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Published on: 01 Sep 2001 - Zoological Science (Zoological Society of Japan)

Topics: Estrone

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Source: Zoological Science, 18(7) : 937-945

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.937

ZOOLOGICAL SCIENCE **18**: 937–945 (2001)

Steroidogenic Pathway to Estradiol-17β Synthesis in the Ovarian Follicles of the Protogynous Wrasse, *Pseudolabrus sieboldi*

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ABSTRACT—The bambooleaf wrasse, *Pseudolabrus sieboldi*, is a diandric protogynous fish with a diurnal rhythm of ovarian development, and females spawn daily during the spawning season. This study investigated the steroidogenic pathway for estradiol-17β (E2) biosynthesis in vitellogenic ovarian follicles of the bambooleaf wrasse. We incubated follicles *in vitro* with radioactively labeled steroid precursors, and measured serum steroid levels using microtiter plate enzyme-linked immunosorbent assays (ELISAs). ELISAs for estrone (E1) and testosterone (T) were developed. The experiments showed that E2 was synthesized from pregnenolone via 17-hydroxypregnenolone, dehydroepiandrosterone, androstenedione, and E1. T was not produced from any radiolabeled precursors, and exogenous T was not converted to E2. During the spawning season, serum levels of E2 and E1 showed similar patterns, with a diurnal rhythm of high levels at 03:00 hr associated with active ovarian vitellogenic follicles. In contrast, serum T levels were constant and relatively low compared to levels of E2 and E1. These results indicate that E2 is synthesized via E1, rather than T, in the ovarian follicles, and suggest that T detected in the blood is likely derived from extra-follicular tissues.

INTRODUCTION

Ovarian development and recrudescence in teleosts are regulated by pituitary gonadotropin (GtH) through the production of ovarian steroid hormones. During oocyte vitellogenesis, the follicular layer of the oocytes synthesizes estradiol-17β (E2), which stimulates the hepatic production of vitellogenin as a yolk precursor (reviewed by Specker and Sullivan, 1994; Tyler and Sumpter, 1996). Many studies have examined E2 production in teleosts. In some salmonids and in the goldfish *Carassius auratus*, it has been confirmed that E2 is produced by the ovarian follicles via testosterone (T) (Kagawa *et al*., 1984, 1985) and that the appearance of both E2 and T in blood is association with vitellogenesis (Kagawa *et al*., 1983a, b). The correlation between E2 and T levels in blood has been found in a variety of teleost species (e.g., Wingfield and Grimm, 1977; Scott *et al*., 1984; Thomas *et al*., 1987; Matsuyama *et al*., 1988; Greeley *et al*., 1988; Prat *et al*., 1990; Barnett and Pankhurst, 1994; King *et al*., 1994; Pankhurst *et al*., 1999). These findings implicate T as a substrate precursor of E2 in most teleosts. In addition, among teleost females, serum E2 and T exert negative or positive feedback effects on GtH synthesis and secretion (reviewed in Linard *et al*., 1995; Schultz *et al*., 1995; Trudeau and Peter, 1995). Thus, T seems to play important roles in endocrine control of oogenesis in many teleosts. However, the E2 synthetic pathway within ovarian follicles has been determined in only a few species.

The bambooleaf wrasse exhibits diandric protogyny, and populations consist of small initial-phase (IP) males, IP females, and large terminal-phase (TP) males (Nakazono, 1979). TP male individuals may arise from one of two processes: sex change in an IP female or role change in an IP male. This transformation of IP into TP is accompanied by changes in body color, gonad morphology, and mating behavior. A single IP female spawns almost daily during the two-month spawning season, and has a diurnal rhythm in oocyte growth, maturation, ovulation and spawning (Matsuyama *et al*., 1998a). These features make the bambooleaf wrasse a good model for studies on endocrine control of oogenesis.

