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Steroidogenesis in Ovarian Follicles of Chub Mackerel, *Scomber japonicus*

Michiya Matsuyama*, Tetsuro Shiraishi, Jitendra K. Sundaray, Md. Anisur Rahman, Kohei Ohta and Akihiko Yamaguchi

Laboratory of Marine Biology, Faculty of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan

ABSTRACT—We incubated different radiolabeled steroid precursors with intact chub mackerel ovarian follicles to clarify the synthetic pathways of steroid hormones during vitellogenesis and following final oocyte maturation (FOM). During vitellogenesis, estradiol-17β (E2) was synthesized from pregnenolone via 17-hydroxypregnenolone, 17-hydroxyprogesterone, androstenedione, and testosterone. The physiological significance of the intermediate metabolites of E2 in the ovarian follicles was examined by comparing follicular steroidogenesis between gonochoric and hermaphroditic fish species. After vitellogenesis, the steroidogenic pathway shifted from E2 to maturation-inducing hormone (MIH) production owing to the inactivation of 17,20-lyase and the activation of 20β-hydroxysteroid dehydrogenase. Of the new steroids produced during FOM, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) was most effective at inducing germinal vesicle breakdown *in vitro*. Circulating levels of 17,20β-P increased specifically around the time of germinal vesicle migration, while another FOM-specific 20β-hydroxylated progestin, 17,20β,21-trihydroxy-4-pregnen-3-one, was present at consistently low levels during FOM. These results indicate that 17,20β-P is the MIH of chub mackerel.

Key words: steroidogenesis, ovarian follicles, vitellogenesis, oocyte maturation, teleost

INTRODUCTION

The chub mackerel, *Scomber japonicus,* is one of the most important pelagic fish resources in the world. In response to increasing demand for this species as a quality food, the cultivation of chub mackerel has recently commenced in the southwestern regions of Japan. In most cases, young or adult fish are caught from wild stocks and cultured for an appropriate period in sea cages. However, the wild fish catch is unreliable and unpredictable, and consequently a stable supply of chub mackerel juveniles or fertilized eggs is highly desirable. Detailed studies of chub mackerel endocrine kinetics regulating oocyte growth and maturation are necessary to enable and stabilize seed production of this species.

Like hatchery-reared broodstock of many commercial fish species (Zohar, 1989), female chub mackerel are unable to complete their reproductive cycle in captivity. In sea cages or indoor tanks under a natural photoperiod and water temperature, female fish retain yolked oocytes in their ovaries throughout the spawning season from April to June. These oocytes never undergo final oocyte maturation (FOM) and ovulation (Shiraishi *et al.*, 2005). The major endocrinological dysfunction in captive chub mackerel seems therefore to involve a lack of luteinizing hormone (LH) secretion from the pituitary, which is responsible for inducing FOM after the completion of vitellogenesis. Human chorionic gonadotropin (hCG), which has LH-like activity, is commonly used for inducing FOM and ovulation in many hatchery-reared fish. Recently we succeeded in inducing FOM and ovulation in captive chub mackerel by a single injection of hCG (Shiraishi *et al.*, 2005).

The objective of this investigation was to analyze the steroidal regulation of oocyte growth and FOM in chub mackerel. We identified the steroid hormones produced in the ovarian follicles, studied their synthetic pathways and circulating profiles, and investigated the ability of various FOM follicle-produced steroids to induce germinal vesicle breakdown (GVBD) *in vitro*.

* Corresponding author. Phone: +81-92-642-2887;
Fax : +81-92-642-2888;
E-mail: rinya_m@agr.kyushu-u.ac.jp

MATERIALS AND METHOD

Chemicals

The nomenclature of the steroids used in the present study is shown in Table 1. Radioactive steroids, [7-^{3}H]pregnenolone (P5,

Table 1. Nomenclature of steroids used in the present study

Systematic name	Trivial name	Abbreviation
3β-hydroxy-5-pregnen-20-one	pregnenololne	P5
3β-hydroxy-5-androsten-17-one	dehydroepiandrosterone	DHEA
4-androstene-3,17-dione	androstenedione	AD
17β-hydroxy-4-androsten-3-one	testosterone	T
3-hydroxy-1,3,5(10)-estratrien-17-one	estrone	E1
1,3,5(10)-estratriene-3,17β-diol	estradiol-17β	E2
3β,17-dihydroxy-5-pregnen-20-one	17-hydroxypregnenolone	17-P5
17-hydroxy-4-pregnene-3,20-dione	17-hydroxyprogesterone	17-P
17,20β-dihydroxy-4-pregnen-3-one	–	17,20β-P
17,21-dihydroxy-4-pregnene-3,20-dione	11-deoxycortisol	17,21-P
17,20β,21-trihydroxy-4-pregnen-3-one	–	20β-S
17-hydroxy-5β-pregnane-3,20-dione	–	17-P-5β
17,20β-dihydroxy-5β-pregnan-3-one	–	17,20β-P-5β
17,21-dihydroxy-5β-pregnane-3,20-dione	–	17,21-P-5β

780.7 GBq/mmol), [1,2-^{3}H]17-hydroxyprogesterone (17-P, 1565.1 GBq/mmol), [4-^{14}C] dehydroepiandrosterone (DHEA, 2.1 GBq/mmol), [4-^{14}C]androstenedione (AD, 2.0 GBq/mmol), and [1,2-^{3}H]11-deoxycortisol (17,21-P, 1739.0 GBq/mmol), were purchased from New England Nuclear (Boston, Mass.), and [1,2-^{3}H]testosterone (T, 2.2 TBq/mmol) was purchased from Amersham (U.K.). [^{3}H]17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) as a precursor was enzymatically synthesized from [^{3}H]17-P (Scott *et al*, 1982). Unlabeled steroids, except 17,20β-dihydroxy-5β-pregnan-3-one (17,20β-P-5β), were obtained either from Sigma (St. Louis, Mo.) or Steraloids Inc. (Wilton, N.H.). 17,20β-P-5β was purchased from Dr. A. Kambegawa (The Kambegawa Laboratory, Tokyo, Japan). Affinity-purified goat anti-rabbit IgG was obtained from CAPPEL (West Chester, PA). Rabbit anti-steroid hormone antibodies, and steroid hormones labeled with horseradish peroxidase were purchased from Cosmo-Bio (Tokyo, Japan). Enzymes, coenzymes, reagents, and solvents were obtained from either Sigma or Wako (Tokyo, Japan). hCG was purchased from Teikoku Hormone (Tokyo, Japan).

