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Endocrine Control of Diurnal Oocyte Maturation in the Kyusen Wrasse, *Halichoeres poecilopterus*

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ABSTRACT—The present study examined diurnal cycles of oocyte development and maturation in the kyusen wrasse, *Halichoeres poecilopterus*, and investigated the sensitivity of oocytes to maturation-inducing hormone (MIH) and gonadotropic hormone (GTH). Female fish were sampled at fixed intervals throughout the day, revealing that final oocyte maturation and ovulation were completed by 6:00 hr, and that spawning occurred daily between 6:00 and 9:00 hr. *In vitro* experiments showed that the steroids 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) were equally potent and highly effective inducers of germinal vesicle breakdown (GVBD) in kyusen wrasse oocytes. Additionally, circulating levels of 17,20β-P and 20β-S increased around the time of GVBD and ovulation, suggesting that 17,20β-P and 20β-S act as MIHs in the kyusen wrasse. Moreover, *in vitro* experiments clearly showed that kyusen wrasse oocytes had a daily developmental cycle of GTH and MIH sensitivity, and that oocytes that completed vitellogenesis acquired GTH-induced maturational competence. An endogenous GTH surge likely occurs between 12:00 and 15:00 hr, and this daily pre-maturational GTH surge probably controls the diurnal maturation cycles of kyusen wrasse oocytes.

Key words: diurnal oocyte maturation, maturation-inducing hormone (MIH), gonadotropic hormone (GTH), wrasse, teleost

INTRODUCTION

Ovarian development and recrudescence in teleosts are regulated by pituitary gonadotropic hormone (GTH) through the production of ovarian steroid hormones. During oocyte vitellogenesis, estradiol-17β is synthesized by the oocyte follicular layer, and this steroid stimulates the liver to produce vitellogenin as a yolk precursor (reviewed by Specker and Sullivan, 1994; Tyler and Sumpter, 1996). When vitellogenesis is complete, GTH stimulates maturation-inducing hormone (MIH) production in the follicular cells, and MIH induces germinal vesicle breakdown (GVBD) in oocytes (Nagahama, 1997).

A number of C21 steroids induce GVBD *in vitro*. In the majority of fish species investigated, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) is the most potent of the steroid inducers (Scott and Canario, 1987), and has been identified as a MIH in the amago salmon *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985), the Indian catfish *Clarias batrachus* (Haider and Rao, 1992), the killifish *Fundulus heteroclitus* (Petrino *et al.,* 1993), the medaka *Oryzias latipes* (Fukada *et al*., 1994), and the yellowtail *Seriola quinqueradiata* (Rahman *et al*., 2002). Another C21 steroid, 17,20β, 21-trihydroxy-4-pregnen-3-one (20β-S), is the major MIH in two sciaenid fishes, the Atlantic croaker *Micropogonias undulatus* and the spotted sea trout *Cynoscion nebulosus* (Trant *et al*., 1986; Trant and Thomas, 1989; Thomas and Trant, 1989; Patino and Thomas, 1990a), in an anadromous perciform, the striped bass *Morone saxatilis* (King *et al*., 1994b; 1997), and in the puffer fish *Takifugu rubripes* (Matsuyama *et al*., 2001). Both steroids, 17,20β-P and 20β-S, are 20β-hydroxylated progestins, and the only structural difference between the two is a hydroxyl group at the 21 position. At present, however, only the species mentioned above have been studied and there is little information on marine fishes.

In addition to its steroidogenic actions, GTH is also involved in final oocyte maturation (FOM). *In vivo* and *in vitro* studies indicate that GTH induces sensitivity to MIH in oocytes (Patino and Thomas, 1990b; Zhu *et al*., 1994; Kagawa *et al*., 1994; Patino *et al*., 2001). The ability of

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oocytes to respond to MIH is termed maturational competence. It has been shown that the MIH receptor concentration in spotted sea trout ovaries increases in response to *in vitro* GTH treatment, and that the oocytes acquire maturational competence concomitantly (Thomas and Patino, 1991). Thus, an increase in MIH receptor is an essential component of the development of GTH-induced maturational competence.

Halichoeres poecilopterus, the kyusen wrasse, is a diandric, protogynous hermaphrodite (Okada, 1962) that is among the most common labrids on coastal sandy bottoms with stones in western Japan. Populations consist of small initial-phase (IP) males (primary males), IP females, and large terminal-phase (TP) males. The ratio of males in IP fish is around 10% (Nakazono, 1978). TP males are derived either from females that have undergone a sex change to become males (secondary males), or from IP males. In captivity, kyusen wrasse spawn daily during an extended June to September spawning season (Kimura and Kiriyama, 1992; Kashiwagi *et al*., 1992), and spawning is typically observed between 7:00 and 10:00 hr. These observations indicate that the kyusen wrasse have a daily rhythm of oocyte development, maturation and spawning.

