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Histological description and endocrine regulation of ovarian maturation in wild and captive white-streaked grouper *Epinephelus ongus*

Tomofumi Yamaguchi [a,b,1], Takayuki Takebe [c,2,3], Masato Nakachi [d], Yuuki Kawabata [e,4], Kazuhisa Teruya [f], Kiyoshi Soyano [a,*,5]

[a] *Institute for East China Sea Research, Organization for Marine Science and Technology, Nagasaki University, Nagasaki 851–2213, Japan*
[b] *Yaeyama Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Okinawa 907–0451, Japan*
[c] *Japan Fisheries Research and Education Agency, Kanagawa 221–8529, Japan*
[d] *Chatan Town Office, Okinawa 904–0192, Japan*
[e] *Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki 852–8521, Japan*
[f] *Kamiura Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Oita 879–2602, Japan*

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ABSTRACT

Information regarding the endocrine regulation of oocyte maturation in cultured species is essential for the artificial seed production. To elucidate endocrine mechanisms that underlie oocyte maturation of the white-streaked grouper, *Epinephelus ongus*, a commercially important fish species, we collected and compared data linked to the ovaries of captive and wild white-streaked grouper specimens, and plasma steroids data were obtained using captive fish. This data allowed for a comparative analysis of the ovarian development of both groups; the comparison was necessary for the acquisition of physiological patterns universally representative of the species at hand. The dynamics of the gonadosomatic index (GSI) and oocyte development of both groups confirmed that vitellogenesis progressed toward the last quarter moon, final oocyte maturation began the day before the last quarter moon, and spawning occurred after the last quarter moon. These results indicate that the changes in ovarian development and GSI in wild and captive fish were synchronous, and that the endocrine changes observed in captive fish represent those that occur in wild fish. The changes in estradiol 17β (E_2) levels in captive fish increased and decreased during the vitellogenic and final oocyte maturation phases, respectively. These dynamics were similar to those of GSI. We found that 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) was consistently detected at concentrations higher than those of 17α, 20β-21-trihydroxy-4-pregnen-3-one (20β-S); this trend was constant across all stages of ovarian development. In contrast, 20β-S was detected at lower concentrations than DHP in all captive individuals, but at higher levels after the onset of final oocyte maturation. These results suggest that 20β-S and/or DHP may be maturation-inducing hormones (MIH) in this species. Our results also highlight the importance of integrative analyses of wild and captive individuals to comprehensively elucidate the endocrine regulation of cultured species of interest to improve aquaculture management practices.

1. Introduction

Groupers (family Serranidae), are a group of protogynous and hermaphroditic fish whose wide distribution spans the tropical and temperate regions of the world. These fish are an important fishery species. Specifically, groupers have high market value due to their taste and the quality of their flesh; and in recent years they have also attracted attention as species for aquaculture and seed production, especially in

* Correspondence to: Institute for East China Sea Research (ECSER), Organization for Marine Science and Technology (OMST), Nagasaki University, 1551–7, Taira-machi, Nagasaki 851–2213, Japan.
E-mail address: soyano@nagasaki-u.ac.jp (K. Soyano).
[1] ORCID: 0000–0003-3096–7864
[2] Deceased 2nd January, 2023
[3] 0000–0003-3913–8931
[4] 0000–0001-8267–5199
[5] 0000–0003-0384–3241

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many Asian countries. Overall, there are at least 47 grouper species and 15 grouper hybrids that have researched for aquaculture or are currently being used in aquaculture (Rimmer and Glamuzina, 2019).

The white-streaked grouper, *Epinephelus ongus*, the main habitat of which is the Yaeyama region in Okinawa, has long been an important fishery species. However, there are concerns about decreases in wild stocks of this species due to fishing pressure. This is because white-streaked groupers form spawning aggregations in specific places during the spawning season, and these aggregations are usually targeted for fishing (Ohta and Ebisawa, 2015). The maturity and spawning of this species is very predictable, as they always spawn in the last quarter of the lunar cycle (Ohta and Ebisawa, 2015). In the Yaeyama region, there is a spawning ground in the Yonara Channel. This species moves from its typical habitat, coral reef ponds, to the spawning ground, and returns to its habitat after spawning (Nanami et al., 2013). As fishing was focused on targeting these aggregations, prohibited fishing periods and areas were set to improve resource management, based on the spawning characteristics of this species. To further accelerate such efforts, it is necessary to understand the spawning characteristics of the target species in detail. For the sustainable use of this species, it is necessary to optimize their use in aquaculture. This optimization will be important within the context of sustainability, as it will result in aquacultural efforts that yield the best outcomes in terms of productivity while having a minimal impact on natural resources. However, for such a result to be achieved, a comprehensive understanding of the physiological mechanisms that underlie the reproductive system of these fish will be necessary. However, the ovarian development and physiological mechanisms of white-streaked groupers during the spawning season have not yet been clarified. Maturation in teleost fish, including groupers, is regulated by the endocrine system of the brain-pituitary-gonadal axis (Lubzens et al., 2010; Nagahama and Yamashita, 2008). It is essential to understand the endocrine changes that accompany gonadal development, as it will allow for an understanding of the vitellogenesis and the final oocyte maturation of white-streaked groupers.

Previously, there have been cases in which the spawning season was determined by histological observations of the gonads using wild white-streaked groupers sourced from fish markets (Ohta and Ebisawa, 2015), but information on their reproductive physiology has not yet been obtained. It is difficult to source this information from wild fish. Therefore, we attempted to clarify the vitellogenesis and final oocyte maturation (i.e., and their corresponding physiological changes) of this species by comparing the gonadal development of wild and captive fish. In addition, changes in the gonadal development and the reproductive endocrine system that controls it, which change daily in relation to the lunar cycle, and which can only be determined under controlled conditions, were investigated, and research to clarify the vitellogenesis and the final oocyte maturation of this species was conducted. The significance of this study transcends the fact that it brings forth insights into the vitellogenesis and the final oocyte maturation of white-streaked groupers. Specifically, this foundational information is essential for the next-generation production and aquaculture of other groupers, for which there are concerns about their ever-decreasing natural stocks. Therefore, by comparing ovarian development in wild and captive fish, in addition to examining the development of sex steroids in captive fish, we provide insight into the histology and endocrinology of ovarian maturation within the period of a month for the white-streaked grouper.