Fish and hCG injection

The chub mackerel, caught by a purse seine off the Goto Islands at autumn, 2001, were kept for following half year in the sea pens of a fish farm. Nearly 250 fish (430–550 g in body weight, 300–330 mm in fork length) were selected and transferred to the Fisheries Research Laboratory, Kyushu University on 7 May, 2002, and moved into outdoor concrete tanks circulated with running sea water. On the next day, fish were anesthetized with 2-phenoxyethanol (100 mg/l), measured the fork length (mm) and body weight (g), sexed by gonadal biopsy using plastic catheter (2 mm internal diameter), and an identification (ID) tag (model TX1400L, Destron-Fearing Co., MN) was embedded into the dorsal muscle. Ovarian tissue sucked were preserved in Ringer's solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 20 mM HEPES, pH adjusted to 7.5 using 1 N NaOH) and the diameter of the most advanced 20 oocytes were measured. After measurement the diameter, Ringer's solution was discarded and a few drops of clearing solution (ethanol:formalin:acetic acid = 6:3:1, v/v) was added to confirm the nucleus by making the cytoplasm transparent. When the nucleus (germinal vesicle, GV) is not observed, which indicating the onset of oocyte atresia (Mylonas *et al*, 1997), the fish was removed from materials. Finally, a total of 80 females, which had ovaries with oocytes at late yolk (LY) stage of 600–650 μm in diameter, were selected. At this stage of oocyte (0 h), ten fish were first sacrificed by decapitation. The remaining females were injected intramuscularly with hCG (500 IU/kg body weight) around at 14:00 h. According to our previous study on inducing FOM of captive chub mackerel with hCG, GVBD and ovulation completed by 30 and 36 hours after hCG injection, respectively (Shiraishi *et al*, 2005). Therefore, at each time of 12, 18, 24, 30, 36 and 42 post-injection, ten to thirteen injected females were sampled after anesthetization. For light microscopy, small ovarian fragments were fixed in Bouin's solution, dehydrated and embedded in Technovit resin (Kulzer, Germany). Sections 4-μm thick were cut and stained with 1% toluidine blue solution. The gonadal maturity of all fish sampled was determined based on the developmental stages of largest oocytes which have been previously reported (Shiraishi *et al*, 2005).

In vitro follicle incubation with radiolabeled steroid precursors

Removed ovaries were placed in ice-cold Leibovitz's L-15 culture medium (Flow Laboratories Ltd., Scotland), pH 7.4, supplemented with 2.5 g of HEPES, 0.1 g of streptomycin, 100,000 IU of penicillin, and 1 g of bovine serum albumin per liter. The ovaries were then cut into small fragments and pipetted to disperse follicle-enclosed oocytes. For *in vitro* follicle incubation with radiolabeled steroid precursors, two hundred follicles at the most advanced stage were gathered into 1.5-ml microtubes, and frozen in liquid nitrogen. These tubes were stored at –80°C until incubation. Our preliminary experiments revealed that there was little difference in the steroid metabolic patterns during the incubation with frozen and intact follicles.

Two hundred follicles were placed in a 10-ml glass tube with 1 ml of sucrose buffer (250 mM sucrose, 20 mM HEPES, pH adjusted to 7.8 with 1 N NaOH). Ten pmol of radiolabeled steroid precursors were dissolved in 150 μl sucrose buffer. Coenzymes (NAD, NADH, NADP, and NADPH; 10 mM each) were dissolved in a solution of 100 μl $MgCl_2$ (20 mM) and 50 μl citrate buffer (5 mM, pH 7.3). At the start of the incubation, both the radiolabeled precursor and the coenzyme solutions were added to the incubation media. Incubations were performed at 20°C for 2 h with constant shaking. At the end of the incubation, steroids were extracted three times from the media with 4 ml dichloromethane. The extracts were concentrated and applied to a thin layer chromatography (TLC) plate (Merck, Darmstadt, Germany) with non-radioactive steroid standards, and then developed in benzene:acetone (4:1). Radioactive steroid metabolites were analyzed with a BAS 1500 bio-imaging analyzer

(Fuji Film, Tokyo), and estrone (E1) and estradiol-17β (E2) standards were visualized by exposure to iodine vapor. Other steroid standards were detected by UV absorption at 254 nm. Radioactive steroids were scraped from the TLC plates and extracted three times with 3 ml diethyl ether. Some radioactive metabolites were further separated in different solvent systems. Radiolabeled steroid metabolites were identified by their chromatographic mobility in TLC and by recrystallization as described by Axelrod *et al* (1965). There was little difference in the patterns of metabolites in follicles from three fish sampled at the same time. Therefore, we used follicles derived from one fish at each stage.