Recently, we studied steroidogenesis in ovarian follicles of this species, and reported the pathway of two maturation-inducing hormones (MIHs), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), and the enzymatic kinetics of their production during final oocyte maturation (FOM) (Ohta and Matsuyama, 2001). It has been suggested that the pre-maturational GtH surge is responsible for the increase in 17,20β-P and 20β-S during FOM via 20β-hydroxysteroid dehydrogenase (20β-HSD) activation in the ovarian follicles of bambooleaf wrasse (Matsuyama *et al*., 1998a, b). In this wrasse, as in other teleosts, the steroid hormones produced by ovarian follicles during vitellogenesis may also exert feedback effects on GtH syn-

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thesis and secretion. However, detailed analysis of steroidogenesis in the ovarian follicles of bambooleaf wrasse during vitellogenesis has not been performed.

Therefore, we investigated the steroidogenic pathway of E2 biosynthesis in the ovarian follicles of bambooleaf wrasse. We determined the synthetic pathway of E2 production, and demonstrated, for the first time in teleosts, that E2 is synthesized via E1, and not T.

MATERIALS AND METHODS

Chemicals

The nomenclature of the steroids used in this study is shown in Table 1. Radioactive steroids, [7-^{3}H]pregnenolone (P5, 780.7 GBq/mmol), [1,2-^{3}H]17-hydroxyprogesterone (17-P, 1565.1 GBq/mmol), [4-^{14}C]dehydroepiandrosterone (DHEA, 2.0535 GBq/mmol), [4-^{14}C]androstenedione (AD, 2.0 GBq/mmol), and [2,4,6,7-^{3}H]E1 (2738 GBq/mmol) were purchased from New England Nuclear (Boston, Mass). [4-^{14}C]T (2.15 GBq/mmol) was purchased from Amersham (U.K.). Unlabeled steroids were obtained either from Sigma (St Louis, Mo) or Steraloids Inc. (Wilton, NH). 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). Coenzymes, reagents and solvents were obtained from either Sigma or Wako (Tokyo, Japan).

Fish and sampling

During the spawning season (late September-mid November), mature female and TP male bambooleaf wrasse were caught by hook and line in coastal waters near the Fisheries Research Laboratory, Kyushu University and transferred to the laboratory. Fish were kept in 500-litre fiberglass tanks with filtered seawater, under natural day-length and water temperature, and fed krill and live hermit crab once a day. After confirming daily spawning, 4–6 female fish (body weight 17.05–59.62 g, total length 113–159 mm) were sampled at 12:00, 18:00, 03:00, and 06:00 hr. Fish were anesthetized with 2-phenoxyethanol (300 ppm), and blood samples were collected from the caudal vessel using syringes fitted with 25-gauge needles and centrifuged at 3,000 rpm for 20 min. The separated serum was stored at –30°C until assayed for steroid level. After blood sampling, fish were killed by decapitation, and the ovaries were dissected out. For ovarian histology, small ovarian fragments were fixed 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 soluton. The developmental stages of oocytes have been previously reported (Matsuyama *et al.*, 1998b).

The developmental stages of the largest oocytes from the fish collected at 12:00, 18:00, and 03:00 hr were tertiary yolk (TY), early migratory nucleus (EMN), and late migratory nucleus (LMN) stages, respectively. The largest follicles from the fish sampled at 06:00 hr, in which germinal vesicle breakdown (GVBD) had already occurred and the cytoplasm was transparent due to yolk proteolysis and hydration, were described as mature (M) stage.

Ovarian follicles collected at 12:00 hr were used for *in vitro* incubation with radiolabeled steroid precursors. After decapitation, the ovaries were removed and placed in ice-cold Ringer's solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.5 mM NaH_2PO_4, 2 mM $NaHCO_3$, 20 mM Hepes, pH adjusted to 7.5 with 1 N NaOH). The largest follicles (n=250) were isolated and gathered with forceps and pipettes. After removing of excess solution, follicles were frozen in liquid nitrogen and stored at –80°C until use. Our preliminary experiments revealed that there was little difference in the steroid metabolic patterns during the incubation with frozen and intact follicles.