2. Materials and methods

2.1. Animal welfare

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 number #15–06). According to these guidelines and policies, all the experiments were conducted without causing severe distress to the fish. To obtain tissue samples for histological observation and blood samples for hormone measurement, the fish were anesthetized for pain reduction. No other surgical operations or drug administration were performed.

2.2. Measuring and sampling wild white-streaked grouper

A total of 75 female specimens of wild white-streaked grouper with vitellogenic oocytes or post-ovulatory ovaries collected from the coastal waters of the Yaeyama Islands in Okinawa, Japan, between 15-Apr and 19-May, 2012, were obtained from the Yaeyama fish market. This study period included the last quarter moon of the 4th lunar calendar month (LCM4); this when the white-streaked grouper spawn (Ohta and Ebisawa, 2015). The lunar cycle during the study period was as follows: new moon on 21-Apr and 21-May, first quarter moon on 29-Apr, full moon on 6-May, and last quarter moon on 13-Apr and 13-May. Sampling was conducted between 10 am and 3 pm, and the total lengths (TL) and the body weights (BW) of the fish on the day the fish were obtained (24.1 $\pm$ 1.9 cm TL, 23.8 $\pm$ 6.1 g BW; Supplementary Table S1). TL was used to confirm that it was larger than that of the smallest mature female (18.9 cm TL), as reported in a previous study (Ohta and Ebisawa, 2016). Then, the ovaries were removed by abdominal incision and their masses measured to calculate the gonadosomatic index (GSI) [(ovarian weight / BW) $\times$ 100]. Ovaries were then subjected to histological examination. All wild fish are captured, killed, and then sold at market the next day. We purchased these fish and used them in our experiments. Therefore, all data of wild fish were treated as if they had been caught a day before the actual sampling date.

2.3. Rearing conditions, measuring and sampling of captive fish

From March to April 2012, a total of 42 wild white-streaked groupers were caught in Nagura Bay and Urasoko Bay, on Ishigaki Island. The sex determination of the collected fish was conducted as follows. Male: individuals with spermiation due to abdominal compression. Mature females: individuals with apparent abdominal swelling. Immature females: individuals that had no abdominal swelling and were not found to be spermiating after abdominal pressure.

Males and mature females were transported to the Yaeyama Station of the Seikai National Fisheries Research Institute (Fisheries and Technology Institute). Captive fish were kept in a large aquarium at the institute. Three sets of 5 kL FRP tanks were prepared; three males and 11 mature females were then added to each tank in 17-Apr 2012. This was done prior to the commencement of the rearing experiment. The fish were then allowed to acclimate to the experimental environment in running seawater under natural water temperature and photoperiod. During the experimental period, fillets of the double-lined fusilier (*Pterocaesio digramma*) were fed to the groupers once every three days.

Three randomly selected females from each tank were sampled every three days from 22-Apr to 22-May. This study period includes the last quarter moon of LCM4 as well as wild fish. These samplings were conducted at the same time of day as wild fish and performed using the following procedure. After the fish were anesthetized with 2-phenoxyethanol, their TL and BW were measured (23.5 $\pm$ 1.8 cm TL, 22.5 $\pm$ 5.6 g BW; Supplementary Table S2). TL was used to identify mature females as well as wild fish. Blood was collected from the caudal blood vessel using 1 ml syringes coated in advance with 1000 IU of sodium heparin (Mochida Pharmaceutical Co., Tokyo, Japan). Plasma was separated from blood by centrifugation (12000 $\times g$ for 10 min at 4 °C) and then frozen at $-$ 80 °C until use in the steroid assay. After blood collection, the ovaries were removed using an abdominal incision, and the ovarian weight was measured. GSI calculations were performed in the same method as for wild fish.

2.4. Gonadal histology

Ovaries were fixed in Bouin's solution for 24–48 h and preserved in

70% ethanol. The preserved ovaries were dehydrated using a graded ethanol and butanol series, and embedded in paraffin according to a standard method. The embedded ovaries were sliced at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) using standard histological techniques. Stained ovarian sections were histologically observed using an optical microscope (BX50F4; Olympus, Tokyo, Japan).

The maturity of each individual was classified according to the stage of the most advanced oocyte in the ovary. The classification of oocyte stages was conducted with reference to previous studies on groupers (Amagai et al., 2022; Ohta and Ebisawa, 2015) and was done as follows: yolk vesicle stage (Yv, Fig. 1A) refers to secondary growth phase of oocytes, with a yolk vesicle (cortical alveolus) in the cytoplasm; primary yolk stage (Py, Fig. 1B) refers to small yolk globules appear in the cytoplasm, and many small oil droplets lie around the germinal vesicle; secondary yolk stage (Sy, Fig. 1C and D) refers to oocytes are filled with slightly larger yolk globules and oil droplets, and the egg membrane thickens; tertiary yolk stage (Ty, Fig. 1E) refers to oocytes have almost completed yolk accumulation, and some large oil droplets lie around the germinal vesicle; migratory nucleus stage (Mn, Fig. 1F and G) refers to the germinal vesicle of the oocyte migrates toward the animal pole, and each of yolk globules and oil droplets begin to fuse; and ripe stage (R, Fig. 1H) refers to complete fusion of each of the yolk globules and oil droplets. In addition, individuals who had experienced ovulation (spawning) were identified by the detection of post-ovulatory follicles (Pof, Fig. 1I).