In vitro GVBD assay

Three females which had vitellogenic oocytes over 600 μm in diameter were collected and injected intramuscularly with a single dose of 200 IU hCG/kg body weight for inducing MIH receptor formation in the oocyte surface. Eighteen hours after injection, the fish were sacrificed, the ovaries removed and placed in ice-cold Leibovitz's L-15 culture medium buffered with 20 mM HEPES at pH 7.6 adjusted by 1N NaOH. Ovaries in the medium were dissected into small pieces and pipetted gently. Approximately 100 ovarian follicles with largest diameter were collected and incubated in individual wells of 24-well culture plates, each containing 1 ml of culture medium supplemented with 200 mg/l gentamycin sulphate. Steroids were dissolved and diluted with ethanol. Ten μl of steroid solution were added to the wells, and 10 μl of ethanol was added as a control for steroids. Three replicates of 100 oocytes were prepared for each dose of steroid. After incubation at 20°C for 18 h, the number of oocytes that had completed GVBD was counted in each well. Oocytes that have undergone GVBD become transparent, and can be easily distinguished (Shiraishi *et al*, 2005).

Measurement of serum steroid levels

Blood samples were collected from the caudal vessel using syringes with 19-gauge needles. Samples were then centrifuged at 1,000 *g* for 20 min, and the separated serum was stored at –30°C until use for the steroid assay. Serum E2 levels were measured using an Estradiol EIA Kit (Cayman Chemical, MI, USA). Serum T, 17,20β-P and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) levels were measured using ELISAs used the same procedure of our previous study (Ohta *et al*, 2001; Matsuyama *et al*, 1998) in which validation of ELISAs have been described. Competition curves for serum collected from chub mackerel were almost parallel to the standard curves (ANCOVA. $P>0.05$).

Statistics

Steroid content results were compared between each ovarian developmental stage and analyzed by one-way ANOVA, followed by Tukey-Kramer test.

RESULTS

Steroid metabolism in vitellogenic follicles

When vitellogenic follicles at LY stage were incubated with [^{3}H]P5 and [^{3}H]17-P, a total of seven radioactive fractions manifested on TLC after development in benzene/acetone (4:1) (Fig. 1). Fractions 1, 2, 4, and 5 corresponded to AD, P5, T, and 17-P5, respectively. Fraction 3 was resolved into DHEA and 17-P following TLC development in chloroform/ethyl acetate (2:1) (data not shown). Two minor polar metabolites (fractions 6 and 7) were found, but no standard steroids co-migrated with them.

To determine the E2 steroidogenic pathway, we used three radiolabeled steroids in addition to [^{3}H]P5 as precursors for *in vitro* incubations of vitellogenic follicles. [^{3}H]17-P was metabolized to AD, T, UN-1, and UN-2 (Fig. 1). [^{14}C]AD was metabolized into T and E2 was produced after incubation with [^{3}H]T.

All metabolites except fractions 6 and 7 were identified

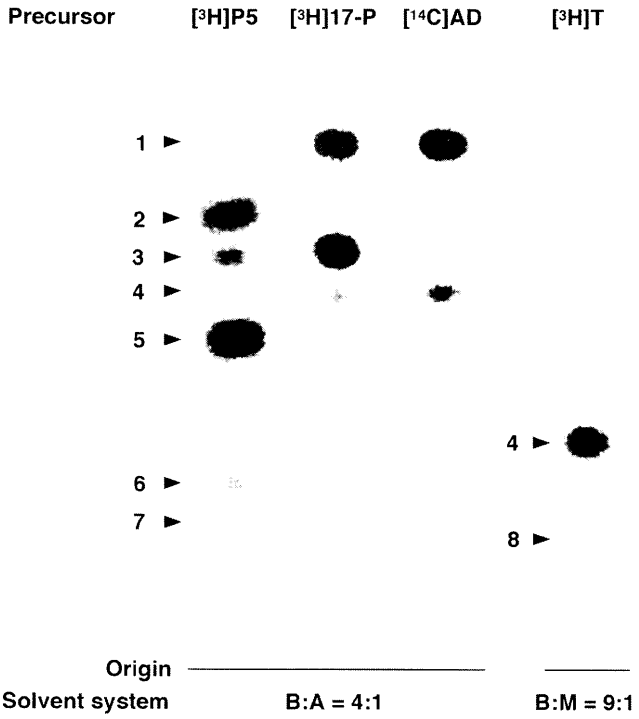

Fig. 1. Autoradiograms of steroid metabolites from vitellogenic ovarian follicles at late yolk stage of the chub mackerel incubated with [7-^{3}H]P5, [1,2-^{3}H]17-P, [4-^{14}C]AD and [1,2-^{3}H]T. Eight metabolites were separated by thin layer chromatography and developed with benzene:acetone (B:A=4:1) or benzene:methanol (B:M=9:1). 1, AD; 2, P5; 3, 17-P and DHEA; 4, T; 5, 17-P5; 6, Unknown-1; 7, Unknown-2; and 8, E2. See Table 1 for steroid abbreviations.

Table 2. Metabolism of various radiolabeled steroids by ovarian follicles of the vitellogenic oocytes of chub mackerel

Metabolite[1]	Precursor			
	[^{3}H]P5	[^{3}H]17-P	[^{14}C]AD	[^{3}H]T
P5	17,327[2]	–[3]	–	–
17-P5	26,675	–	–	–
17-P	11,288	201,156	–	–
DHEA	1,317	–	–	–
AD	–	25,100	5,200	–
T	–	1,395	600	1,100,540
E2	–	–	–	13,110
UN1[4]	3,680	2,554	–	–
UN2[5]	3,440	1,259	–	–

[1] See table 1 for systematic names.
[2] Radioactivities (dpm).
[3] Not detectable.
[4, 5] Unknown fractions corresponding to respective fractions 6 and 7 in Fig. 1.

by chromatographic mobility in TLC using different solvent systems and recrystallization. Quantitative data are shown in Table 2.