Although many economically important marine teleosts, such as the red seabream (Matsuura *et al*., 1988), spawn daily, studies of single female spawning records are scarce. The kyusen wrasse is easily caught and is resistant to handling and other stresses. Unlike many other marine fish, it readily spawns in captivity without any environmental or hormonal treatment. These features make this wrasse a good model for studying the endocrine control of daily oocyte development and maturation in marine teleosts.

In this study, we examined ovarian development in the kyusen wrasse at three-hour intervals during the spawning season to elucidate the daily reproductive cycle. The fish were either captured in the field or kept in captivity. Second, the relative GVBD-inducing effectiveness of nine steroids was compared to identify the presumed MIH of the kyusen wrasse. Additionally, the daily circulating levels of two 20β-hydroxylated progestins showing high GVBD-inducing potency *in vitro* were investigated. Finally, the responsiveness of oocytes to GTH and steroids at different times of day was investigated.

In the present study we refer to our previously published results on the bambooleaf wrasse. Recently, bambooleaf wrasses were divided into two species, *Pseudolabrus sieboldi* and *P. eoethinus*, based on morphological characteristics and mitochondrial DNA sequences (Mabuchi and Nakabo, 1997; Mabuchi *et al*., 2000). The population that we studied clearly exhibited the morphological characteristics of *P. sieboldi*, and therefore, we use the term bambooleaf wrasse to indicate *P. sieboldi*.

MATERIALS AND METHODS

Animals

In this study, we regard the spawning rhythm of kyusen wrasse in the field and captivity as being the same. Fish were caught by angling in the coastal waters of the Fisheries Research Laboratory of Mie University in Ago Bay from mid July to early August. Fish were caught at three-hour intervals during the day (around 6:00, 9:00, 12:00, 15:00, and 18:00 hr), and seven to ten IP fish were collected at each time. A portion of catch in late July, including IP and TP fish, was transferred to the laboratory and reared in a concrete tank (200×150×50 cm) with running seawater and natural day length and water temperature. The average water temperature of the concrete tank during the experiment was 25°C, and it showed similar value to that of field where the fish were sampled. Three concrete blocks were placed on the bottom to provide territories for the TP males. Fish were fed twice a day with shucked live shellfish. After daily spawning was confirmed, seven to ten IP fish were sampled during the night at 21:00, 0:00, and 3:00 hr. Immediately after being caught or sampled, fish were anesthetized with 2-phenoxyethanol (300 ppm), measured, and blood samples were taken from the caudal vessel using syringes with 21-gauge needles. Blood samples were then centrifuged at 3,000 rpm for 20 min, and the separated serum was stored at –30°C until used for steroid assay. The fish were sacrificed by decapitation, the gonads were dissected out, and their sex was determined. Gonads were then weighed and the gonadosomatic index (GSI) was calculated using the following equation: GSI=(gonad weight/body weight)×100. Ovarian tissue samples were immersed in Bouin's solution, dehydrated and embedded in Technovit resin (Kulzer, Wehrheim). For light microscopy, 4 μm-thick sections were cut and stained with 1% toluidine blue solution. The developmental stages of the oocytes and the degenerative stages of the postovulatory follicles (POF) were classified according to the schema used for the Japanese whiting (kisu) *Sillago japonica* (Matsuyama *et al*., 1990), with a slight modification.

Chemicals

The 20β-S was purchased from Steraloids, Inc., and all other steroids and reagents were obtained either from Sigma or Wako Chemicals. Rabbit anti-steroid hormone antibodies, and steroid hormones labeled with horseradish peroxidase were purchased from Cosmo-Bio.

Culture technique

First, to investigate the relative GVBD-inducing effectiveness of the 9 steroids in kyusen wrasse oocytes *in vitro*, three females were sampled from the tank at 16:00 hr. At this time, GVBD was inducible in only the largest oocytes. Second, to investigate the sensitivity of oocytes to GTH and steroids, two females were sampled from the tank at 3-hour intervals (16 fish in total). These fish were sampled after daily spawning was confirmed. Ovaries were removed and placed in ice-cold Leibovitz's L-15 culture medium (GIBCO), buffered with 0.02 M HEPES at pH 7.6 (adjusted with 1N NaOH). Gentamycin sulfate (200 mg/l) was added at the beginning of the assay. The ovaries were cut into small pieces weighing approximately 20 mg and containing 50–80 of the largest oocytes, and were transferred separately into the wells of a 24-well plastic culture dish (Falcon) containing 1 ml/well of incubation medium.

Steroids were dissolved in ethanol, and human chorionic gonadotropin (HCG) was dissolved in and diluted with incubation medium. Hormone solution (10 μl) was added to the wells, and 10 μl of ethanol was used as a control. Cultures were maintained for 20 hr at 25°C in a temperature-controlled incubator in air. The incubation temperature was based on the average water temperature when fish were collected. Our preliminary study showed that the *in*

vitro response of GVBD and ovulation to steroid at 25°C reached a plateau after an incubation of 16 hr.

Maturation criteria

The response of each piece of ovary to the various hormone concentrations was judged as follows: 0=no response, 1=some oocytes underwent GVBD but no ovulation, 2 = one to 15 oocytes ovulated, 3=16 to 30 oocytes ovulated, 4=31 to 45 oocytes ovulated, 5=more than 46 oocytes ovulated.

