Lunar-related maturation and spawning migration in the honeycomb grouper, *Epinephelus merra*

Ryosuke Murata1,2, Takafumi Amagai1, Daisuke Izumida1,3, Yuji Mushirobira1, Ryo Nozu2,4,5, and Kiyoshi Soyano*1

1 Institute for East China Sea Research, Organization for Marine Science and Technology, Nagasaki University, 1551–7 Taira-machi, Nagasaki 851–2213, Japan
2 Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905–0227, Japan
3 Kushiro Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, Katsurakoi, Kushiro, Hokkaido 085–0802, Japan
4 Research Center, Okinawa Churashima Foundation, 888 Ishikawa, Motobu, Okinawa 905–0206, Japan
5 Present address: Department of Biological Science and Technology, Kumamoto University, Kumamoto 860–8555, Japan

* Corresponding author: K. Soyano  E-mail: soyano@nagasaki-u.ac.jp

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**Abstract** To better understand the eco-physiological characteristics of reproduction in the coral reef small honeycomb grouper, *Epinephelus merra*, we investigated their spawning migration using biotelemetry, in addition to reproductive physiology analysis. Histological observations indicated that final ovarian maturation was not completed in honeycomb grouper collected from the coral reef pond (CRP), even during their spawning season. Additionally, our visual observations revealed that fish numbers decreased in the CRP after a full moon, which is thought to be their spawning time, suggesting their spawning migration. Next, we investigated the migration of honeycomb grouper during their spawning season using biotelemetry. Our investigations indicated that honeycomb grouper migrated from the inside of the CRP to the outside after a full moon, and then back to the inside again a few days later. These results strongly suggested that honeycomb grouper migrate to spawning sites located outside of the CRP, attain final ovarian maturation, and spawn after a full moon in the spawning season.

**Keywords** Coral reef fish, Eco-physiology, Biotelemetry, Spawning site, Ovary

**Introduction**

Fish exhibit diversity not only in terms of reproductive styles, but also in the sexual maturation process (Kagawa 2013; Kobayashi et al. 2013). Many studies regarding reproductive phenomena or sexual maturation processes have been conducted using a wide range of fishes with diverse reproductive styles (Devlin and Nagahama 2002; Wootton and Smith 2014). As a result, morphological and endocrinological characteristics of gametogenesis have been revealed. Additionally, expression profiles of genes related to sexual maturation have also been clarified, as described in reviews providing detailed information on fish reproductive physiology (Nagahama 1994; Munakata and Kobayashi 2010). Many of ecological researches regarding fishes reproductions have been also conducted to date (Wootton and Smith 2014; Bobori et al. 2018; Akongyuure 2020). Unfortunately, there is only a limited number of combined research of ecological research and physiological research concerning gonadal development in wild fish (Desjardins et al. 2011). Environmental factors such as water temperature, day length, photoperiod, and lunar cycle have strong effects on gametogenesis in wild teleosts (Lam 1983). In addition to these environmental factors, ecological characteristics such as population density, migration to suitable environments, and physical characteristics, such as spawning ground shape...
and water flow, may have some effects on the series of developmental steps from final maturation to spawning. Thus, to completely understand sexual maturation in teleosts, physiological and endocrinological studies associated with environmental, ecological, and ethological investigations regarding gonadal differentiation and maturation are needed.

Recently, biotelemetry has been actively used for ethological analyses in fish (Metcalfe et al. 1993; Acolas et al. 2004; Kawabe et al. 2004; Windle and Rose 2005). Physiological investigations of ethological data collected by biotelemetry have also been conducted (Mitamura et al. 2005; Ueda 2011). Biotelemetry is thought to be an effective tool for understanding several physiological aspects of spawning behaviors in fish. We recently clarified lunar-related spawning and gonadal development in the coral reef small honeycomb grouper, *Epinephelus merra* (Bloch, 1793). Our results demonstrated that honeycomb groupers show not only lunar-related spawning, but also migration to their spawning site before the full moon (Soyano et al. 2003). Thus, we commenced eco-physiological research concerning their spawning behavior and physiological phenomena using biotelemetry. Here, we provide reproductive characteristics and ethological knowledge collected using biotelemetry in the honeycomb grouper.