Steroid metabolism in maturing follicles

The incubation of maturing follicles at the germinal vesicle migration (GVM) stage with [^{3}H]P5 resulted in a total of nine radioactive fractions on the TLC plate after development in benzene/acetone (4:1) (Fig. 2). Fractions 1, 2, 3, 4, 5, and 9 corresponded to AD, P5, 17-P, 17-P5, 17,20β-P-5β, and 20β-S, respectively. Fraction 6 was separated into 17,20β-P and 17,21-P by TLC development in benzene/chloroform/diethyl ether/methanol (2:2:1:1). Two polar fractions observed in vitellogenic follicles (UN-1 and UN-2 in Fig. 1) were also found in maturing follicles as fractions 7 and 8, respectively. When [^{3}H]17-P was used as a precursor, 17-P, 17-P-5β, AD, 17,20β-P, 17,21-P, 20β-S, UK-1, and UK-2 were synthesized (data not shown).

In order to clarify the synthetic pathway by which 20β-S was produced, follicles at the GVM stage were incubated with [^{3}H]17,20β-P and [^{3}H]17,21-P. [^{3}H]17,20β-P was converted largely into 17,20β-P-5β, along with a small quantity of a new, unidentified metabolite (fraction 10, Fig. 2). 20β-S was synthesized, along with 17,21-P-5β and another two unidentified metabolites (fractions 12 and 13), when [^{3}H]17,21-P was used as the precursor.

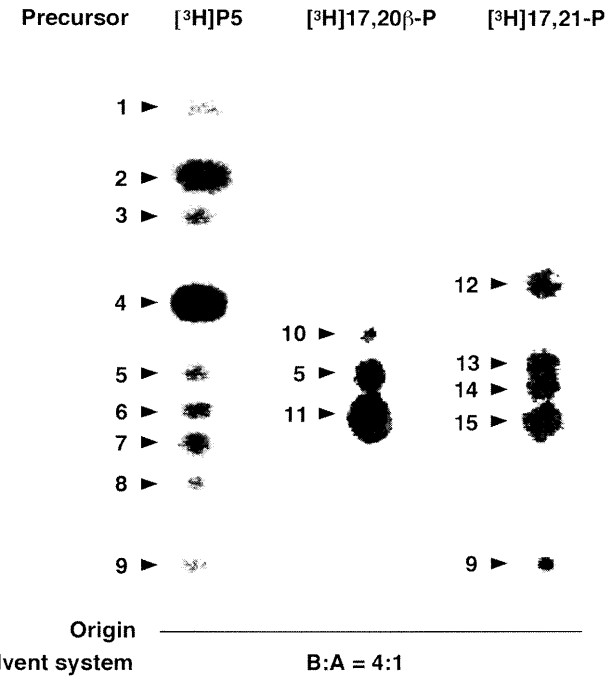

Fig. 2. Autoradiograms of steroid metabolites from maturational ovarian follicles at germinal vesicle migration stage of the chub mackerel incubated with [7-^{3}H]P5, [1,2-^{3}H]17,20β-P and [1,2-^{3}H]17,21-P. Fifteen metabolites were separated by thin layer chromatography and developed with benzene:acetone (B:A=4:1). 1, AD; 2, P5; 3, 17-P ; 4, 17-P5; 5, 17,20β-P-5β; 6, 17,20β-P and 17,21-P; 7, Unknown-1; 8, Unknown-2; 9, 20β-S; 10, Unknown-3; 11, 17,20β-P; 12, Unknown-4; 13, Unknown-5; 14, 17,21-P-5β; and 15, 17,21-P. See Table 1 for steroid abbreviations.

Table 3. Metabolism of various radiolabeled steroids by ovarian follicles of the maturing oocytes of chub mackerel

Metabolite[1]	Precursor			
	[^{3}H]P5	[^{3}H]17-P	[^{3}H]17,20β-P	[^{3}H]S
P5	12,354[2]	–[3]	–	–
17-P5	27,725	–	–	–
17-P	8,406	587,066	–	–
17-P-5β	–	165,871		–
AD	5,850	9,120	–	–
17,20β-P	3,030	1,420	343,269	–
17,20β-P-5β	3,035	1,105	36,738	–
S	6,110	11,135	–	172,500
S-5β	–	–	–	18,330
20β-S	3,203	1,501	–	2,070
UN1[4]	4,221	10,000	–	–
UN2[5]	3,500	12,080	–	–
UN3[6]	–	–	3,000	–
UN4[7]	–	–	–	2,080
UN5[8]	–	–	–	4,000

[1] See Table 1 for systematic names.
[2] Radioactivities (dpm).
[3] Not detectable.
[4–8] Unknown fractions corresponding to respective fractions 7, 8, 10, 12 and 13 in Fig. 2.
[4, 5] Showed similar co-migration with unknown-1 and -2 fractions in Fig. 1, respectively.

With the exceptions of fractions 7, 8, 10, 12, and 13, the metabolites were identified by chromatographic mobility in TLC using different solvent systems and recrystallization. Quantitative data are shown in Table 3.

In vitro GVBD assay

The relative effectiveness of the four major steroids produced during FOM at inducing GVBD is summarized in Table 4. Of these, 17,20β-P was most effective at inducing

Table 4. *In vitro* effectiveness of various steroid hormones on the percentage of the chub mackerel ovarian follicles to complete GVBD

Steroids[1]	Dose				
	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	0 M
17,20β-P	50.5±7.7	22.7±2.9	0	0	
17,20β-P-5β	14.7±1.2	10.1±2.7	0	0	
20β-S	15.4±6.0	11.0±2.8	0	0	
17,21-P	0	0	0	0	
Control[2]					0

[1] Synthetic name of steroid hormones:
17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one
17,20β-P-5β, 17,20β-dihydroxy-5β-pregnan-3-one
20β-S, 17,20β,21-trihydroxy-4-pregnen-3-one
17,21-P, 17,21-dihydroxy-4-pregnene-3,20-dione
[2] Control medium contains 1% ethanol.

