocillado et al., 2007; Vidal et al., 2004; Yaron et al., 2003), this catecholamine is likely involved in controlling LH release from the pituitary. Moreover, recent reports have elucidated that melatonin treatment upregulates the expression levels of gonadotropin-inhibitory hormone (GnIH) in the brain of the Nile tilapia *Oreochromis mossambicus* (Kim et al., 2017). Although GnIH was not measured in the honeycomb grouper in the present study, GnIH likely has an inhibitory effect on the synthesis of GtH in this species, as the transcription levels of hypothalamic *gnih* decreased in accordance with oocyte development in the orange-spotted grouper (Wang et al., 2015).

We concluded that hormones secreted from the respective endocrine organs of the HPG axis repeat lunar-related fluctuations during the spawning season and that melatonin functions as a key mediator and transducer of moonlight information. Based on these fundamental findings, we suggest that in the honeycomb grouper, transient changes in brightness at night from the new moon (dark night) to the full moon (bright night) reduce the quantitative levels of melatonin in the brain and, subsequently, interlocking activation of the HPG axis occurs toward the spawning lunar phase. Because lunar-related oscillation has been observed in some clock genes in fish (Fukushiro et al., 2011; Takeuchi et al., 2018; Toda et al., 2014), further studies are needed to verify the involvement of biological clocks in the entrainment of spawning to species-selective moon phases.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Authors' contribution

KF designed and carried out the entire experiments and analyzed all the data obtained in the present study. FY, NO, HM and YT contributed to prepare samples and accomplish experiments. CY contributed to store fish used in this study. They participated in preparing the manuscript. AT was a collaborator and supervisor in analyzing the data and writing the manuscript. All authors have read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113211.

References

Aizen, J., Meiri, I., Tzchori, I., Levavi-Sivan, B., Rosenfeld, H., 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen. Comp. Endocrinol. 142, 212–221. https://doi.org/10.1016/j.ygcen.2005.01.002.

Alvarado, M.V., Carrillo, M., Felip, A., 2013. Expression of kisspeptins and their receptors, *gnrh-1/gnrhr-II-1a* and gonadotropin genes in the brain of adult male and female European sea bass during different gonadal stages. Gen. Comp. Endocrinol. 187, 104–116. https://doi.org/10.1016/j.ygcen.2013.03.030.

Alvarado, M.V., Carrillo, M., Felip, A., 2015. Melatonin-induced changes in *kiss/gnrh* gene expression patterns in the brain of male sea bass during spermatogenesis. Comp. Biochem. Physiol. A 185, 69–79. https://doi.org/10.1016/j.cbpa.2015.03.010.

Amano, M., Oka, Y., Yamanome, T., Okuzawa, K., Yamamori, K., 2002. Three GnRH systems in the brain and pituitary of a pleuronectiform fish, the barfin flounder *Verasper moseri*. Cell Tissue Res. 309, 323–329. https://doi.org/10.1007/s00441-002-0594-z.

Badruzzaman, M., Bapary, M.A.J., Takemura, A., 2013. Possible roles of photoperiod and melatonin in reproductive activity via changes in dopaminergic activity in the brain of a tropical damselfish, *Chrysiptera cyanea*. Gen. Comp. Endocrinol. 194, 240–247. https://doi.org/10.1016/j.ygcen.2013.09.012.

Bayarri, M.J., Madrid, J.A., Sánchez-Vázquez, F.J., 2002. Influence of light intensity, spectrum and orientation on sea bass plasma and ocular melatonin. J. Pineal Res. 32, 34–40. https://doi.org/10.1034/j.1600-079x.2002.10806.x.

Bhandari, R.K., Komuro, H., Nakamura, S., Higa, M., Nakamura, M., 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zool. Sci. 20, 1399–1404. https://doi.org/10.2108/zsj.20.1399.

Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol. Reprod. Dev. 67, 303–307. https://doi.org/10.1002/mrd.20027.

Bijoux, J., Dagorn, L., Cowley, P.D., Simier, M., Adam, P.A., Robinson, J., 2013. Spawning aggregation dynamics of brown-marbled grouper and camouflage grouper at a remote Indian Ocean atoll. Endang. Species Res. 22, 145–157. https://doi.org/10.3354/esr00534.