1. Ovarian development and spawning in honeycomb grouper

Honeycomb grouper (*E. merra*) is a small fish species widely distributed in tropical and sub-tropical coral reef areas. It is known that they prefer coastal coral reef areas as their habitat, and they exhibit protogynous hermaphroditism in territorial male fish; however, more detailed ecological characteristics are still unclear.

Honeycomb groupers begin ovarian development as the water temperature increases in early spring and, in Okinawa, they usually spawn from April to July. We investigated the ovarian development of honeycomb grouper collected from Sesoko Beach on Sesoko Island in the northern area of Okinawa, Japan, over several years since 2001. Our results showed that rapid ovarian develop-

![Fig. 1](image) Changes in gonadosomatic index (GSI) in honeycomb grouper collected from Sesoko Beach during spawning season (A). Histological images of the ovaries consisted of perinucleolus stage oocytes in early May (B), vitellogenic oocytes in late May (C), tertiary yolk-stage oocytes in early June (D), and perinucleolus or yolk vesicle-stage oocytes at just after the full moon in middle June (E). Pn, perinucleolus stage oocyte; Vt, vitellogenic oocyte; Ty, tertiary yolk-stage oocyte. Scale bars = 200 μm.
opment was observed in May, associated with an increase in the gonadosomatic index (GSI) (Fig. 1). Our results also demonstrated that GSI gradually increased after the full moon in early May, and then reached its highest level at the full moon of early June, after which GSI rapidly decreased (Fig. 1A). Subsequent studies demonstrated similar patterns of GSI levels in 2002 and 2003 (unpublished data). Additionally, histological observations of the gonads revealed that immature ovaries consisted of perinucleolus or yolk vesicle-stage oocytes, before the onset of vitellogenesis, in early May (Fig. 1B). Vitellogenic oocytes first appeared in late May, with increased GSI; then, the ovaries were filled with tertiary yolk-stage oocytes just before the full moon of early June (Fig. 1C and D). Fish collected from the inside of the coral reef pond (CRP) immediately after the full moon had immature ovaries consisting only of perinucleolus stage or vitellogenic oocytes (Fig. 1E). These results clearly indicated that vitellogenesis is completed in approximately 1 month in honeycomb grouper, and they spawn just after the full moon. Our subsequent investigations over the next few years have demonstrated that honeycomb grouper distributed in Sesoko Beach usually first spawn in the year just after the full moon of June, or early May in some cases. Thus, we have revealed lunar-synchronized spawning in the honeycomb grouper, consistent with the other related reports (Lee et al. 2002; Soyano et al. 2003).

However, neither fish with oocytes at the final mature stage, nor ovulated eggs, were collected during our survey involving the CRP at Sesoko Beach. Oocytes that have completed egg yolk formation usually begin final maturation by germinal vesicle migration, and then breakdown to attain fertilizability (Nagahama 1994). Later, oocytes are ovulated from follicle cell layers and kept in an ovarian or body cavity. It has been reported in other groupers that ovulation occurs at 36–48 h after the completion of egg yolk formation, followed by pairing of females and males, and spawning (Donaldson 1989; Okumura et al. 2002; Shein et al. 2004). Interestingly, in our study, physiological preparations for the final maturation or spawning were not observed in the fish collected from the CRP, which is the main habitat for honeycomb grouper, consistent with the other related report (Lee et al. 2002).