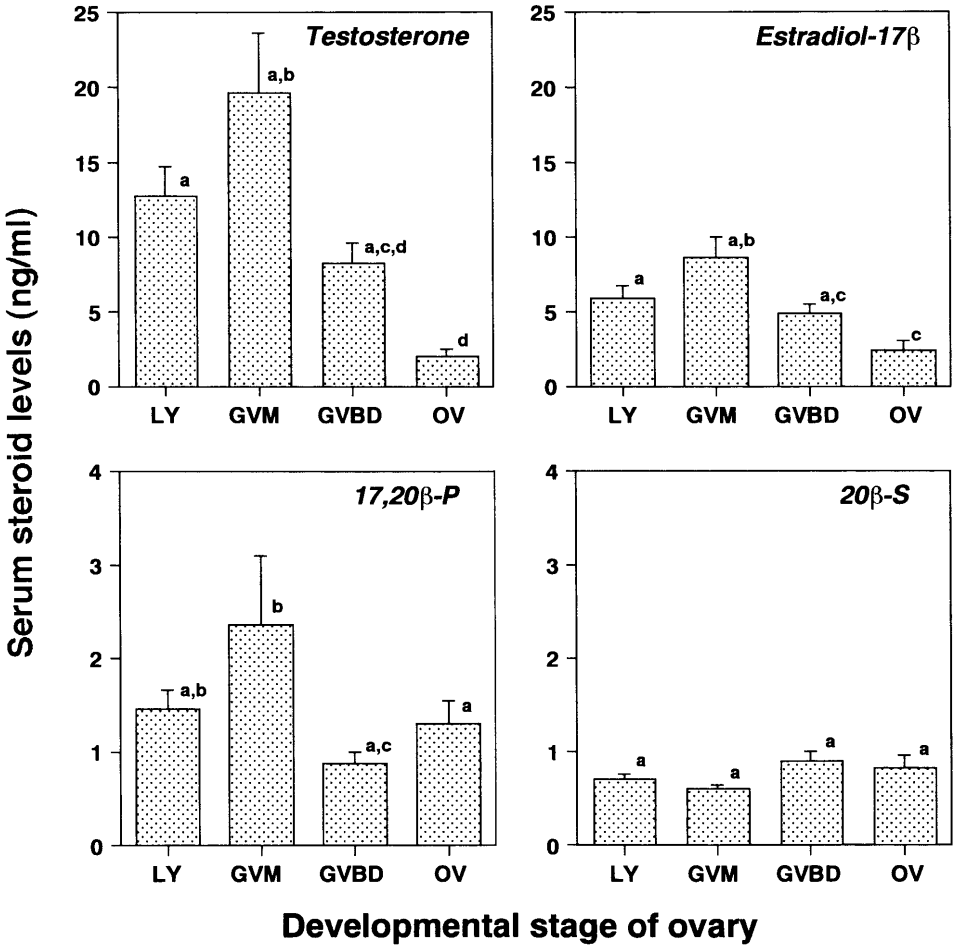

Fig. 3. Changes in serum levels of testosterone, estradiol-17β, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and17,20β,21-tihydroxy-4-pregnen-3-one (20β-S) in female chub mackerel sampled at different stages of ovarian development. Ovarian stage is represented by the developmental stage of the most advanced oocytes. Numbers of sample size are 32, 8, 18 and 34 at LY, GVM, GVBD and OV stage, respectively. Each value is the mean±SEM. Statistically significant differences between groups are shown by different letters ($P<0.05$). LY, late yolk; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown; and OV, ovulation.

GVBD at concentrations between 10^{-5} and 10^{-6} M, while 20β-S and 17,20β-P-5β were much less effective at each concentration. 17,21-P did not appear to induce GVBD at any concentration.

Serum steroid hormone levels

Although their absolute values differed, serum levels of T and E2 had similar profiles from vitellogenesis to ovulation (Fig. 3). When the developmental stage of the largest oocytes was GVM, serum T and E2 concentrations were high (T = 19.51 ng/ml, E2 = 8.54 ng/ml). Just after ovulation, the concentrations dropped to the lowest recorded for these steroids (T = 2.56 ng/ml, E2 = 2.45 ng/ml). There was a stage-specific increase in serum concentrations of 17,20β-P at the GVM stage (17,20β-P = 2.35 ng/ml), while 20β-S concentrations in serum were consistently maintained throughout vitellogenesis to ovulation (20β-S = 0.58–0.88 ng/ml).

DISCUSSION

This study identified the metabolites of steroids and clarified their biosynthetic pathways in chub mackerel ovarian follicles during vitellogenesis and FOM (Fig. 4).

When vitellogenic follicles were incubated with [^{3}H]P5, the major metabolites produced were 17-P5, 17-P, and DHEA. However, the radioactivity of 17-P from [^{3}H]P5 was 8.6 times that of DHEA from [^{3}H]P5, indicating that P5 is largely metabolized via 17-P5 to 17-P but not to DHEA. Along with the results for another three different radioactive precursors, this led to the conclusion that E2 was synthesized through a major pathway from P5 via 17-P5, 17-P, AD, and T (Fig. 4). Although there are a number of *in vivo* and *in vitro* studies of follicular steroidogenesis in various teleost species, the complete steroidogenic pathway from P5 to E2 has been determined only in four other species: medaka *Oryzias latipes* (Kobayashi *et al.*, 1996), bambooleaf wrasse *Pseudolabrus sieboldi* (Ohta *et al.*, 2001), red seabream