Carnevali, O., Gioacchini, G., Maradonna, F., Olivotto, I., Migliarini, B., 2011. Melatonin induces follicle maturation in *Danio rerio*. PLoS ONE 6, e19978. https://doi.org/10.1371/journal.pone.0019978.

Chai, K., Liu, X., Zhang, Y., Lin, H., 2013. Day-night and reproductive cycle profiles of *melatonin receptor*, *kiss*, and *gnrh* expression in orange-spotted grouper (*Epinephelus coioides*). Mol. Reprod. Dev. 80, 535–548. https://doi.org/10.1002/mrd.22191.

Falcón, J., Migaud, H., Muñoz-Cueto, J.-A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469–482. https://doi.org/10.1016/j.ygcen.2009.04.026.

Fukushiro, M., Takeuchi, T., Takeuchi, Y., Hur, S.-P., Sugama, N., Takemura, A., Kubo, Y., Okano, K., Okano, T., 2011. Lunar phase-dependent expression of cryptochrome and a photoperiodic mechanism for lunar phase-recognition in a reef fish, goldlined spinefoot. PLoS ONE 6, e28643. https://doi.org/10.1371/journal.pone.0028643.

Gen, K., Yamaguchi, S., Okuzawa, K., Kumakura, N., Tanaka, H., Kagawa, H., 2003. Physiological roles of FSH and LH in red seabream, *Pagrus major*. Fish Physiol. Biochem. 28, 77–80. https://doi.org/10.1023/B:FISH.0000030480.97947.ba.

González-Martínez, D., Madigou, T., Zmora, N., Anglade, I., Zanuy, S., Zohar, Y., Elizur, A., Muñoz-Cueto, J.-A., Kah, O., 2001. Differential expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). J. Comp. Neurol. 429, 144–155. https://doi.org/10.1002/1096-9861(20000101)429:1 < 144::AID-CNE11 > 3.0.CO;2-B.

Hoque, M.M., Takemura, A., Takano, K., 1998. Annual changes in oocyte development and serum vitellogenin level in the rabbitfish *Siganus canaliculatus* (Park) in Okinawa, Southern Japan. Fisheries Sci. 64, 44–51. https://doi.org/10.2331/fishsci.64.44.

Huang, H., Zhang, Y., Huang, W.R., Li, S.S., Zhu, P., Liu, Y., Yin, S.W., Liu, X.-C., Lin, H.-R., 2009. Molecular characterization of marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles during artificially induced gonadal development. Gen. Comp. Endocrinol. 162, 192–202. https://doi.org/10.1016/j.ygcen.2009.02.012.

Ikegami, T., Takeuchi, Y., Hur, S.-P., Takemura, A., 2014. Impacts of moonlight on fish reproduction. Mar. Genomics 14, 59–66. https://doi.org/10.1016/j.margen.2013.11.007.

Johnson, K., Thomas, P., Wilson, R.R., 1998. Seasonal cycles of gonadal development and plasma sex steroid levels in *Epinephelus morio*, a protogynous grouper in the eastern Gulf of Mexico. J. Fish Biol. 52, 502–518. https://doi.org/10.1111/j.1095-8649.1998.tb02013.x.

Kajimura, S., Yoshiura, Y., Suzuki, M., Aida, K., 2001. cDNA cloning of two gonadotropin β subunits (GTH-Iβ and -IIβ) and their expression profiles during gametogenesis in the Japanese flounder (*Paralichthys olivaceus*). Gen. Comp. Endocrinol. 122, 117–129. https://doi.org/10.1006/gcen.2000.7610.

Kanemaru, T., Nakamura, M., Murata, R., Kuroki, K., Horie, H., Uchida, K., Senthilkumaran, B., Kagawa, H., 2012. Induction of sexual maturation of the female honeycomb grouper, *Epinephelus merra*, in the non-breeding season by modulating environmental factors with GnRH analogue implantation. Aquaculture 358–359, 85–91. https://doi.org/10.1016/j.aquaculture.2012.06.026.

Karigo, T., Kanda, S., Takahashi, A., Abe, H., Okubo, K., Oka, Y., 2012. Time-of-day-dependent changes in GnRH1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka. Endocrinology 153, 3394–3404. https://doi.org/10.1210/en.2011-2022.