Steroid measurement

Serum 17,20β-P and 20β-S levels were measured by ELISA as previously described (Matsuyama *et al*., 1998b). The intra- and interassay coefficients of variation were determined close to the 50% binding point (10.9% and 13.9% for 17,20β-P; 9.4% and 13.3% for 20β-S; N=4, duplicate). Antisera of 17,20β-P had the following cross reactivities: 5% for 17-hydroxyprogesterone, 3.6% for 17,20α-dihydroxy-4-pregnen-3-one, 1.2% for 20β-S, 0.4% for testosterone, 0.4% for androstendione, 0.18% for 11-deoxycortisol, 0.15% for pregnenolone, 0.07% for 20β-hydroxyprogesterone, 0.05% for 17-hydroxypregnenolone 0.02% for cortisol, 0.02% for corticosterone, less than 0.01% for progesterone, and less than 0.01% for estradiol-17β. Antisera of 20β-S had the following cross reactivities: 1.1% for testosterone, 1% for progesterone, 0.9% for cortisol, 0.8% for 17,20α-dihydroxy-4-pregnen-3-one, 0.28% for 17,20α,21-trihydroxy-4-pregnen-3-one, 0.14% for 11-deoxycortisol, 0.07% for deoxycorticosterone, less than 0.01% for 17-hydroxyprogesterone, less than 0.01% for 17,20β-P, and less than 0.01% for estradiol-17β. Competition curves for kyusen wrasse sera were almost parallel to the standard curves (ANCOVA, $p>0.05$).

Statistics

The Student's t-test was used to compare means between treated and control groups in the GVBD assay. GSI values were compared between each sampling time and steroid content results were compared between each oocyte developmental stage, and analyzed by one-way ANOVA, followed by a Tukey-Kramer test.

Table 1. Developmental stages of the ovary and degenerative stages of postovulatory follicles (POF) in the kyusen wrasse ovary sampled at different times of the day during the spawning season

0:00 hr		3:00 hr		6:00 hr		9:00 hr	
Stage[1]	POF[2]	Stage	POF	Stage	POF	Stage	POF
EMN	III	EM	–	LM	–	SY	II
LMN	–[3]	EM	–	OV[4]	I	SY	II
LMN	–	EM	–	OV	I	SY	II
LMN	–	EM	–	OV	I	SY	II
LMN	–	EM	–	OV	I	SY	II
LMN	–	EM	–	OV	I	SY	II
		EM	–				
		EM	–				
		SY	–				
		SY	–				

12:00 hr		15:00 hr		18:00 hr		21:00 hr	
Stage	POF	Stage	POF	Stage	POF	Stage	POF
SY	II	TY	II	EMN	III	EMN	III
SY	II	TY	II	EMN	III	EMN	III
SY	II	TY	II	EMN	III	EMN	III
SY	II	TY	II	EMN	III	EMN	III
SY	II	EMN	III	EMN	III	LMN	–
SY	II	EMN	III	EMN	III	LMN	–
TY	II	EMN	III	EMN	III	LMN	–
EMN	III	EMN	III			LMN	–
		EMN	III			LMN	–

[1]The ovarian stage was represented by the developmental stage of the largest oocytes in the ovary. SY, secondary yolk stage; TY, tertiary yolk stage; EMN, early migratory nucleus stage; LMN, late migratory nucleus stage; EM, early mature stage; LM, late mature stage.

[2]See Matsuyama *et al*. (1990) for degenerative stages of POF. I, newly-formed; II, follicular lumen becomes greatly reduced in size or completely closed; III, degeneration of POF advanced, and become indistinguishable from the ovarian tissue stroma.

[3]POF were absent from the ovary.

[4]Ovulated; mature eggs discharged from follicular cells are present in the ovarian cavity.

RESULTS

Daily cycle of oocyte development

The kyusen wrasse has asynchronous ovaries, i.e. oocytes at different stages of development are present simultaneously. Consequently, the ovarian stage was represented by the developmental stage of the largest oocytes in the ovary. The ovarian stages of fish sampled at eight different times of the day are shown in Table 1. The ovaries of fish sampled at any one time of day were similar to one another. Fish sampled at 9:00 hr were in the post-spawning stage, had no ovulated eggs in the ovarian cavity and their POF were at stage II. Between 9:00 and 15:00 hr, yolk accumulation in the largest oocytes was in progress (stage SY) or complete (stage TY). At 15:00 hr, oocytes in five of nine fish were at the early stage of germinal vesicle migration (stage EMN), when the germinal vesicle is situated between the center of oocyte and half way to the animal pole. At 18:00 hr, all seven fish had oocytes at stage EMN. At 21:00 hr, four of nine fish had oocytes at stage EMN, and the remaining five fish had oocytes at the late stage of germinal vesicle migration (stage LMN), when the germinal vesicle is located near the zona radiata at the micropyle. The POF continued to degenerate and finally began to disappear from the ovary at stage LMN. Germinal vesicle migration occurred from 15:00 to 0:00 hr, and GVBD (stage EM) was first observed at 3:00 hr. GVBD had just occurred in oocytes at stage EM, and they had a translucent appearance likely due to the progression of yolk proteolysis. At 6:00 hr, one of six fish had transparent oocytes at stage LM in which yolk proteolysis was complete. Five of six fish sampled at 6:00 hr had ovulated (stage OV), and ovulated oocytes were present in the ovarian cavity. Newly-formed POF were observed in the same ovaries. These results clearly indicate that GVBD and ovulation occurred around 3:00 hr and 6:00 hr, respectively, and that spawning occurred daily between 6:00 and 9:00 hr.