2. Reproduction-related behavior in honeycomb grouper

2.1 Visual observation in the coral reef pond

Our previous observations revealed that honeycomb grouper exhibited neither final maturated oocytes, nor spawning, in the CRP of Sesoko Beach. Based on these results, we hypothesized that honeycomb grouper intend to spawn migrate to the outside of the CRP during the spawning period just after the full moon. To test this hypothesis, we investigated the number of maturated fish distributed in the CRP daily during their spawning period by visual observation. We visually observed and counted the number of fish in the CRP by skin-diving for 1 h. Our results showed a trend of decreasing numbers of fish at 2 d after the full moon of early June, and then no fish were observed from 3 to 5 d after the full moon (Fig. 2). Additionally, the number of mature fish decreased at the same time in July (unpublished data). These results suggested that honeycomb groupers migrate out of the CRP to spawn during the spawning period. Our results also indicated that final maturation of oocytes and ovulation surely occur during migration from their habitat to the spawning site, or after the completion of migration. Col-
lection and observation of honeycomb grouper undergoing migration or staying at the spawning site would be needed to clarify the physiological characteristics of final maturation and spawning. Next, to specify the spawning site and migration route, we used biotelemetry methods.

2.2 Behavioral investigation using biotelemetry

We used a transmitter and receiver (V8SC-6L-R256 coded pinger and VR2 receiver, Vemco, Bedford, Canada) in this experiment. We embedded a small coded pinger in the body cavity of mature honeycomb grouper, which were collected from the inside of the CRP of Sesoko Beach before the full moon. Anesthetized fish were incised approximately 1 cm on the posterior side of the abdomen with a scalpel, and then the coded pingers, after being disinfected with 70% ethanol, were embedded. The notch was closed using surgical sutures and surgical adhesive (Fig. 3). In addition, antibiotics were applied to protect the notch, and the individual was released to the location where it was captured. Receivers were installed at 4 sites that did not dry out at low tide inside the CRP, and 4 places outside of the CRP (Fig. 4). The spatial installation interval of the receivers outside the reef edge was approximately 200 m, according to the manufacturer’s instructions. However, in the inside of the coral reef lagoon, we installed receivers at intervals of 50 to 100 m due to limitations in receiving range resulting from obstructing reef lagoons or rocks.

All receivers were recovered 10 d after the full moon, and we analyzed the recorded data. Our results clarified that the honeycomb grouper initially in the CRP underwent migration out of the CRP after the full moon, and then several days later, the fish returned to the inside (Soyano and Nakamura 2006). This result was associated with the visual observation results (Fig. 2). The fish appeared within the sensitive range of the receivers outside the CRP; however, the concentrated appearance of spawning was not observed at midnight. We still have not collected enough information regarding the spawning site to date, and thus further studies are needed to specify the spawning site and migration route. However, in this
study, we successfully provided new and valuable insights regarding reproduction-related behavior in wild groupers using biotelemetry.

3. Evidence of spawning after full moon

Although we clarified that the honeycomb grouper inhabiting Sesoko Beach migrate to the outside of the reef lagoon after the full moon, spawning and spawning behavior was not confirmed. Thus, we investigated spawning after the full moon in captivity using the fish collected about 2 months before spawning from Sesoko Beach. Female (n=22) and male (n=5) honeycomb groupers were kept in a 60 T concrete tank, and spawning was observed and confirmed. The fish usually spawn at midnight, and thus we collected and measured eggs floating in the tank the next morning to confirm their spawning in the tank. In this study, we collected floating eggs in the tank in the morning after 3 d from full moon, and thus we successfully confirmed that honeycomb groupers spawned for several days from midnight 2 d after the full moon (Fig. 5). These results were consistent with their migration period to the CRP.