Kim, J.-H., Park, J.-W., Jin, Y.-H., Kim, D.-J., Kwon, J.-Y., 2017. Effect of melatonin on GnIH precursor gene expression in Nile tilapia, *Oreochromis niloticus*. Biol. Rhythm Res. 49, 303–313. https://doi.org/10.1080/09291016.2017.1357336.

Kobayashi, Y., Alam, M.A., Horiguchi, R., Shimizu, A., Nakamura, M., 2010. Sexually dimorphic expression of gonadotropin subunits in the pituitary of protogynous honeycomb grouper (*Epinephelus merra*): evidence that follicle-stimulating hormone (FSH) induces gonadal sex change. Biol. Reprod. 82, 1030–1036. https://doi.org/10.1095/biolreprod.109.080986.

Lee, L., Lee, C.-H., Hur, S.-W., Song, Y.-B., Takano, K., Takemura, A., Lee, Y.-D., 2010. Effect of 11-ketotestosterone (11-KT) on gonadal sex reversal and spermatogenesis of honeycomb grouper *Epinephelus merra*. Dev. Reprod. 14, 1–5.

Lee, Y.-D., Park, S.-H., Takemura, A., Takano, K., 2002. Histological observations of seasonal reproductive and lunar-related spawning cycles in the female honeycomb grouper *Epinephelus merra* in Okinawan waters. Fisheries Sci. 68, 872–877. https://doi.org/10.1046/j.1444-2906.2002.00505.x.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437. https://doi.org/10.1016/j.ygcen.2009.07.019.

Li, C.-J., Zhou, L., Wang, Y., Hong, Y.-H., Gui, J.-F., 2005. Molecular and expression characterization of three gonadotropin subunits common α, FSHβ and LHβ in groupers. Mol. Cell. Endocrinol. 233, 33–46. https://doi.org/10.1016/j.mce.2005.01.010.

Migaud, H., Davie, A., Taylor, J.F., 2010. Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. J. Fish Biol. 76, 27–68. https://doi.org/10.1111/j.1095-8649.2009.02500.x.

Nanami, A., Sato, T., Kawabata, Y., Okuyama, J., 2017. Spawning aggregation of white-streaked grouper *Epinephelus ongus*: spatial distribution and annual variation in the fish density within a spawning ground. PeerJ 5, e3000. https://doi.org/10.7717/peerj.3000.

Nocillado, J.N., Levavi-Sivan, B., Carrick, F., Elizur, A., 2007. Temporal expression of G-protein-coupled receptor 54 (GPR54), gonadotropin-releasing hormones (GnRH), and dopamine receptor D2 (drd2) in pubertal female grey mullet, *Mugil cephalus*. Gen. Comp. Endocrinol. 150, 278–287. https://doi.org/10.1016/j.ygcen.2006.09.008.

Okuzawa, K., Granneman, J., Bogerd, J., Goos, H., Zohar, Y., Kagawa, H., 1997. Distinct expression of GnRH genes in the red seabream brain. Fish Physiol. Biochem. 17, 71–79. https://doi.org/10.1023/A:1007760329837.

Oliveira, C., Dinis, M.T., Soares, F., Cabrita, E., Pousão-Ferreira, P., Sánchez-Vázquez, F.J., 2009. Lunar and daily spawning rhythms of Senegal sole *Solea senegalensis*. J. Fish Biol. 75, 61–74. https://doi.org/10.1111/j.1095-8649.2009.02263.x.

Oliveira, C., Duncan, N., Pousão-Ferreira, P., Mañanós, E., Sánchez-Vázquez, F.J., 2010. Influence of the lunar cycle on plasma melatonin, vitellogenin and sex steroids rhythms in Senegal sole, *Solea senegalensis*. Aquaculture 306, 343–347. https://doi.org/10.1016/j.aquaculture.2010.05.003.

Oliveira, C., Ortega, A., López-Olmeda, J.F., Vera, L.M., Sánchez-Vázquez, F.J., 2007. Influence of constant light and darkness, light intensity, and light spectrum on plasma melatonin rhythms in senegal sole. Chronobiol. Int. 24, 615–627. https://doi.org/10.1080/07420520701534657.

Pankhurst, N.W., Munday, P.L., 2011. Effects of climate change on fish reproduction and early life history stages. Mar. Freshwater Res. 62, 1015–1026. https://doi.org/10.1071/MF10269.