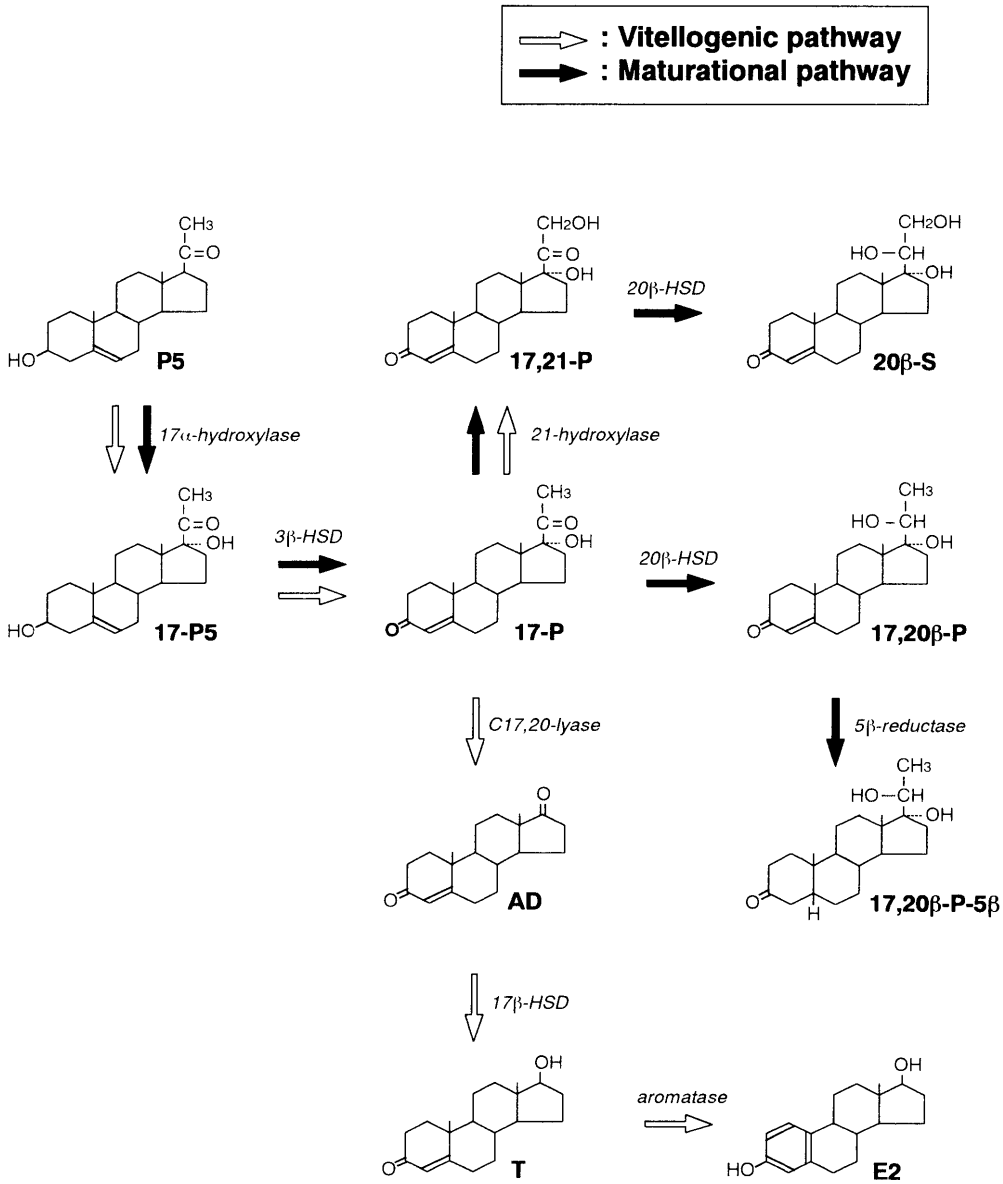

Fig. 4. Steroidogenic pathways in chub mackerel ovarian follicles during vitellogenesis and final oocyte maturation.

Pagrus major (Ohta *et al.*, 2002a), and Japanese yellowtail *Seriola quinqueradiata* (Rahman *et al.*, 2002a). Chub mackerel, medaka, and yellowtail are gonochoric, while bambooleaf wrasse and red seabream have a bisexual phase in their life history. The bambooleaf wrasse exhibits a diandric protogyny in which functional females change sex to secondary males with terminal-phase appearance (Nakazono, 1979). Red seabream are gonochoric with a bisexual juvenile stage; testes originate from the underdeveloped ovary via a bisexual juvenile gonad (Matsuyama *et al.*, 1988). Interestingly, T is the substrate precursor of E2 in vitellogenic ovarian follicles of medaka, yellowtail, and chub mackerel (although AD is converted from DHEA in yellowtail; Rahman *et al.*, 2002a). In contrast, E2 is synthesized via estrone (E1) rather than T in bambooleaf wrasse and red seabream (Ohta *et al.*, 2001, 2002a). Gonadal steroid hormones are likely to play an important role in sex changes of hermaphroditic fish (Baroiller *et al.*, 1999; Devlin and Nagahama, 2002). In protogynous species, androgen treatment has generally been shown to be effective in inducing female-to-male inversions (Reinboth, 1975; Kramer *et al.*, 1988; Grober *et al.*, 1991). We have also succeeded in inducing a gonadal sex change from female to male in bambooleaf wrasse by the implantation of T and 11-ketotestosterone (11-KT, an active androgen in male teleosts; Sakai *et al.*, 2004). Thus, despite limited information, we hypothesize that low levels or lack of production of T in ovarian follicles of red seabream and bambooleaf wrasse may be related to sex reversals in their life cycles. In other words, ovarian T production (over a certain threshold) may affect the mainte-

nance of ovarian structure and function in the female phase of hermaphroditic species. In the ovary of gonochoric species including chub mackerel and in other teleost groups, E2 is converted from AD via T. Recently, we confirmed the above hypothesis in studies of steroidogenesis in the ovarian follicles of the pufferfish *Takifugu rubripes* and kyusen wrasse *Halichoeres poecilopterus*. In the vitellogenic ovarian follicles of gonochoric pufferfish, E2 was converted from AD via T, while in the protogynous kyusen wrasse, E2 was converted from AD via E1 (unpublished data).