The developmental stages of the second largest oocytes found in ovaries with the largest oocytes are shown in Table 2. In ovaries where the largest oocytes were at stage SY or TY, the second largest oocytes were at stage PY, representing the beginning of vitellogenesis. In ovaries where the largest oocytes were undergoing germinal vesicle migration (stages EMN and LMN) and GVBD was complete (stages EM and LM), the second largest oocytes were in the course of active vitellogenesis (stage SY).

Fig. 1 shows the GSI of fish sampled at different times of the day. The GSI increased rapidly and peaked at 6:00 hr when hydration was complete and the fish ovulated. At 9:00 hr, when spawning was complete, the GSI drastically decreased due to the release of ovulated oocytes. No further changes in GSI were found between 9:00 and 3:00 hr. Thus, the GSI values reflected the condition of the ovaries, particularly hydration and ovulation, in the diurnal reproductive cycle of the kyusen wrasse.

Table 2. Developmental stages of the second-largest oocytes in the ovaries with largest oocytes at different developmental stages in kyusen wrasse

Largest oocytes	Second-largest oocytes
SY	PY
TY	PY
EMN	SY
LMN	SY
EM	SY
LM	SY
OV	SY

PY, primary yolk stage; SY, secondary yolk stage; TY, tertiary yolk stage; EMN, early migratory nucleus stage; LMN, late migratory nucleus stage; EM, early mature stage; LM, late mature stage; OV, ovulation stage.

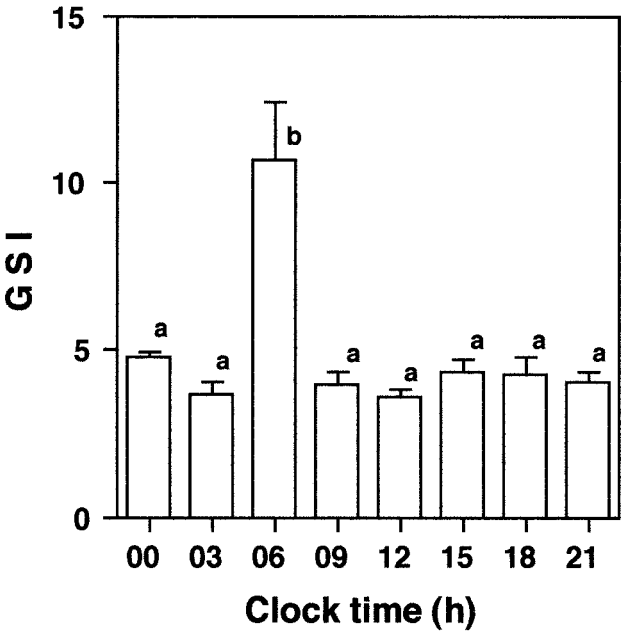

Fig. 1. Changes in the gonadosomatic index (GSI) throughout the day. Bars represent the mean±SEM. Different letters represent statistically different values ($p<0.05$).

In vitro effects of various steroids on GVBD and ovulation

The relative effectiveness of the nine steroids tested is summarized in Table 3. Pregnenolone, progesterone, 17-hydroxyprogesterone (17-P), 17,20β-P, and 20β-S were effective inducers of oocyte maturation *in vitro*. At 10 ng/ml, 17-P was less effective than 17,20β-P or 20β-S. At a concentration of 1 ng/ml, 17,20β-P, and 20β-S were the most potent inducers of GVBD and ovulation. In contrast, 17,20α-dihydroxy-4-pregne-3-one (17,20α-P), testosterone, 11-ketotestosterne, and estradiol-17β were totally ineffective at concentrations of up to 10 ng/ml.

Serum steroid levels

During most of the maturation cycle, the ovarian devel-

Table 3. *In vitro* effectiveness of various steroid hormones on GVBD and ovulation in kyusen wrasse oocytes. Numbers are mean response of oocytes for three test on each of four fish. See text for criteria for oocyte maturation.