2.5. Measurement of plasma steroid hormones

The plasma estradiol-17β (E_2) level was measured following the

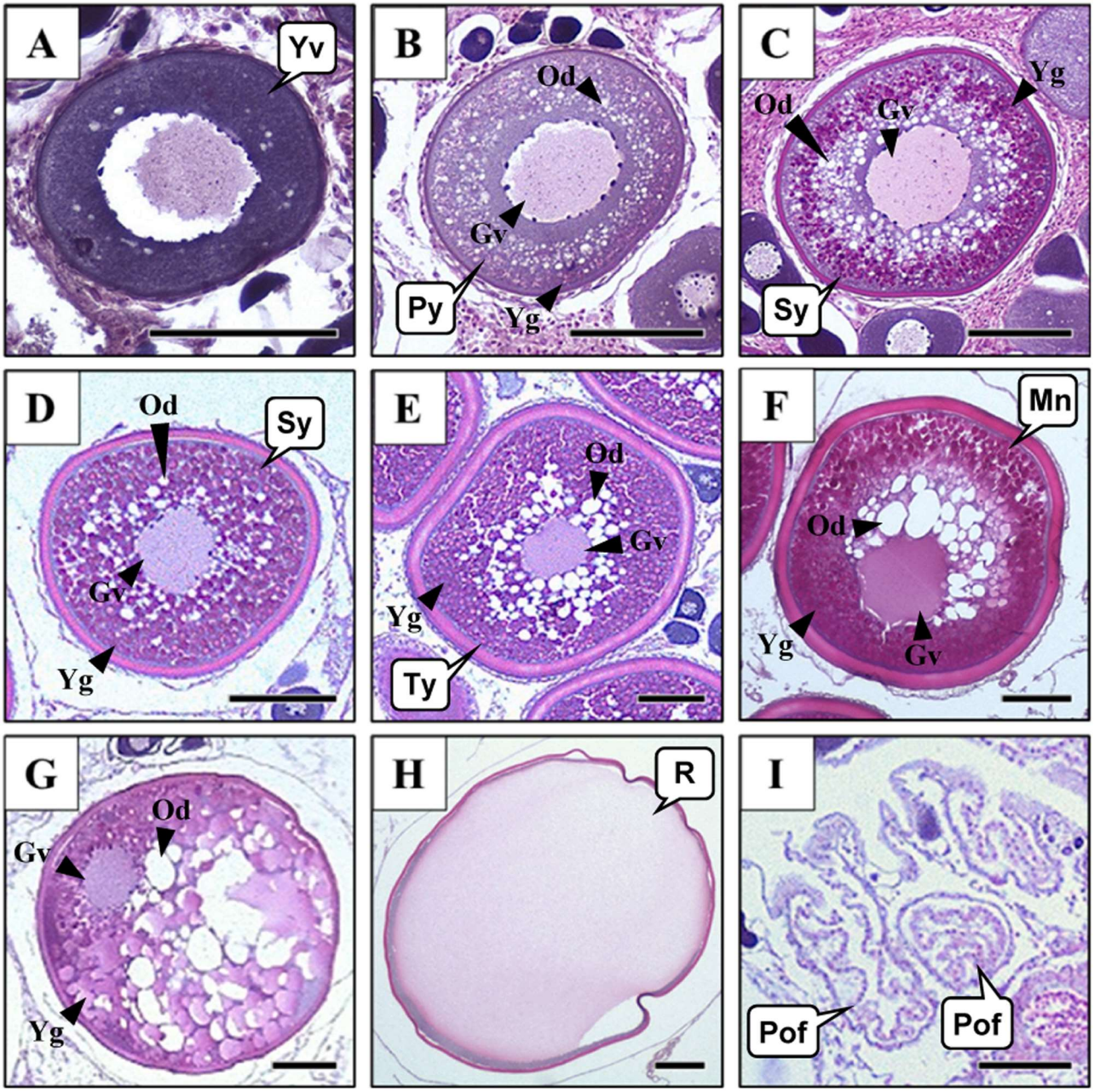

Fig. 1. Oocyte development of white-streaked grouper. A, york vesicle stage (Yv); B, primary yolk stage (Py); C, D, secondary yolk stage (Sy); E, tertiary yolk stage (Ty); F, G, migratory nucleus stage (Mn); H, ripe stage (R); I, post-ovulatory follicles (Pof). Od, oil droplet; Yg, yolk globule; Gv, germinal vesicle. Scale bars = 100 μm.

manufacturer's protocol using an estradiol EIA kit (Cayman Chem. Co., MI, USA). Plasma 17α, 20β-21-trihydroxy-4-pregnen-3-one (20β-S) and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) levels were measured using enzyme-linked immunosorbent assay (ELISA), according to the method described in (Asahina et al., 1995). Antibodies (anti-20β-S and anti-DHP) and steroid hormones labeled with horseradish peroxidase for the determination of the plasma 20β-S and DHP levels were purchased from Cosmo Bio, Tokyo, Japan.

2.6. Statistical analysis

Statistical analyses were performed using the Microsoft Excel add-in software Statcel4 (OMS Ltd, Saitama, Japan). These analyses were conducted to examine changes in GSI for wild and captive fish, and three steroid hormones for captive fish during the experimental sampling period. All data were tested using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine if there were significant differences in each stage of the most developed oocytes ($p < 0.05$ was significantly different). The same statistical analyses were also performed to test the association between GSI and the most developed oocytes in wild and captive fish, and between sex steroids and the most developed oocytes in captive fish.

3. Results

3.1. Changes in the ovarian development of wild and captive fish

The number of wild and captive individuals at the confirmed stages of oocyte development are shown for each study day in Tables 1 and 2,

Table 1
Changes in the stages of oocytes in wild white-streaked grouper.