Park, Y.-J., Park, J.-G., Takeuchi, Y., Hur, S.-P., Lee, Y.-D., Kim, S.-J., Takemura, A., 2014. Influence of moonlight on mRNA expression patterns of melatonin receptor subtypes in the pineal organ of a tropical fish. Mar. Genomics 14, 67–70. https://doi.org/10.1016/j.margen.2013.10.006.

Patino, R., Sullivan, C.V., 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol. Biochem. 26, 57–70. https://doi.org/10.1023/A:1023311613987.

Popek, W., Luszczek-Trojnar, E., Drag-Kozak, E., Rzasa, J., Epler, P., 2006. Effect of melatonin on dopamine secretion in the hypothalamus of mature female common carp, *Cyprinus carpio* L. Acta Ichthyol. Piscat. 36, 135–141. https://doi.org/10.3750/aip2006.36.2.07.

Prat, F., Sumpter, J.P., Tyler, C.R., 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus Mykiss*). Biol. Reprod. 54, 1375–1382. https://doi.org/10.1095/biolreprod54.6.1375.

Rahman, M.S., Kim, B.-H., Takemura, A., Park, C.-B., Lee, Y.-D., 2004. Influence of light–dark and lunar cycles on the ocular melatonin rhythms in the seagrass rabbitfish, a lunar-synchronized spawner. J. Pineal Res. 37, 122–128. https://doi.org/10.1111/j.1600-079X.2004.00147.x.

Rahman, M.S., Takemura, A., Takano, K., 2000. Correlation between plasma steroid hormones and vitellogenin profiles and lunar periodicity in the female golden rabbitfish, *Siganus guttatus* (Bloch). Comp. Biochem. Physiol. B 127, 113–122. https://doi.org/10.1016/S0305-0491(00)00240-6.

Rahman, M.S., Takemura, A., Takano, K., 2001. Lunar synchronization of testicular development and steroidogenesis in rabbitfish. Comp. Biochem. Physiol. B 129, 367–373. https://doi.org/10.1016/S1096-4959(01)00323-2.

Rahman, M.S., Takemura, A., Takano, K., 2002. Lunar synchronization of *in vitro* steroidogenesis in ovaries of the golden rabbitfish, *Siganus guttatus* (Bloch). Gen. Comp. Endocrinol. 125, 1–8. https://doi.org/10.1006/gcen.2001.7708.

Saavedra, M., Pousao-Ferreira, P., 2006. A preliminary study on the effect of lunar cycles on the spawning behaviour of the gilt-head sea bream, *Sparus aurata*. J. Mar. Biol. Assoc. UK 86, 899–901. https://doi.org/10.1017/S0025315406013841.

Sébert, M.E., Legros, C., Weltzien, F.-A., Malpaux, B., Chemineau, P., Dufour, S., 2008a. Melatonin activates brain dopaminergic systems in the eel with an inhibitory impact on reproductive function. J. Neuroendocrinol. 20, 917–929. https://doi.org/10.1111/j.1365-2826.2008.01744.x.

Sébert, M.E., Weltzien, F.-A., Moisan, C., Pasqualini, C., Dufour, S., 2008b. Dopaminergic systems in the European eel: characterization, brain distribution, and potential role in migration and reproduction. Hydrobiologia 602, 27–46. https://doi.org/10.1007/s10750-008-9288-1.

Senthilkumaran, B., Okuzawa, K., Gen, K., Ookura, T., Kagawa, H., 1999. Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. J. Neuroendocrinol. 11, 181–186. https://doi.org/10.1046/j.1365-2826.1999.00304.x.

Servili, A., Lethimonier, C., Lareyre, J.-J., Lopez-Olmeda, J., Sánchez-Vázquez, F.J., Kah, O., Muñoz-Cueto, J.-A., 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the european sea bass *Dicentrarchus labrax*. Endocrinology 151, 2265–2275. https://doi.org/10.1210/en.2009-1207.

Shein, N.L., Chuda, H., Arakawa, T., Mizuno, K., Soyano, K., 2004. Ovarian development and final oocyte maturation in cultured sevenband grouper *Epinephelus septemfasciatus*. Fisheries Sci. 70, 360–365. https://doi.org/10.1111/j.1444-2906.2004.00814.x.