Steroids[1]	Dose (ng/ml)					
	100	10	1	0.1	0.01	0[2]
Pregnenolone	1.6*	1.0	0.3	0.6	0.3	0.6
Progesterone	1.3*	1.0	0.6	0.6	0.3	0.6
17-P	1.6*	1.3*	1.0	0.6	0.6	0.6
17,20α-P	1.0	0.6	0.6	0.6	0.3	0.6
17,20β-P	3.0**	2.6*	1.6*	0.6	1.0	0.6
20β-S	4.5**	3.6*	2.3*	1.0	0.6	0.6
Testosterne	0.3	0.3	0.3	1.0	0.6	0.6
11-Ketotestosterone	1.0	1.0	0.6	1.0	1.0	0.6
Estradiol-17β	0.3	1.0	0.6	1.0	1.0	1.0

[1]Synthetic name of steroid hormones:
Pregnenolone, 3β-hydroxy-5-pregnen-20-one
Progesterone, 4-pregnene-3,20-dione
17-P, 17-hydroxy-4-pregnene-3,20-dione
17,20α-P, 17,20α-dihydroxy-4-pregnen-3-one
17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one
20β-S, 17,20β,21-trihydroxy-4-pregnen-3-one
Testosterone, 17β-hydroxy-4-androstene-3-one
11-Ketotestosterone, 17β-hydroxy-4-androstene-3,11-dione
Estradiol-17β, 1,3,5(10)-estratriene-3,17β-diol
[2]Control medium contains 1% ethanol.
Asterisk(s) indicates the statistical difference (*p<0.05, **p<0.01) to control.

opment stage, as represented by the stage of the largest oocyte, was consistent in groups of fish sampled at the same time (Table 1). However, there was less consistency with respect to the developmental stage of the ovary in fish sampled at the same time. The serum hormone levels reflect the developmental stage of ovary. Therefore, serum 17,20β-P and 20β-S levels were established from fish grouped according to gonadal stage (Fig. 2). Additionally, the number of fish at stage LM was so small that data from fish at EM and LM stages were combined as the mature stage (stage M).

Serum 17,20β-P peaked at stage M (0.50±0.17 ng/ml, $p<0.05$ vs. SY and EMN), remained at a high level at ovulation (stage OV), then decreased to the lowest value (0.13 ±0.02 ng/ml) at stage SY after spawning. Low levels (0.15–0.24 ng/ml) were found between stages TY and LMN.

The serum 20β-S level was lowest (0.26±0.02 ng/ml) at EMN, and increased at LMN (0.55±0.06 ng/ml, $p<0.05$ vs. EMN). The level was highest (0.73–0.85 ng/ml) during GVBD (stage M) and following ovulation, and it decreased at stage SY (0.45±0.09 ng/ml, $p<0.05$ vs. OV).

Thus, both serum 17,20β-P and 20β-S levels showed significant increases at mature and ovulation stages, and decreased markedly at stage SY after spawning.

Development of sensitivity to GTH and MIH

Since 17,20β-P and 20β-S showed almost the same GVBD-inducing potency in kyusen wrasse oocytes *in vitro*,