Date	Lunar phase	Number of fish examined	Number of fish with most developed oocyte stage*				
			Yv	Sy	Ty	Mn	R
15-Apr		4			4		
17-Apr		3		3			
19-Apr	→ 21-Apr ●	2		1	1		
22-Apr		4		4			
24-Apr		1		1			
26-Apr		1		1			
28-Apr	→ 29-Apr ◐	1			1		
30-Apr		2		1	1		
5-May	→ 6-May ○	4		1	3		
7-May		1		1			
9-May		10			10		
10-May		3			3		
12-May	→ 13-May ◑	10			7	3	
14-May		23			2 (1)	7 (1)	14 (7)
17-May		4				4 (4)	
19-May		3	1 (1)			2	

●, New moon; ◐, First quarter moon; ○, Full moon; ◑, Last quarter moon.
The numbers in parentheses indicate the number of individuals observed post-ovulatory follicles.
Yv, yolk vesicle stage; Sy, secondary yolk stage; Ty, tertiary yolk stage; Mn, migratory nucleus stage; R, ripe stage.
* The categorization is based on the classification shown in Fig. 1.

Table 2
Changes in the stages of oocytes in captive white-streaked grouper.

Date	Lunar phase	Number of fish examined	Number of fish with most developed oocyte stage*				
			Yv	Sy	Ty	Mn	R
22-Apr		3			2		1
25-Apr		3		1	2		
28-Apr	→ 29-Apr ◐	2 *		1	1		
1-May		2 *			2		
4-May	→ 6-May ○	3			3		
7-May		3			3		
10-May		3			3		
13-May	→ 13-May ◑	3			2		1
16-May		3				3	
19-May	→ 21-May ●	3		1 (1)		1 (1)	1
22-May		3	1 (1)	1 (1)	1 (1)		

●, New moon; ◐, First quarter moon; ○, Full moon; ◑, Last quarter moon.
* Exclusion of an individual with immature oocytes.
The numbers in parentheses indicate the number of individuals observed post-ovulatory follicles.
Yv, yolk vesicle stage; Sy, secondary yolk stage; Ty, tertiary yolk stage; Mn, migratory nucleus stage; R, ripe stage.
* The categorization is based on the classification shown in Fig. 1.

respectively. The histological findings representative of the entire study period are also shown in Supplementary Fig. S1 and S2. Wild fish ovaries showed Ty on 15 and 19-Apr, but showed Sy from 17-Apr to 7-May (Table 1). After 28-Apr, Ty oocytes were confirmed again, and the ovaries of all individuals showed Ty on 9-May. On 12-May, the day before the last quarter moon, the ovaries of some fish showed Mn, but did not show R and Pof. On 14-May, the day after the last quarter moon, the ovaries of more than half of the fish showed R, and Pof was present in the ovaries of nine fish. On 19-May, in addition to a fish with Mn, Yv stage fish with Pof was identified.

On 22-Apr, the day after the new moon, one of the ovaries of captive fish showed R (Table 2). Although the captive fish ovaries showed Sy on 25 and 28-Apr, Ty oocytes were confirmed until the last quarter moon on 13-May. Mn or R of the FOM stage was observed between the last quarter moon and six days later. In addition, 6 days after the last quarter moon, Pof was present in the ovaries of all but one fish that showed R.

3.2. Changes in GSI and its relationship to maturity of wild and captive fish

The GSI values of wild and captive fish are shown in Fig. 2. The GSI of wild fish was significantly different in 22-Apr and from 9-May to 14-May, with higher values observed before and after the last quarter moon (13-May). Thereafter, the GSI of wild fish declined sharply and was significantly lower on 19-May. The GSI of captive fish did not change significantly from 21-Apr to 13-May, reaching its maximum on 13-May. Thereafter, the GSI of captive fish remained high until 16-May, then it declined sharply and was significantly lower on 22-May. The developmental stage of the most developed oocyte was classified as the developmental stage of the individual, and the mean and range of GSI for each stage is shown in Tables 3 and 4. Of these, individuals with confirmed Pof were separately classified as Pof, and the GSI for each developmental stage and Pof were compared. The GSI of Ty was significantly higher than that of Sy in both wild and captive fishes. GSI increased as oocytes matured from Ty to Mn and R in both wild and captive fish, with R being significantly higher than Ty. As shown in Tables 1 and 2, Pof included some individuals that had Ty, Mn, or R oocytes remaining that would be spawned after one or more spawnings, and some that did not.

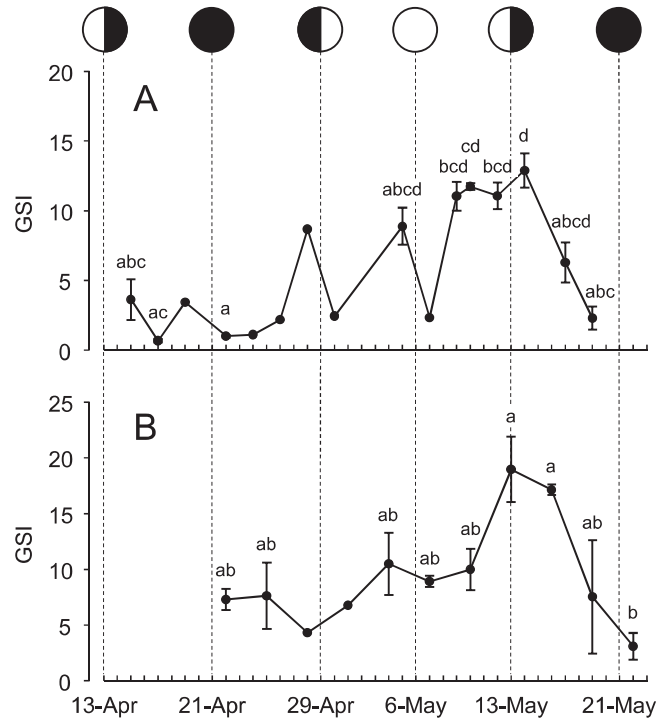

Fig. 2. Changes in GSI of wild (A) and captive (B) white-streaked grouper. ●, New moon; ◐, First quarter moon; ○, Full moon; ◑, Last quarter moon. Mean ± SEM. Different letters on the graph indicate statistical significant differences ($p < 0.05$).