In vitro follicle incubation with radiolabeled steroid precursors

250 follicles were placed in a 10-ml glass tube with 1 ml of sucrose buffer (250 mM sucrose, 20 mM Hepes, pH adjusted to 7.6 with 1 N NaOH). Ten pmol of [^{3}H]P5, [^{3}H]17-P, [^{14}C]DHEA, [^{14}C]AD, [^{14}C]T, or [^{3}H]E1 were dissolved in 150 μl sucrose buffer. Coenzymes (NAD, NADH, NADP, and NADPH; 10 mM each) were dissolved in a solution that consisted of 100 μl $MgCl_2$ (20 mM) and 50 μl citrate buffer (5 mM, pH 7.3). At the start of incubation, both radiolabeled precursor and coenzymes solutions were added to the incubation media. Incubations were performed at 20°C for 2 hr with constant shaking. At the end of incubation, steroids were extracted three times from the media with 4 ml dichloromethane. The extract was concentrated and applied to a thin layer chromatography (TLC) plate (60F_{254}; Merck, Darmstadt, Germany) with non-radioactive standard steroids, i.e., E1, E2, AD, T, progesterone, 17-P, and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), and then developed in benzene:acetone (4:1). Radioactive steroid metabolites were analyzed with a BAS 1500 bioimaging analyzer (Fuji Film, Tokyo), and standard E1 and E2 were visualized by exposure to iodine vapor. Other standard steroids 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).

Table 1. Nomenclature of steroids used in the present study

Systematic name	Trival name	Abbreviation
3β-hydroxy-5-pregnen-20-one	pregnenolone	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
5-pregnene-3β,17,20β-triol	–	17,20β-P5
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

Measurement of serum steroid levels

Serum E2 and AD were measured using Estradiol EIA Kit (Cayman Chemical, MI) and androstenedione enzyme immunoassay kit (Oxford Biomedical Research Inc., MI), respectively. ELISAs of T and E1 were performed according to the same procedure for 17,20β-P (Matsuyama *et al*., 1998b) and described below briefly.

Stock solutions: Coating buffer, 0.05 M carbonate buffer pH 8.4, containing 0.05% NaN_3; washing solution, 0.85% NaCl; blocking solution, 0.05 M PBS containing 0.1% BSA, 3% sucrose, and 0.005% thimerosal; assay buffer, 0.05 M borate buffer, pH 7.8, containing 0.1% BSA and 0.01% thimerosal; substrate solution, 0.2 M citrate buffer, pH 4.5, containing 0.01% H_2O_2, added 0.5% o-phenylenediamine immediately before use; stopping solution, 6 N H_2SO_4. All the solutions except for the stopping solution were stored at 4°C.

Second Antibody-coated Microtiter Plates: Microtiter plates (MS-3596 F/H plate, Sumitomo Bakelite Co.) were coated with 100 μl per well of goat anti-rabbit IgG (15 μg/ml in coating buffer). The plate was sealed and incubated at 4°C for 48 hr. After removal of unbound antibody, the wells were washed three times with washing solution and dried. Blocking solution (200 μl) was added to each well, which was then sealed and incubated at 4°C for 24 hr. The wells were emptied and dried by leaving the plates in a refrigerator for 24 hr.

Assay procedure: Serum steroids were extracted 2-fold of diethyl ether twice. The extracts were evaporated and the residue was reconstituted with assay buffer. The wells of a second antibody-coated plate were loaded with 50 μl of standard or sample, 50 μl of diluted steroid-enzyme conjugate solution and anti-steroid solution (all dissolved in assay buffer), in this sequence. Samples and standards were applied in duplicate to each plate. After incubation at 20°C for 2 hr, the plate was drained and washed three times with washing solution. 150 μl of substrate solution was added to each well, and the plate was incubated at 20°C for 40 min. Color development was stopped by adding 50 μl of stopping solution to each well. The absorbance of each well was measured at 492 nm with a microtiter plate analyzer (model 2550, Biorad).