E2 production pathways differ between gonochoric species. Chub mackerel ovarian follicles synthesize E2 using the same pathways as medaka, namely P5, 17-P5, 17-P, AD, T, and E2 (Kobayashi *et al.*, 1996). In yellowtail and pufferfish, AD is synthesized from 17-P5 via DHEA but not 17-P (Rahman *et al.*, 2002a). The reason for these species-specific differences in E2 synthetic pathways is unknown. Recent studies have shown that E2 and T in female teleost serum exert negative and/or positive feedback effects on pituitary gonadotropin (GtH) synthesis and secretion (reviewed in Linard *et al.*, 1995; Schultz *et al.*, 1995; Tredeau and Peter, 1995). In addition, AD produced in the vitellogenic follicles of the goldfish *Carassius auratus* has been reported to act as a primary pheromone inducing agonistic behavior among males (Poling *et al.*, 2001). Future studies should look at potential physiological roles for intermediate products other than T in the E2 synthetic pathway, such as steroid feedback or pheromonal activity.

The profiles of the serum concentrations of four steroid hormones at developmental stages of oocytes after hCG injection are summarized in Fig. 5. Serum T and E2 concentrations peaked as the first clutch of oocytes began GVM, ~24 h after hCG injection. At the GVM stage, steroidogenesis in the ovarian follicles has shifted from E2 to MIH pro-

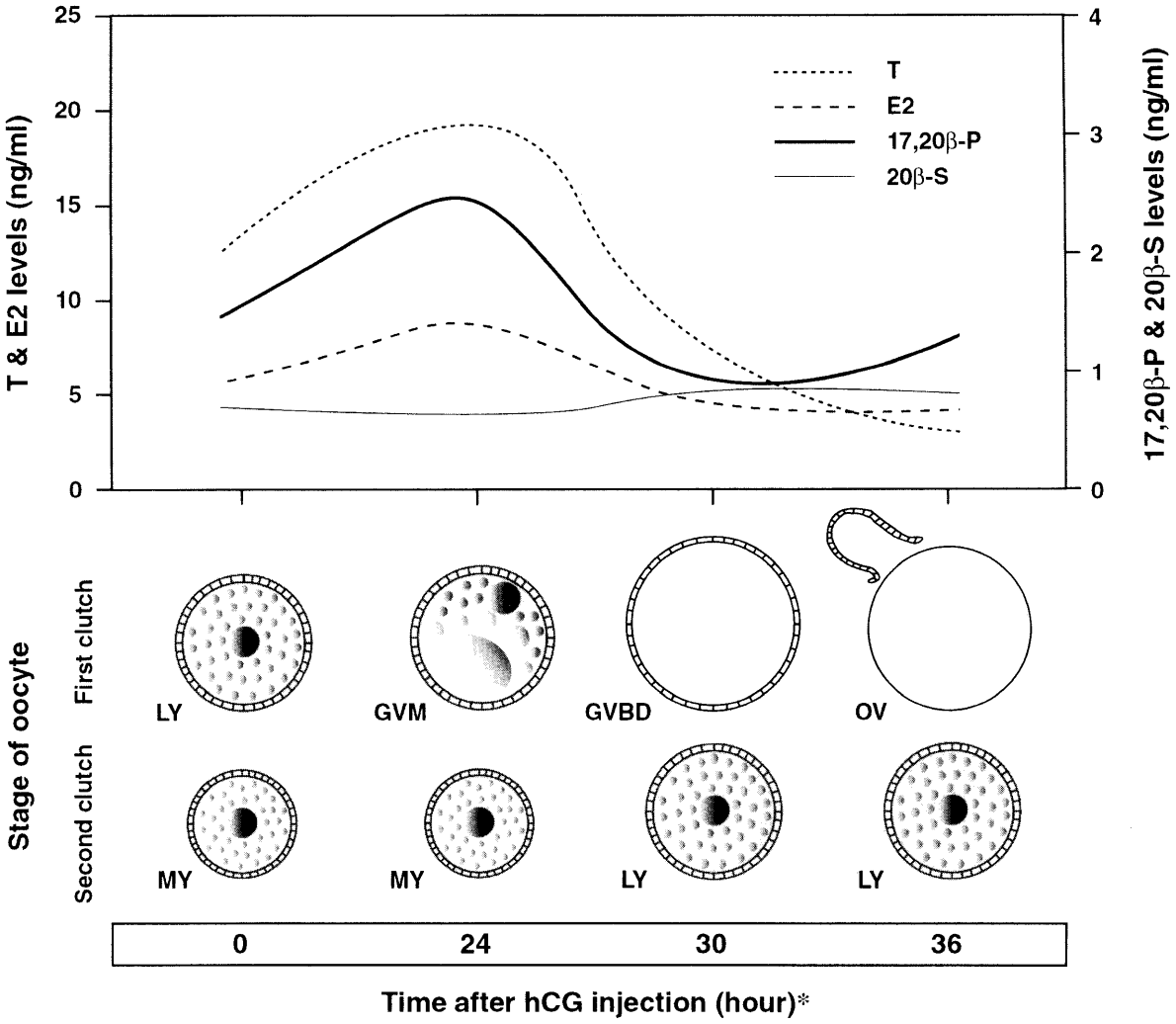

Fig. 5. Diagrammatic representation of changes of oocyte composition and serum levels of testosterone (T), estradiol-17β (E2), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-tihydroxy-4-pregnen-3-one (20β-S) after hCG injection. MY, mid yolk; LY, late yolk; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown; and OV, ovulation. * Time course of ovarian development after hCG injection is cited from Shiraishi *et al.* (2005).

duction; thus, the observed increases in serum concentrations of T and E2 are probably produced by the second clutch of oocytes, which are by then at mid-yolk (MY) stage and are actively vitellogenic.