Table 3
GSI at each stage of the most developed oocytes of wild white-streaked grouper.

Stage of the most developed oocytes	Number of fish examined	GSI						
		Mean ± SD				Range		
York vesicle stage (Yv)	1	0.6				0.6		
Secondary yolk stage (Sy)	13	1.7	±	1.4	a	0.5	-	5.7
Tertiary yolk stage (Ty)	32	9.3	±	3.6	b	1.3	-	14.1
Migratory nucleus stage (Mn)	16	9.9	±	4.9	bc	2.6	-	18.8
Ripe stage (R)	14	13.4	±	7.1	c	2.4	-	30.3
Post-ovulatory follicles (Pof)*	14	8.0	±	4.4	b	0.6	-	14.7

Different letters (a, b, and c) indicate that there was a significant difference between each stage of the most developed oocytes according to one-way ANOVA, followed by Tukey's multiple comparison test ($p < 0.05$). Yv was excluded from the analysis due to the small number of samples.
* Pof individuals overlap with individuals from other stages.

3.3. Changes in the plasma steroid concentrations of captive fish

Changes in the three plasma steroid concentrations in captive fish are shown in Fig. 3. The E_2 concentration remained high; specifically, it remained between 2.9 and 7.4 ng/ml from 21-Apr to 10-May, peaking on 13-May. However, this increase was not significant (Fig. 3A). Subsequently, E_2 concentrations decreased significantly from May 13 to May 19. The 20β-S concentration was low until the last quarter moon on 13-May, although a few outliers were detected (Fig. 3B). However, the 20β-S concentrations in most fish increased rapidly after May 16 and were significantly higher on May 22. DHP was detected at concentrations higher than 20β-S, although no noticeable changes in the DHP concentrations were observed during the study period (Fig. 3C).

The mean and range of the three steroid concentrations in the plasma

Table 4
GSI at each stage of the most developed oocytes of captive white-streaked grouper.

Stage of the most developed oocytes	Number of fish examined	GSI						
		Mean ± SD				Range		
York vesicle stage (Yv)	1	2.0				2.0		
Secondary yolk stage (Sy)	4	1.6	±	0.4	a	1.0	-	1.9
Tertiary yolk stage (Ty)	19	9.5	±	3.5	b	5.0	-	16.2
Migratory nucleus stage (Mn)	4	13.6	±	7.1	bc	3.0	-	18.0
Ripe stage (R)	3	17.1	±	8.0	c	8.9	-	24.8
Post-ovulatory follicles (Pof)*	5	2.8	±	1.6	a	1.8	-	5.5

Different letters (a, b, and c) indicate that there was a significant difference between each stage of the most developed oocytes according to one-way ANOVA, followed by Tukey's multiple comparison test ($p < 0.05$). Yv was excluded from the analysis due to the small number of samples.
* Pof individuals overlap with individuals from other stages.

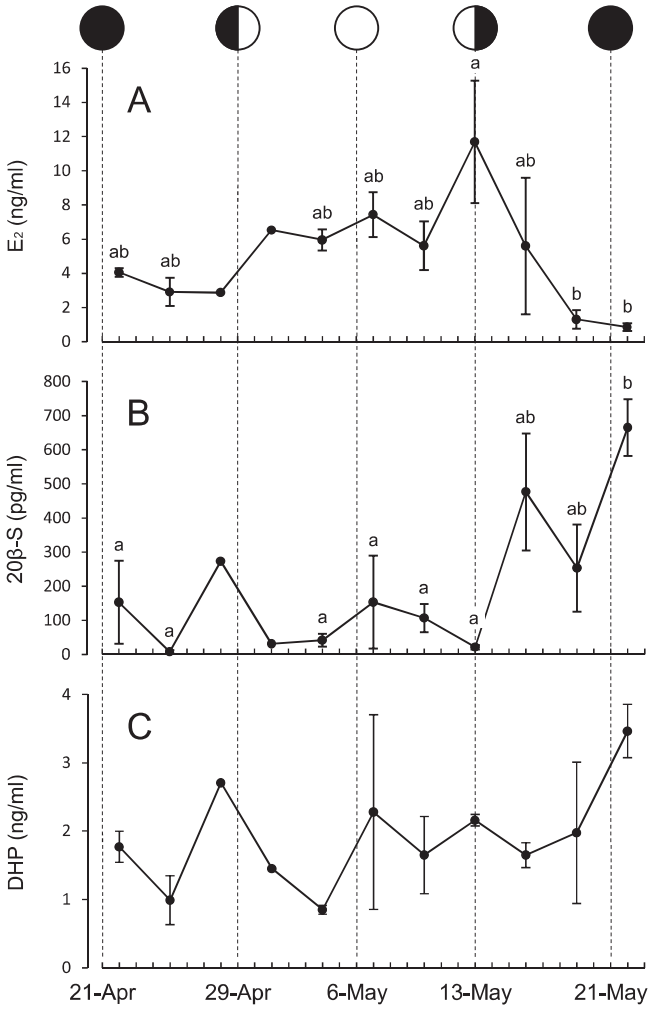

Fig. 3. Changes in sex steroid concentrations of captive white-streaked grouper. A, E_2; B, 20β-S; C, DHP. ●, New moon; ◐, First quarter moon; ○, Full moon; ◑, Last quarter moon. Mean ± SEM. Different letters on the graph indicate statistical significant differences ($p < 0.05$).