Takemura, A., Rahman, M.S., Park, Y.J., 2010. External and internal controls of lunar-related reproductive rhythms in fishes. J. Fish Biol. 76, 7–26. https://doi.org/10.1111/j.1095-8649.2009.02481.x.

Takemura, A., Sri Susilo, E., Rahman, M.S., Morita, M., 2004. Perception and possible utilization of moonlight intensity for reproductive activities in a lunar-synchronized spawner, the golden rabbitfish. J. Exp. Zool. A 301A, 844–851. https://doi.org/10.1002/jez.a.105.

Takemura, A., Ueda, S., Hiyakawa, N., Nikaido, Y., 2006. A direct influence of moonlight intensity on changes in melatonin production by cultured pineal glands of the golden rabbitfish, *Siganus guttatus*. J. Pineal Res. 40, 236–241. https://doi.org/10.1111/j.1600-079X.2005.00306.x.

Takeuchi, Y., Imamura, S., Sawada, Y., Hur, S.-P., Takemura, A., 2014. Effects of different colors of light on melatonin suppression and expression analysis of *Aanat1* and melanopsin in the eye of a tropical damselfish. Gen. Comp. Endocrinol. 204, 158–165. https://doi.org/10.1016/j.ygcen.2014.05.015.

Takeuchi, Y., Kabutomori, R., Yamauchi, C., Miyagi, H., Takemura, A., Okano, K., Okano, T., 2018. Moonlight controls lunar-phase-dependency and regular oscillation of clock gene expressions in a lunar-synchronized spawner fish, Goldlined spinefoot. Sci. Rep. 8, 6208. https://doi.org/10.1038/s41598-018-24538-1.

Teruya, K., Masuma, S., Hondo, Y., Hamasaki, K., 2008. Spawning season, lunar-related spawning and mating systems in the camouflage grouper *Epinephelus polyphekadion* at Ishigaki island, Japan. Aquaculture Sci. 56, 359–368. https://doi.org/10.11233/aquaculturesci.56.359.

Toda, R., Okano, K., Takeuchi, Y., Yamauchi, C., Fukushiro, M., Takemura, A., Okano, T., 2014. Hypothalamic expression and moonlight-independent changes of *Cry3* and *Per4* implicate their roles in lunar clock oscillators of the lunar-responsive goldlined spinefoot. PLoS ONE 9, e109119. https://doi.org/10.1371/journal.pone.0109119.

Tuz-Sulub, A., Brulé, T., 2015. Spawning aggregations of three protogynous groupers in the southern Gulf of Mexico. J. Fish Biol. 86, 162–185. https://doi.org/10.1111/jfb.12555.

Vidal, B., Pasqualini, C., Le Belle, N., Holland, M.C.H., Sbaihi, M., Vernier, P., Zohar, Y., Dufour, S., 2004. Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: a neuroendocrine lock for the onset of puberty. Biol. Reprod. 71, 1491–1500. https://doi.org/10.1095/biolreprod.104.030627.

Wallace, R., Selman, K., 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Am. Zool. 21, 325–343. https://doi.org/10.1093/icb/21.2.325.

Wang, Q., Qi, X., Guo, Y., Li, S., Zhang, Y., Liu, X., Lin, H., 2015. Molecular identification of GnIH/GnIHR signal and its reproductive function in protogynous hermaphroditic orange-spotted grouper (*Epinephelus coioides*). Gen. Comp. Endocrinol. 216, 9–23. https://doi.org/10.1016/j.ygcen.2015.04.016.

White, S.A., Kasten, T.L., Bond, C.T., Adelman, J.P., Fernald, R., 1995. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. Proc. Natl. Acad. Sci. U.S.A. 92, 8363–8367. https://doi.org/10.1073/pnas.92.18.8363.

Yamada, H., Chiba, H., Amano, M., Iigo, M., Iwata, M., 2002. Rainbow trout eyed-stage embryos demonstrate melatonin rhythms under light–dark conditions as measured by a newly developed time-resolved fluoroimmunoassay. Gen. Comp. Endocrinol. 125, 41–46. https://doi.org/10.1006/gcen.2001.7731.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225, 131–185. https://doi.org/10.1016/S0074-7696(05)25004-0.

Zohar, Y., Muñoz-Cueto, J.-A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438–455. https://doi.org/10.1016/j.ygcen.2009.04.017.