4. Physiological mechanism creating a lunar rhythm

It is well known that marine organisms frequently exhibit tidally-related spawning and gonadal development (Numata and Helm 2014). It has been reported that chiton, crab, and grass puffer show half-lunar cycle-related spawning or larva release (Saigusa 1980; Yoshioka 1988; Yamahira 1997). These organisms with the half-lunar related reproductive cycle are thought to be affected mainly by tidal changes, and some other related environmental factors such as lunar illumination, or sunrise and sunset times. However, our results demonstrated that honeycomb grouper show a full moon spring tide-related reproductive cycle, including oocyte maturation, spawning, and migration to spawning sites. Although monthly spawning has already been reported in several other groupers or rabbitfishes, these fishes exhibit a new moon-, not full moon-related spawning (Hamamoto 1986; Masuma et al. 1993; Rahman et al. 2000; Harahap et al. 2001). In the white-streaked grouper Epinephelus ongus (Bloch, 1790), which is distributed in shallow coral reefs, spawning biology (e.g. gonadal development, spawning behaviors, and spawning aggregation) have been investigated at a spawning ground, the Yonara Channel, Ishigaki Island, Okinawa, Japan (Nanami et al. 2013, 2015, 2017; Ohta and Ebisawa 2015). Behavioral investigation of white-streaked grouper using biotelemetry revealed that they form spawning aggregations around the last quarter moon (Nanami et al. 2017). These facts suggest the diversity of environmental factors and sensitivity inducing ovarian maturation or spawning among fish species; however, we predict that all fish species might have a common endocrine mechanism creating a lunar-related reproductive cycle. Recently, the involvement of clock genes of the brain on the lunar-related spawning was reported in the honeycomb grouper (Fukunaga et al. 2020). Thus, we hypothesized that the pineal gland, which is one of the photoreceptive organs in fish, may receive a monthly change in lunar illumination, and then the lunar rhythm might be transmitted to gonads through the hypothalamus-pituitary gonadal axis, which is a well-known endocrine pathway regulating fish reproduction. We also predicted that pituitary thyroid hormones might trigger reproductive behavior or migration to spawning sites; however, further studies are needed to prove our hypothesis. Unfortunately, it is difficult to continue investigations involving the honeycomb grouper at Sesoko Beach because the wild population of the fish has decreased there. However, we recently discovered

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**Fig. 5** Number of surface-floating eggs collected from honeycomb grouper in the tank during their spawning season.
the spawning site of honeycomb grouper near Sesoko Island. We are now investigating using biotelemetry to better understand the timing of spawning aggregation and physiological changes during spawning in honeycomb grouper. It has been reported that the white-streaked groupers select spawning aggregation sites with a greater current velocity to widely spread egg to the outside of the spawning site as well as to avoid egg predation (Nanami et al. 2017). We predict that the honeycomb groupers also show the spawning aggregation to the out of CRP around full moon period to use the greater ebb current to transport egg, as well as to avoid predation by the many of predator inhabiting in the CRP, however, further study would be needed, to prove this hypothesis.

5. Necessity of eco-physiological studies using biotelemetry

There are several environmental factors affecting the internal lunar rhythm of fish, other than lunar illuminance, such as changes in water depth and vibrations accompanying tidal changes. To understand the physiological changes regulated by the lunar rhythm, we need to identify the endocrine pathway from a receptive organ to the brain. First, for that purpose, we have to begin a search for environmental factors causing the internal lunar rhythm in fish. The main factor that creates the internal lunar rhythm is revealed by investigations of the physiological changes affected by certain environmental factors, such as lunar illuminance, tidal changes, or vibrations, under artificial conditions. Simultaneously, we will need to confirm the effects of these factors on the appearance of the internal lunar rhythm, as well as in wild individuals. However, it is difficult to investigate physiological conditions of wild individuals, especially in the honeycomb grouper, because their migration is synchronized with the lunar rhythm. Therefore, biotelemetry methods are needed that are effective for eco-physiological studies in the field, as described above. The data logger, which has developed remarkably over recent years, is also an essential tool for understanding the environmental characteristics of fish habitats. To accurately understand physiological phenomenon in marine wild organisms, other than honeycomb grouper, a biotelemetry method should be actively utilized in future studies.

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Compliance

All experimental procedures involving animals were conducted in compliance with the Animal Care and Use Committee of the Institute for East China Sea Research, Nagasaki University, Japan (permit no. 15–06)

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