Table 5
E_2 concentration at each stage of the most developed oocytes of captive white-streaked grouper.

Stage of the most developed oocytes	Number of fish examined	E_2 (ng/ml) Mean ±SD	Range
York vesicle stage (Yv)	1	0.4	0.4
Secondary yolk stage (Sy)	4	2.1 ± 1.5	0.4 - 3.6
Tertiary yolk stage (Ty)	19	6.0 ± 3.9	0.9 - 18.9
Migratory nucleus stage (Mn)	4	4.8 ± 5.9	1.1 - 13.6
Ripe stage (R)	3	4.3 ± 3.4	1.3 - 7.9
Post-ovulatory follicles (Pof)*	5	1.0 ± 0.8	0.4 - 2.3

* Pof individuals overlap with individuals from other stages.

Table 6
20β-S concentration at each stage of the most developed oocytes of captive white-streaked grouper.

Stage of the most developed oocytes	Number of fish examined	20β-S (pg/ml) Mean ± SD		Range
York vesicle stage (Yv)	1	575.7		575.7
Secondary yolk stage (Sy)	4	340.1 ± 406.4	ab	5.7 - 829.9
Tertiary yolk stage (Ty)	19	89.4 ± 155.8	a	0.6 - 589.1
Migratory nucleus stage (Mn)	4	465.0 ± 244.5	b	297.0 - 820.5
Ripe stage (R)	3	250.0 ± 194.0	ab	30.0 - 396.5
Post-ovulatory follicles (Pof)*	5	486.3 ± 304.3	b	5.9 - 829.9

Different letters (a and b) indicate that there was a significant difference between each stage of the most developed oocytes according to one-way ANOVA, followed by Tukey's multiple comparison test ($p < 0.05$). Yv was excluded from the analysis due to the small number of samples.
* Pof individuals overlap with individuals from other stages.

Table 7
DHP concentration at each stage of the most developed oocytes of captive white-streaked grouper.

Stage of the most developed oocytes	Number of fish examined	DHP (ng/ml) Mean ± SD	Range
York vesicle stage (Yv)	1	3.3	3.3
Secondary yolk stage (Sy)	4	2.0 ± 1.7	0.4 - 4.2
Tertiary yolk stage (Ty)	19	1.8 ± 1.1	0.5 - 5.1
Migratory nucleus stage (Mn)	4	1.5 ± 0.4	1.0 - 1.9
Ripe stage (R)	3	2.5 ± 1.4	1.4 - 4.0
Post-ovulatory follicles (Pof)*	5	2.5 ± 1.5	0.9 - 4.2

* Pof individuals overlap with individuals from other stages.

of captive fish are shown for each of the ovarian stages classified in Tables 5, 6 and 7. The mean values of E_2 did not differ significantly between all stages, but were the highest at Ty and the lowest at Pof. In contrast, the mean values of 20β-S were the lowest at Ty and highest at Pof, and the mean values of Mn and Pof were significantly higher than those of Ty. The mean values of DHP did not differ significantly between the stages.

4. Discussion

This study is the first to provide previously unknown physiological and endocrinological information on the white-streaked grouper, for which a wealth of knowledge has been accumulated on spawning characteristics, such as the reproductive cycle (Ohta and Ebisawa, 2015), spawning migration (Nanami et al., 2014), spawning behavior (Nanami et al., 2013; Okuyama and Yamaguchi, 2022), and its links to the lunar cycle (Ohta and Ebisawa, 2015). In particular, by using wild and captive fish simultaneously, endocrinological information associated with maturation, which is difficult to obtain from wild fish alone, could be obtained. As a result, the details of the gonadal development and accompanying physiological changes in this species were clarified.

It is clear that this species begins spawning in the last quarter of the moon, as evidenced by the calculation of GSI and observations of tissue changes in the gonads. These results are similar to those of a previous report using wild fish (Ohta and Ebisawa, 2015), and verify that this species is a lunar-related spawner. Although this report documented monthly and weekly gonadal development over several months, we observed changes in ovarian development every 2–3 days, whenever possible, throughout the month of the spawning season. Vitellogenesis was found to progress between 15-Apr (2 days after the last quarter moon in the previous month) and 10-May (3 days before the last quarter moon in the following month), when Sy or Ty was observed. This result was the same for both the wild and captive fish. The time period when the fish had Sy, a particularly active stage of vitellogenesis, was 21 days, from 17-Apr to 7-May in wild fish, but only between 25-Apr and 28-Apr in captive fish, which otherwise had Ty oocytes. This difference between wild and captive fish is a noteworthy distinction. In wild fish, the number of oocytes at Ty stage increased rapidly between 9-May and 12-May. The fact that captive fish continue to have oocytes in the Ty stage for a longer period than wild fish indicates that vitellogenesis in captive fish progresses and is completed faster than in wild fish. This is most likely due to the stable water temperature and food intake in captivity, but no studies have examined and compared wild and captive fish simultaneously, and it remains to be seen whether the above factors have an impact.

Although gonadal development in captive grouper species has been studied for several species (Ni Lar Shein et al., 2004; Phạm et al., 2022), many of these studies examined gonadal development over a scale of a year them. This has translated to developmental patterns across shorter time intervals (e.g., a few days) not being as clearly understood. For example, in the case of the honeycomb grouper, *E. merra* (i.e., which like the subject of this study is a lunar spawner), its gonadal development has mostly been studied on individuals captured from the wild (Murata et al., 2022). In contrast, when captive *E. merra* individuals are concerned, only fragmentary and less comprehensive insights into their gonadal development have been brought forth. However, since captive fish have been shown to spawn in an aquarium at the same time as wild fish, it is likely that their gonadal development (i.e., and the endocrine system that coordinates it) are not different. In our study, although the timing of spawning captive fish coincided with that of wild fish, we noted that there is a difference between the two groups in the length of time for the preparation of development of the oocytes. It is possible that similar differences may occur in other groupers cannot be ruled out.