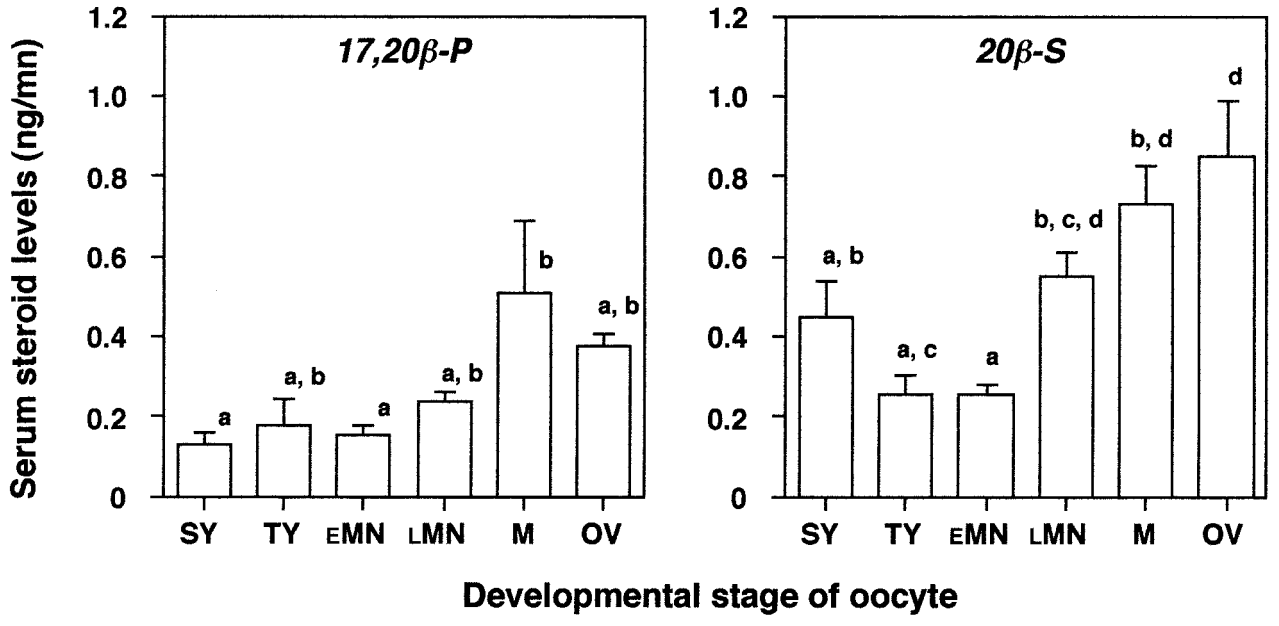

Fig. 2. Changes in serum levels of 17,20β-P and 20β-S in female kyusen wrasses sampled at different times of the day during the spawning season. Bars represent the mean±SEM. SY, secondary yolk stage; TY, tertiary yolk stage; EMN, early migratory nucleus stage; LMN, late migratory nucleus stage; M, mature stage; OV, ovulation stage; data from fish at early mature and late mature stages were combined as the mature stage. Different letters represent statistically different values ($p<0.05$).

Table 4. Development of sensitivity to HCG and 17,20β-P in kyusen wrasse oocytes. Numbers are average response of oocytes duplicate test. See text for criteria for oocyte maturation.

Time (hr)	Individual No.	HCG (IU/ml)					17,20β-P (ng/ml)				
		100	10	1	0.1	0	10	1	0.1	0.01	0
0:00	1	3	3	3	3	3	3	3	3	3	2
	2	3	3	3	2.5	2.5	3	3	3	2.5	2.5
3:00	3	3	3	3	3	3	3	3	3	3	3
	4	3	3	3	3	3	3	3	3	3	3
6:00	5	*									
	6										
9:00	7										
	8										
12:00	9										
	10	2	1								
15:00	11	2	1								
	12	3	2.5	1			2	1			
18:00	13	3	3	2	2		2	2	2	1	
	14	3	3	2	1		3	2			
21:00	15	3	3	3	3	2	3	3	3	2.5	2.5
	16	3	3	2.5	2	2	3	2.5	2.5	2.5	2

* blank represents no response.

only the 17,20β-P results are presented here. Changes in oocyte sensitivity to HCG and 17,20β-P are shown in Table 4.

Neither HCG nor 17,20β-P induced GVBD and ovulation in fish sampled at 6:00 and 9:00 hr. Two fish sampled at 12:00 (No. 10) and 15:00 hr (No. 11) responded to HCG at high concentrations, but not to 17,20β-P. GVBD and ovulation were induced in three fish sampled at 15:00 (No. 12) and 18:00 hr (No. 13 and 14) by both HCG and 17,20β-P, but no GVBD was seen in the absence of HCG or 17,20β-P. GVBD and ovulation were spontaneous in all fish sampled at 21:00, 0:00, and 3:00 hr without hormonal treatment.

The ovarian oocytes of the kyusen wrasse can be classified into three stages based on their responsiveness to HCG and 17,20β-P. In oocytes sampled at 12:00 and 15:00 hr, FOM was induced by GTH, but not by steroid; vitellogenesis was complete, and these oocytes were at stage TY. In oocytes collected at 18:00 hr, FOM was induced by both GTH and steroid; these oocytes were at an early stage of germinal vesicle migration (EMN). In oocytes collected at 21:00, 0:00, and 3:00 hr, FOM occurred spontaneously without any hormonal treatment; these oocytes were between the late stage of germinal vesicle migration (stage LMN) and a mature stage (EM or LM).

DISCUSSION

The present study demonstrates that oocyte development and MIH production are diurnal in kyusen wrasses, and further clarifies temporal differences in oocyte responsiveness to GTH and MIH. Diurnal changes in two clutches of oocytes, the serum levels of two presumed MIHs, and the sensitivity of the largest oocytes to hormones are summarized in Fig. 3.

FOM and ovulation were completed by 6:00 hr, and the fish spawned between 6:00 and 9:00 hr. Of 10 fish sampled at 3:00 hr, two had ovaries containing stage SY oocytes without mature oocytes (Table 1). There were no POF in these ovaries. It is likely that these fish would fail to mature during this period. As shown in the present study, a single female kyusen wrasse usually spawns daily, although some fish will not spawn for one or several days following various stresses. The progress of POF degeneration in the daily reproductive cycle of kyusen wrasses suggests that newly formed POF disappear within one day. Similar POF resorption has been reported in daily-spawning marine fishes, including red seabream (Matsuyama *et al*., 1988), Japanese whiting (Matsuyama *et al*., 1990), and bambooleaf wrasse (Matsuyama *et al*., 1998a).

Two C19 steroids (testosterone and 11-ketotestosterone) and a C18 steroid (estradiol-17β) failed to induce FOM in kyusen wrasses. In agreement with structure-activity studies in teleosts (Scott and Canario, 1987), only C21 steroids hydroxylated at position 20β group had high potency. Our results show that 17,20β-P and 20β-S are equally potent and highly effective GVBD inducers in kyusen wrasse oocytes *in vitro*. In addition, circulating levels of 17,20β-P and 20β-S increased around the time of GVBD and ovulation. These results suggest that 17,20β-P and 20β-S act as MIHs in the kyusen wrasse.