Two 20β-hydroxylated progestins are known to act as maturation-inducing hormones (MIH) in teleosts: 17,20β-P and 20β-S. Of the steroids produced in ovarian follicles undergoing FOM in the present study, only 17,20β-P was highly effective for induction of GVBD *in vitro*. Although 20β-S was synthesized from 17-P via 17,21-P to the same extent as was 17,20β-P (without passing through 17,20β-P) in maturing chub mackerel follicles, the ability of 20β-S to induce GVBD *in vitro* was much lower than that of 17,20β-P. Moreover, serum levels of 17,20β-P increased specifically around the time of GVM, while 20β-S was present in serum at consistently low levels throughout follicle development. These results strongly suggest that 17,20β-P acts as MIH in chub mackerel.

MIH induces FOM by acting directly through a plasma membrane receptor on the oocyte surface (Nagahama, 1997; Thomas *et al.*, 2002). This receptor, unrelated to the classical steroid nuclear receptors, mediates rapid non-genomic steroid activity. Ovarian 20β-S membrane receptors have been identified in the spotted sea trout *Cynoscion nebulosus* (Patino and Thomas, 1990) and striped bass *Morone saxatilis* (King *et al.*, 1997). An oocyte 17,20β-P receptor was found on ovarian plasma membranes of the rainbow trout *Oncorhynchus mykiss* (Yoshikuni *et al.*, 1993) and has also been identified and characterized in yellowtail (Rahman *et al.*, 2002b). More recently, membrane progestin receptors (mPRs) that mediate FOM-inducing activities of progestins have been cloned and characterized in the spotted sea trout (Zhu *et al.*, 2003), which is the first identification of a gene with mPR characteristics. Studies of this new class of steroid receptors with respect to the molecular basis of rapid intracellular responses to MIH cell surface activity are underway.

We observed a distinct shift in steroidogenesis from E2 to 20β-hydroxylated steroids during follicle development. The MIH of chub mackerel, 17,20β-P, has also been identified in some other teleosts. However, our research has confirmed the importance of common enzymatic kinetics in the steroidogenic shift involving decreased 17,20-lyase activity and increased 20β-HSD activity at stages from vitellogenesis through FOM. It has been suggested that GtH secretion just before FOM causes the *de novo* synthesis of 20β-HSD in salmonids (Nagahama, 1997). Several studies in mammals have shown that C17,20-lyase activity is regulated by P450 oxidoreductase, cytochrome *b*5, or Ser/Thr phosphorylation (Miller, 1997). The molecular mechanism underlying this process remains unclear. Future studies should investigate how GtH and other hormonal factors act on ovarian follicles during FOM to control the expression of these specific genes at specific times.

When ^{3}H-labeled P5 or 17-P was incubated with maturing follicles, the production of 17,20β-P-5β (which is the 5β-reduced form of 17,20β-P) increased with 17,20β-P production. The 17,20β-P-5β was much less effective than 17,20β-P at FOM induction in chub mackerel. The quick conversion of 17,20β-P observed in the chub mackerel probably represents the inactivation process of MIH. Similar 5β-reduction of 17,20β-P has been observed in maturing follicles of medaka (Fukada *et al.*, 1994), yellowtail (Rahman *et al.*, 2001), bambooleaf wrasse (Ohta and Matsuyama, 2002), and red seabream (Ohta *et al.*, 2002b).

When ^{3}H-labeled 17-P was incubated with maturing follicles, 17,20β-P and 20β-S were produced by 20β-HSD at similar radioactivities. In addition, 20β-S was synthesized from [^{3}H]17,21-P but not from [^{3}H]17,20β-P. These results clearly demonstrate that 20β-S is synthesized from 17-P via 17,21-P but not via 17,20β-P, suggesting substrate specificity for 21-hydroxylase in chub mackerel ovarian follicles. Substrate specificity of 21-hydroxylase has previously been found during FOM in ovarian follicles of pufferfish (Matsuyama *et al.*, 2001), bambooleaf wrasse (Ohta and Matsuyama, 2002), and red seabream (Ohta *et al.*, 2002b). However, the physiological role of 20β-S synthesized via 17,21-P with substrate-specific 21-hydroxylase in maturing follicles of chub mackerel is not known. Both 17,20β-P and 20β-S are synthesized in maturing follicles of bambooleaf wrasse and red seabream and are highly effective in inducing GVBD *in vitro*, indicating the two 20β-hydroxylated progestins act as MIH in these species (Ohta and Matsuyama, 2002; Ohta *et al.*, 2002b). In addition, recent studies have shown that 20β-hydrtoxylated progestins induce ovulation under genomic control, probably via the nuclear progestin receptor (Pinter and Thomas, 1995, 1999). This suggests that progestins produced in the ovarian follicles induce maturation and ovulation by different mechanisms, via the membrane and nuclear receptors, respectively. In order to identify the precise roles of 17,20β-P and 20β-S in fish species including chub mackerel, it is necessary to investigate the characteristics of nuclear progestin receptors as well as mPRs.

This is the first report from chub mackerel of steroid hormone production, steroid hormone synthetic pathways in ovarian follicles, and the circulating profiles of major steroids throughout vitellogenesis and FOM. The results of this study will be indispensable in future research into seed production not only for chub mackerel but also for other scombrid fish such as tuna and bonito.

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