Another major difference between wild and captive fish is whether they migrate to spawning grounds. When we attached transmitters to 33 individuals of this species that were captured in the same area and year as this study, and observed their behavior during the spawning period, females began their migration to the spawning grounds 4–5 days before the last quarter moon (13-May) (Nanami et al., 2014). The change from Sy to Ty was just prior to this migration to the spawning grounds, and it is inferred that all individuals migrating to the spawning grounds had Ty

oocytes capable of initiating FOM. This is a major difference from captive fish, which do not migrate.

In wild white-streaked groupers, three individuals with Mn oocytes was observed on the day before the last quarter moon (12-May). However, it is unlikely that they spawned on this day, as no R or Pof were identified in the ovaries at this time. The oocytes in the Mn stage are considered to be in the final maturation process, as they spawn on the following day, the last quarter moon (13-May). In groupers, artificial treatment with gonadotropin (GtH) is known to induce ovulation 36–48 h after treatment, and the process from nucleus migration to full maturity is completed in ~24 h (Donaldson, 1989; Ni Lar Shein et al., 2004; Okumura et al., 2002; Soyano et al., 2008). Although there have been no reports of artificially induced ovulation in the white-streaked grouper, if the ovulation of this species is similar to that of other groupers, it is highly likely that individuals with Mn oocytes on 12-May will ovulate on 13-May. In this study, even though oocytes could not be collected on 13-May, it is very likely that the fish spawned on 13-May, as Pof was confirmed on 14-May. As described above, FOM had not been completed by 12-May in wild white-streaked grouper, and Pof was observed on 14-May, suggesting that the first spawning started on the last quarter moon (13-May). Considering that individuals with the migratory nucleus stage spawn the following day, the spawning period for this phase was found to be eight days, from 13-May to 20-May. The spawning conditions of captive fish were also similar. This study clarified the ovarian development in the white-streaked grouper on a daily basis, thus expanding on the report of Ohta and Ebisawa (2015), and providing a more detailed understanding of the spawning characteristics of this species.

Interestingly, an individual with R was identified in captive white-streaked grouper on 22-Apr. This may be due to the fact that vitellogenesis in the captive fish progressed earlier than in the wild fish, i.e., between the time of capture to before the start of this study, and that the Ty oocyte developed to final maturity more quickly. However, only one fish was observed displaying this pattern, and it is unclear whether it was involved in the spawning process.

The GSI is an important indicator of the developmental status of gonads and reproductive activity (Rizzo and Bazzoli, 2020). During the vitellogenic phase, the GSI of wild fish increased significantly from April 15 to May 12 (Table 1 and Fig. 2A). On the other hand, the GSI of captive fish increased slightly. However, the increase was not significant (Table 2 and Fig. 2B). This difference can be attributed to the fact that the increase in the GSI of wild fish was confirmed via the oocytes changing from Sy to Ty (Table 1 and Fig. 2A), whereas Ty oocytes appeared earlier in captive fish than in wild fish (Tables 1 and 2). Specifically, the comparison of GSIs for Sy and Ty in wild and captive fish, respectively, supports this possibility, as significant differences were detected between Sy and Ty in both fish (Tables 3 and 4). While there were differences in GSI between wild and captive fish during the vitellogenic phase, it was observed that GSI in both fish peaked during the FOM phase (Mn and R) and decreased after spawning. Moreover, this peak in GSI was almost synchronous in wild and captive white-streaked groupers, and coincided with the last quarter moon. Similar changes in GSI are known in the honeycomb grouper, which spawns at the full moon (Fukunaga et al., 2019; Lee et al., 2002; Murata et al., 2022).

White-streaked groupers are batch spawners, in which one individual can spawn once or multiple times after the last quarter month (Ohta and Ebisawa, 2015). In fact, Okuyama and Yamaguchi (2022) reported that the average number of spawning days for one female of this species was 2.1 ± 1.2 days, with variation among individuals, based on the measurement of spawning behavior using data loggers. In the present study, two or more of the three spawning indicators (Mn for spawning the next day, R for spawning today, and Pof indicating that they had already spawned) were found in the ovaries of many individuals. These results indicate that one female spawns multiple times during a lunar cycle, which is consistent with previous reports. Similarly, honeycomb groupers spawn multiple times per lunar spawning period (Amagai et al., 2022). This is probably a common characteristic of grouper species that are lunar-related spawners.

Another point that should be confirmed regarding the spawning characteristics of white-streaked groupers is whether this species also spawns in the following month. From our results, since no vitellogenic oocytes were found in the ovaries of wild white-streaked groupers after spawning, it was considered unlikely that the same individual would spawn in the following lunar phase. However, in captive fish, some Sy oocytes remained in the ovaries after spawning, indicating the possibility of the same individual spawning in the following month. This is a major difference between wild and captive fish, which has not been reported other fish species. However, captive fish are managed in a rearing environment that differs from that of wild fish (e.g., water temperature fluctuations, abundant food intake, and no energy-costly spawning migrations). Therefore, it might be possible for them to perform multiple monthly spawning cycles if conditions are suitable. Further detailed research is required to fully understand this phenomenon.