Validation of assay: An antiserums were raised against 6-oxo-estrone-6-carboxymethyloxime-BSA and testosterone-3(E)-carboxymethyloxime-BSA, respectively. A steep standard curve covering 1.5–192 pg/well (30–3,840 pg/ml) for each steroid was obtained with the present ELISA (antiserum dilution: E1, 1:10,000; T, 1:100,000, labeled hormone dilution: E1, 1:20,000; T, 1:20,000). The intra- and interassay coefficients of variation were determined close to the 50% binding point (for E1, 4.8% and 11.0%; for T, 11.3% and 16.6%; N = 4, duplicate). The sensitivities of these assays were 12.5 pg/ml for E1 and 9.5 pg/ml for T. These antibodies had the following cross reactivities: for E1 antibody, E2, 5%; estriol, 1.2%; estrone-3-sulfate, 0.4%; estrone-3-glucuronide, 0.15%; 2-methoxyestrone, 0.05%; P5, 0%; progesterone, 0%; cortisol, 0%; cortisone, 0%; AD 0%; T, 0%; DHEA, 0%; 5α-dihydrotestosterone, 0%; and for T antibody, 5α-dihydrotestosterone, 7.3%; AD, 2.1%; androsterone, 0.28%; 5-androstene-3β,17β-diol, 0.15%; 5α-androstane-3α,17β-diol, 0.10%; 5β-androstane-3α,17β-diol, 0.09%; cortisol, 0.02%; corticosterone, 0.01%; progesterone, 0.01%; P5, <0.01%; 17-hydroxypregnenolone (17-P5), <0.01%; aldosterone, <0.01%; DHEA, <0.01%; E2, <0.01%. Competition curves for serum collected from bambooleaf wrasse were almost parallel to the standard curves (ANCOVA. $P>0.05$). These results indicate that the present ELISAs are very specific for E1 and T, respectively, and serum levels can be measured after simple collection.

Statistics

The results of steroid contents were compared between each oocyte developmental stage and analyzed by one-way ANOVA followed by Turkey-Kramer test.

RESULTS

***In vitro* follicle incubation with radiolabeled steroid precursors**

When vitellogenic follicles were incubated with [^{3}H]P5, 8 major radioactive fractions appeared on thin-layer chromatography (TLC) after development in benzene:acetone (4:1) (Fig. 1). Fraction 4 was divided into DHEA and 17-P by TLC development in chloroform:ethyl acetate (2:1). Fraction 7 was also divided into 17,20β-P and 17,21-dihydroxy-4-pregnene-3,20-dione (17,21-P) by TLC in benzene:chloroform: diethyl ether:methanol (2:2:1:1). Fractions 1, 2, 3, 5, 6, and 8 corresponded to E1, AD, P5, E2, 17-P5, and 5-pregnene-3β,17,20β-