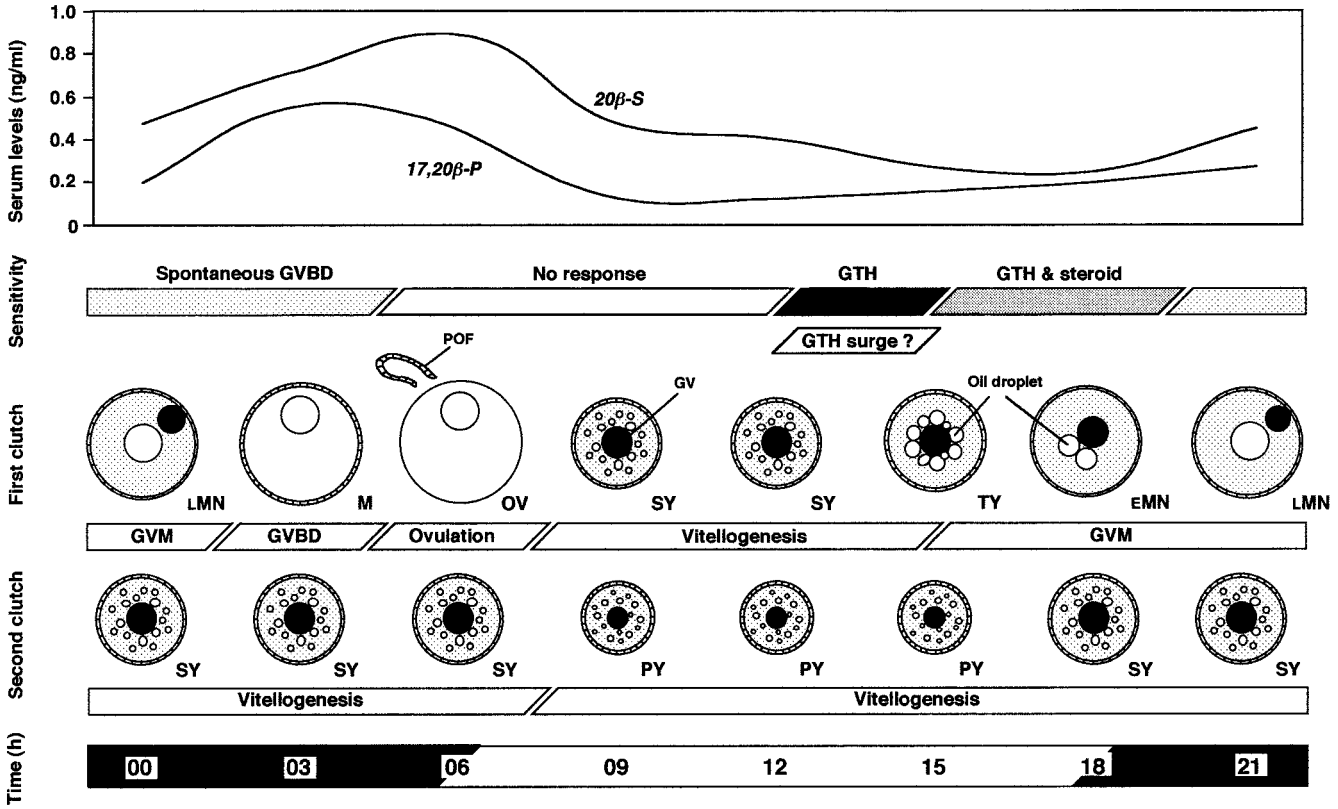

Fig. 3. Diagrammatic representation of the diurnal rhythm of oocyte development, sensitivity of the first clutch of oocytes to gonadotropin (GTH) and maturation-inducing hormone (17,20β-P), and serum levels of 17,20β-P and 20β-S, (presumed MIH of the kyusen wrasse). See Table 2 for an explanation of oocyte stage. GV, germinal vesicle; GVBD, germinal vesicle breakdown; GVM, germinal vesicle migration; POF, postovulatory follicle.

In the striped bass (King *et al*., 1994a, 1994b), the bambooleaf wrasse (Ohta and Matsuyama 2002) and the red seabream (Ohta *et al*., 2002), both 17,20β-P and 20β-S are synthesized in the ovarian follicles during FOM, and show equally high GVBD potency *in vitro*, with increased serum levels around FOM. By examining the binding affinities of various steroids produced, 20β-S was identified as the MIH in the striped bass. A single class of 20β-S binding sites on membranes from striped bass ovaries undergoing FOM was detected, whereas 17,20β-P showed low, nonsaturable binding to ovarian membranes, and was approximately 250 times less effective than 20β-S at displacing 20β-S from ovarian membranes (King *et al*., 1997). Although the results of ligand specificity experiments in the striped bass contradicted the potency of 17,20β-P found in the *in vitro* GVBD assay, it was suggested that the pool of 17,20β-P is utilized as a substrate to produce bioactive 20β-S (King *et al*., 1997). However, for the bambooleaf wrasse and the red seabream, the oocyte MIH receptor has not yet been characterized, although incubation of ovarian follicles with radiolabeled precursors clearly showed that 17,20β-P is not utilized as a substrate pool for 20β-S. These results indicate that the bambooleaf wrasse and red seabream possess not one, but two MIHs, the 20β-hydroxylated progestins 17,20β-P and 20β-S. Although our preliminary experiments on steroidogenesis using radiolabeled precursors showed that the MIH of the kyusen wrasse is analogous to that of the bambooleaf wrasse and red seabream (unpublished), further studies are necessary to determine the true MIH of the kyusen wrasse.