Hormone measurements of captive white-streaked groupers in this study showed that E_2 remained high between the new moon to the last quarter moon, reaching its peak in the last quarter moon. Thereafter, the E_2 levels decreased rapidly. This change was synchronized with GSI and ovarian tissue changes, with an increase during vitellogenesis and decrease during FOM. A generally known function of E_2 in fish is oocyte growth via the synthesis of vitellogenin, which is a precursor of yolk proteins, and the uptake of yolk proteins (Hiramatsu et al., 2015; Kucherka et al., 2006; Manning et al., 2008; Matsuyama et al., 1991). It is clear that the functions of E_2 in this species are similar. This is also the case in other grouper species, with an increase during the vitellogenic phase and a significant decrease during the FOM phase in Gag, *Mycteroperca microlepis* (Carolina and Carolina, 1999) and blacktip grouper, *E. fasciatus* (Phạm et al., 2022). However, in the present study, the E_2 levels were high in some individuals during the FOM stage. This phenomenon is caused by oocytes from the oogenesis period being maintained even during the FOM period in multi-spawning fish, as has been reported in the red grouper, *E. morio* (Johnson et al., 1998). A similar phenomenon observed in some individuals provides evidence that the white-streaked grouper is a multiple spawner.

Maturation-inducing hormone (MIH), a hormone that induces FOM, has been identified as either or both 20β-S and DHP which differ structurally, among fish species. In bambooleaf wrasse, *Pseudolabrus japonicus*, 20β-S and DHP increased in oocytes during GVBD and rapidly decreased just before ovulation (Matsuyama et al., 1998). In the Japanese yellowtail, *Seriola quinqueradiata*, DHP is the most effective inducer of GVBD during the FOM process after human chorionic gonadotropin (hCG) administration, but 20β-S is not detected (Rahman et al., 2001). In the Atlantic croaker, *Micropogonias undulatus*, 20β-S was detected at higher concentrations than DHP after hCG administration (Trant and Thomas, 1989). However, MIH has not been identified in groupers, and which hormone functions as the MIH is a subject of debate (e.g. blacktip grouper (Hwang et al., 2012), and orange-spotted grouper, *E. coioides* (Tang et al., 2019). In orange-spotted groupers, when oocytes at different stages were cultured with MIH (20β-S and DHP), both induced oocyte maturation at the end of vitellogenesis and at the beginning of nuclear migration (Tang et al., 2020), and both had the ability to act as MIH. In our study, when the levels of the two MIH candidate steroids were measured, DHP was detected at higher concentrations than 20β-S. However, no significant changes were observed during ovarian development. In contrast, 20β-S was detected at lower concentrations than DHP, but at higher levels after the onset of FOM. However, the results did not provide a clear answer, because some individuals showed high levels of 20β-S even during vitellogenesis, whereas others showed low levels of 20β-S even when Pof was present. MIH production is stimulated by a surge in LH, which is accompanied by a rapid inactivation process and a diurnal rhythm (Matsuyama et al., 1998). Therefore, it is undeniable that detection may have been difficult in our study due to the

timing of sampling. In addition, MIH in oocytes is drastically reduced just before ovulation, but MIH in blood remains after ovulation and may be detected at high levels even in post-ovulatory individuals (Matsuyama et al., 1998). In fact, the concentration of 20β-S was significantly higher on the last day of our study, when all individuals had Pof (Table 2 and Fig. 3B). As mentioned above, MIH in groupers varies greatly among species, but this may be a characteristic of MIH in groupers.

MIH in the blood of groupers, including this species, had no clear association with FOM. However, based on the results of the culture experiments described above (Tang et al., 2020), MIH has the ability to serve as a hormone that induces FOM in groupers. If an increase or decrease in hormone concentration is not the trigger that induces FOM, is it not the expression of the MIH receptor that controls it? For FOM to be triggered after the completion of vitellogenesis, oocytes must acquire sensitivity to MIH, which is called the acquisition of oocyte maturational competence (OMC) (Yamamoto and Yoshizaki, 2008). The acquisition of OMC involves the expression of the MIH receptor. In groupers, when ovulation is artificially induced by GtH, it occurs 36–48 h after hormone treatment (Soyano et al., 2008). Thus, 36–48 h before spawning, a fish has acquired OMC. In white-streaked groupers, OMC may also be acquired up to two days before the last quarter moon. As there is no clear correlation between blood levels of MIH, which implies synthesis and secretion, and FOM in groupers, including this species, it is possible that FOM is regulated by changes in MIH receptor expression rather than by increases or decreases in blood MIH.

5. Conclusion

This is the first study to simultaneously compare ovarian development in wild and captive grouper fish. We found that ovarian development and reproductive endocrine changes were synchronized in wild and captive fish, suggesting that the endocrine changes observed in captive fish reflect changes occurring in wild fish. The results showed that reproduction in this species is lunar-related, that it begins spawning in the last quarter moon, and spawns multiple times in a single lunar phase. This reproductive endocrinological information will contribute to the artificial induction of maturation, spawning, and seed production in groupers, including the white-streaked grouper. Furthermore, many grouper species are targeted for seed production and aquaculture, but there are many variations in spawning seasons, maturation mechanisms, and spawning frequency, and information on these variations is needed. In addition, many of the species targeted for aquaculture are large, and adult/mature fish are extremely valuable, making it difficult to collect information on maturation. This report provides information on the maturation of problematic groupers. Furthermore, this study also showed that wild and captive fish show slight differences in gonad development and spawning. It is important to understand the differences in the reproductive characteristics of wild and captive fish to better manage seed production and aquaculture.

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CRediT authorship contribution statement

Tomofumi Yamaguchi: Conceptualization, data curation, formal analysis, investigation, resources, visualization, writing–original draft, writing–review, and editing. **Takayuki Takebe**: Conceptualization, investigation, methodology, resources, writing–review, and editing. **Masato Nakachi**: Conceptualization, formal analysis, investigation, resources, writing–review, and editing. **Yuuki Kawabata**: Conceptualization, investigation, resources, writing–review, and editing. **Kazuhisa Teruya**: Conceptualization, methodology, resources, writing–review, editing. **Kiyoshi Soyano**: Conceptualization, resources, supervision, project administration, funding acquisition, investigation, writing–original draft, writing–review, and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101865.

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