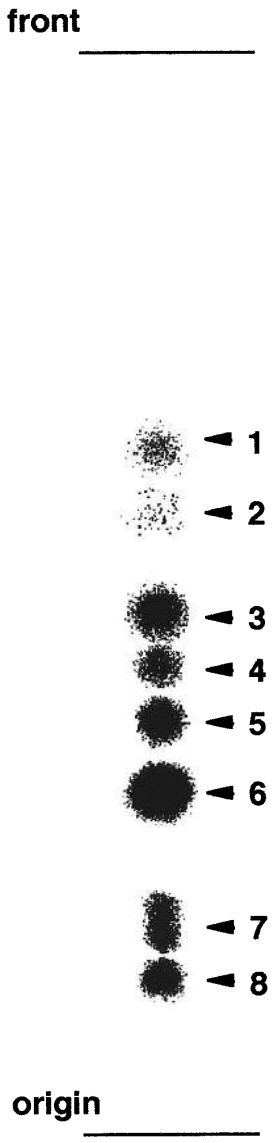

Fig. 1. Autoradiograms of steroid metabolites from vitellogenic ovarian follicles of the bambooleaf wrasse incubated with [7-^{3}H]pregnenolone. Eight metabolites were separated by thin layer chromatography and developed with benzene:acetone (4:1). 1, E1; 2, AD; 3, P5; 4, DHEA & 17-P; 5, E2; 6, 17-P5; 7, 17,20β-P & 17,21-P; and 8, 17,20β-P5. See Table 1 for steroid abbreviations.

Table 2. Steroid metabolites and specific activity of the crystals

Precursor	Metabolite[1]	Specific activities of crystals (dpm/mg)			
		1st	2nd	3rd	before crystallization
[^{3}H]P5	P5	761	808	751	801
	DHEA	330	325	308	373
	AD	528	512	508	536
	17-P5	1145	1168	1187	960
	17-P	189	204	206	238
	17,20β-P5	171	177	169	961
	17,20β-P	446	445	450	842
	17,21-P	890	853	828	876
[^{3}H]E1	E1	940	936	874	1079
	E2	1643	1640	1597	1739

[1] See Table 1 for systematic names.

Table 3. Metabolism of various radiolabeled steroids by ovarian follicles of the bambooleaf wrasse

Metabolite[1]	Precursor					
	P5	17-P	DHEA	AD	E1	T
P5	538[2]	ND[3]	ND	ND	ND	ND
17-P5	2047	ND	ND	ND	ND	ND
17-P	33	1013	ND	ND	ND	ND
DHEA	249	ND	3492	ND	ND	ND
AD	46	136	1839	5656	ND	ND
T	ND	ND	ND	ND	ND	3243
E1	201	ND	424	556	3491	ND
E2	429	ND	1127	1484	1480	ND
17,20β-P5	506	ND	ND	ND	ND	ND
17,20β-P	336	87	ND	ND	ND	ND
17,21-P	104	4055	ND	ND	ND	ND

[1] See Table 1 for systematic names.
[2] 10^{-3} pmoles.
[3] Not detectable.