Recent studies suggest that 17,20β-P and 20β-S induce not only oocyte maturation but also ovulation (Theofan and Goetz, 1981; Pinter and Thomas, 1999). Interestingly, the induction of ovulation is under genomic control, probably via the nuclear progestin receptor (Theofan and Goetz, 1981; Pinter and Thomas, 1995, 1999), while the induction of maturation is non-genomic, via the oocyte plasma membrane receptor. The binding affinity of the nuclear progestin receptor in spotted seatrout ovaries differs from that of the membrane progestin receptor (Pinter and Thomas, 1999). Of the various steroids, 17,20β-P had a higher affinity for nuclear receptors and the ability to induce ovulation, whereas 20β-S had a much higher affinity for membrane receptors and the ability to induce maturation. These results indicate that the progestins produced during FOM induce maturation and ovulation by different mechanisms via the membrane and nuclear receptors, respectively. In spotted seatrout, however, 20β-S has been suggested to induce both maturation and ovulation, because 17,20β-P is not produced in the ovaries (Thomas and Trant, 1989). By contrast, 17,20β-P and 20β-S are synthesized

simultaneously in the ovarian follicles of the kyusen wrasse. In this species, therefore, both 17,20β-P and 20β-S may play roles not only in maturation but also in ovulation. Although the serum levels of 17,20β-P and 20β-S throughout the day showed similar patterns, circulating levels of 17,20β-P peaked at the mature (=GVBD) stage, while 20β-S peaked at the ovulation stage. This slight difference in the serum levels of the two progestins may reflect their respective physiological roles. In order to identify the precise roles of both 17,20β-P and 20β-S in oocyte maturation and ovulation, it is necessary to investigate the ability of these progestins to induce GVBD and ovulation and the characteristics of membrane and nuclear progestin receptors.

The sensitivity of kyusen wrasse oocytes to GTH and 17,20β-P changed depending on the time of day, indicating that there is a daily cycle of hormone sensitivity. Oocytes collected at 12:00 and 15:00 hr underwent GVBD and ovulation after HCG treatment, but not after 17,20β-P treatment. Recent *in vivo* and *in vitro* studies suggest that the GTH-dependant increase in MIH receptor activity is responsible for the enhanced oocyte sensitivity to MIH that defines the acquisition of maturational competence (reviewed by Patino *et al*., 2001). Therefore, the results obtained with the oocytes collected at 12:00 and 15:00 hr suggest that follicles have already acquired GTH receptors, and that GTH stimulates production of MIH in the follicles, while inducing MIH receptor activity in the oocytes. In oocytes collected at 15:00 and 18:00 hr, both HCG and 17,20β-P induced FOM. This suggests that these oocytes had already acquired receptors for both GTH and MIH. In oocytes collected at 21:00, 0:00, and 3:00 hr, FOM was spontaneous, indicating that the oocytes had already been exposed to a surge of endogenous GTH and MIH. These results suggest that a GTH surge occurs between 12:00 and 15:00 hr, about 18 hr prior to spawning, since kyusen wrasse oocytes began to acquire MIH sensitivity during this period (Fig. 3). This daily pre-maturational GTH surge probably controls the diurnal maturation cycles of kyusen wrasse oocytes.

The results presented here indicate a marked increase in the production of MIH(s), coincident with FOM. Therefore, the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), which converts 17-P and 11-deoxycortisol to 17,20β-P and 20β-S, respectively, is likely to be high during this period. It has been reported that follicular 20β-HSD activity is stimulated by GTH via the gonadotropin receptor-adenylate cyclase-cAMP-dependent step (Nagahama and Yamashita, 1989). As described above, an endogenous GTH surge is estimated to occur in the kyusen wrasse between 12:00 and 15:00 hr, exactly when the most advanced oocytes completed vitellogenesis. Thus, it is likely that this GTH surge is responsible for the increase in 17,20β-P and 20β-S production via 20β-HSD activation in kyusen wrasse ovarian follicles.

Ovarian development and recrudescence in teleosts are regulated by GTH through the production of ovarian steroid hormones. In vertebrates, including teleosts, GTH release is primarily under the stimulatory control of gonadotropin-releasing hormone that is synthesized and secreted by the hypothalamus (Chang and Jobin, 1994). The daily ovarian development cycle in the kyusen wrasse is useful experimentally because physiologically different brain, pituitary and oocytes can be obtained on the same day as demand dictates. The kyusen wrasse is therefore a good model for future studies of the endocrine control of oogenesis.

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