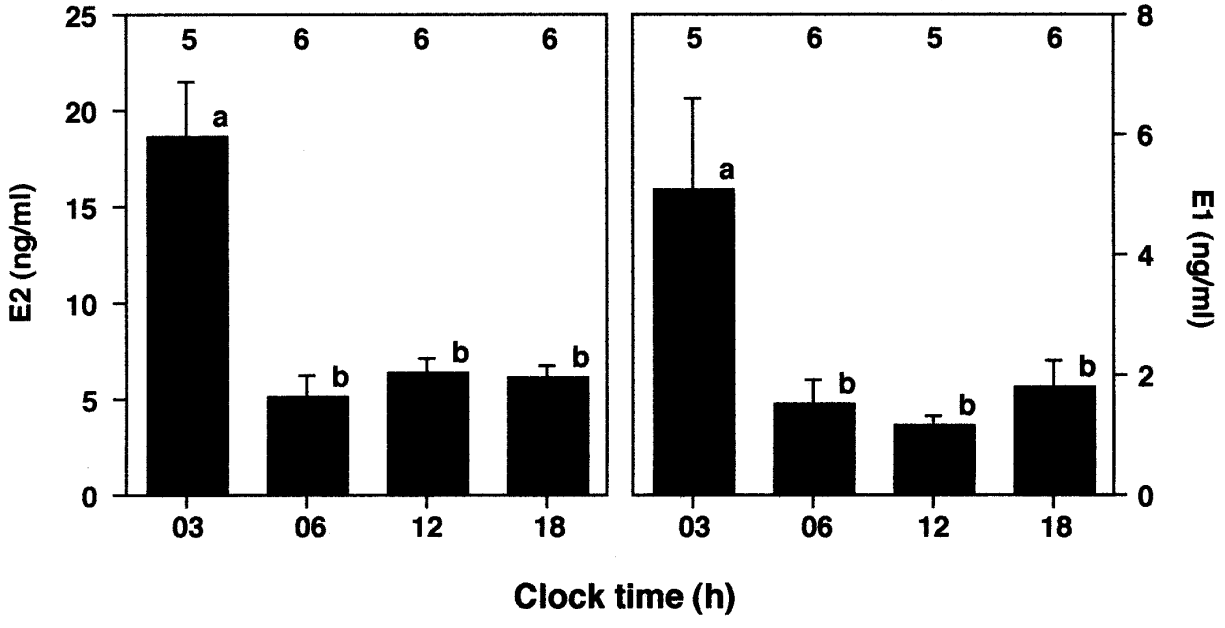

Fig. 2. Changes in the serum levels of estradiol-17β (E2) and estrone (E1) in female bambooleaf wrasse sampled at different times of a day during the spawning season. Numbers indicate sample size. Each value is the mean±SEM. Statistically significant differences between groups are shown by different letters (P<0.05).

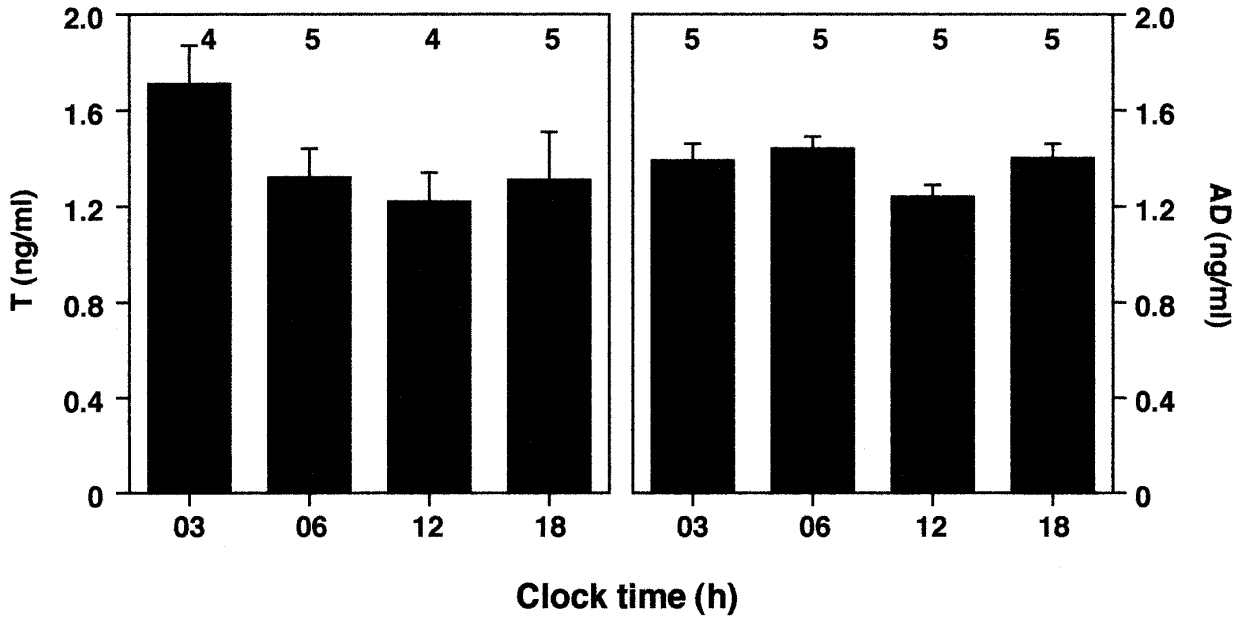

Fig. 3. Changes in serum levels of testosterone (T) and androstenedione (AD) in female bambooleaf wrasse sampled at different times of a day during the spawning season. Numbers indicate sample size. Each value is the mean±SEM.

triol (17,20β-P5), respectively. E1 and E2 from [^{3}H]P5 were twice confirmed by their mobility in TLC (solvent systems: chloroform:ethyl acetate=2: 1; benzene:methanol=9: 1), but recrystallization could not be performed due to their low radioactivity and less stableness. The other metabolites from [^{3}H]P5 and E1 and E2 from [^{3}H]E1 were confirmed by recrystallization to constant specific activity (Table 2). The quantitative data are shown in Table 3.

To determine the steroidogenic pathway of E2 synthesis, five other radiolabeled steroids were used as precursors for *in vitro* incubation of follicles. Of these five precursors, E2 was synthesized from [^{14}C]DHEA, [^{14}C]AD, and [^{3}H]E1. When follicles were incubated with [^{3}H]17-P, E2 was not detected as the metabolite, and a small amount of AD was produced. In all incubations, T production could not be detected, and [^{14}C]T was not entirely converted to E2.

Measurement of serum steroid levels

Serum levels of E2 and E1 showed similar profiles in a day of spawning season (Fig. 2). At 03:00 hr, when the developmental stage of the largest oocytes was LMN, serum E2 and E1 levels were significantly high (E2=18.52 ng/ml, E1=5.08 ng/ml). But by 06:00 hr, when the stage of the largest oocytes was M, the levels dropped (E2=5.03 ng/ml, E1=1.50 ng/ml) and remained constant levels at 12:00 and 18:00 hr, when the stages of the largest oocytes were TY and EMN, respectively (E2=6.00–6.27 ng/ml, E1=1.15–1.79 ng/ml). However, T and AD levels were constant (T=1.22–1.71 ng/ml, AD=1.23–1.43 ng/ml) throughout the day and did not differ significantly among ovarian stages (Fig. 3).

DISCUSSION

This study reveals the steroidogenic pathway to E2 synthesis in the ovarian follicles of bambooleaf wrasse (Fig. 4, vitellogenic pathway). Over the last two decades, a number of studies on steroidogenesis during vitellogenesis, in various teleosts, have incubated intact ovarian follicles or fragments or measured blood steroid levels. *In vitro* incubation of ovarian follicles (amago salmon *Oncorhynchus rhodurus*, Kagawa *et al*., 1982; goldfish, Kagawa *et al*., 1984; guppy *Poecilia reticulata*, Venkatesh *et al*., 1992; medaka *Oryzias latipes*, Kobayashi *et al*., 1996) or ovarian fragments (e.g., Atlantic croaker *Micropogonias undulatus*, Trant and Thomas, 1989) demonstrated that E2 is synthesized via T. In numerous species, it has been shown that blood E2 levels increase during vitellogenesis, along with T levels (e.g., plaice *Pleuronectes platessa*, Wingfield and Grimm, 1977; white sucker *Catostomus commersori*, Scott *et al*., 1984; spotted seatrout *Cynoscion nebulosus*, Thomas *et al*., 1987; red seabream *Pagrus major*, Matsuyama *et al*., 1988; gulf killifish *Fundulus grandis*, Greeley *et al*., 1988; sea bass *Dicentrarchus labrax*, Prat *et al*., 1990; demoiselles *Chromis dispilus*, Barnett and Pankhurst, 1994; striped bass *Morone saxatilis*, King *et al*., 1994; spiny damselfish *Acanthochromis polyacanthus*, Pankhurst *et al*., 1999). These studies have led us to the accepted view that T is a precursor for E2 production in the teleost ovary. In our study, however, T was not produced by vitellogenic follicles after incubation with radiolabeled precursors, and exogenous T was not converted to E2 in the follicles. These results clearly indicate that in bambooleaf wrasse T is not the precursor for E2 production in the vitellogenic follicles. In contrast, E1 was produced instead of T from P5, DHEA, and AD, and exogenous E1 was converted directly

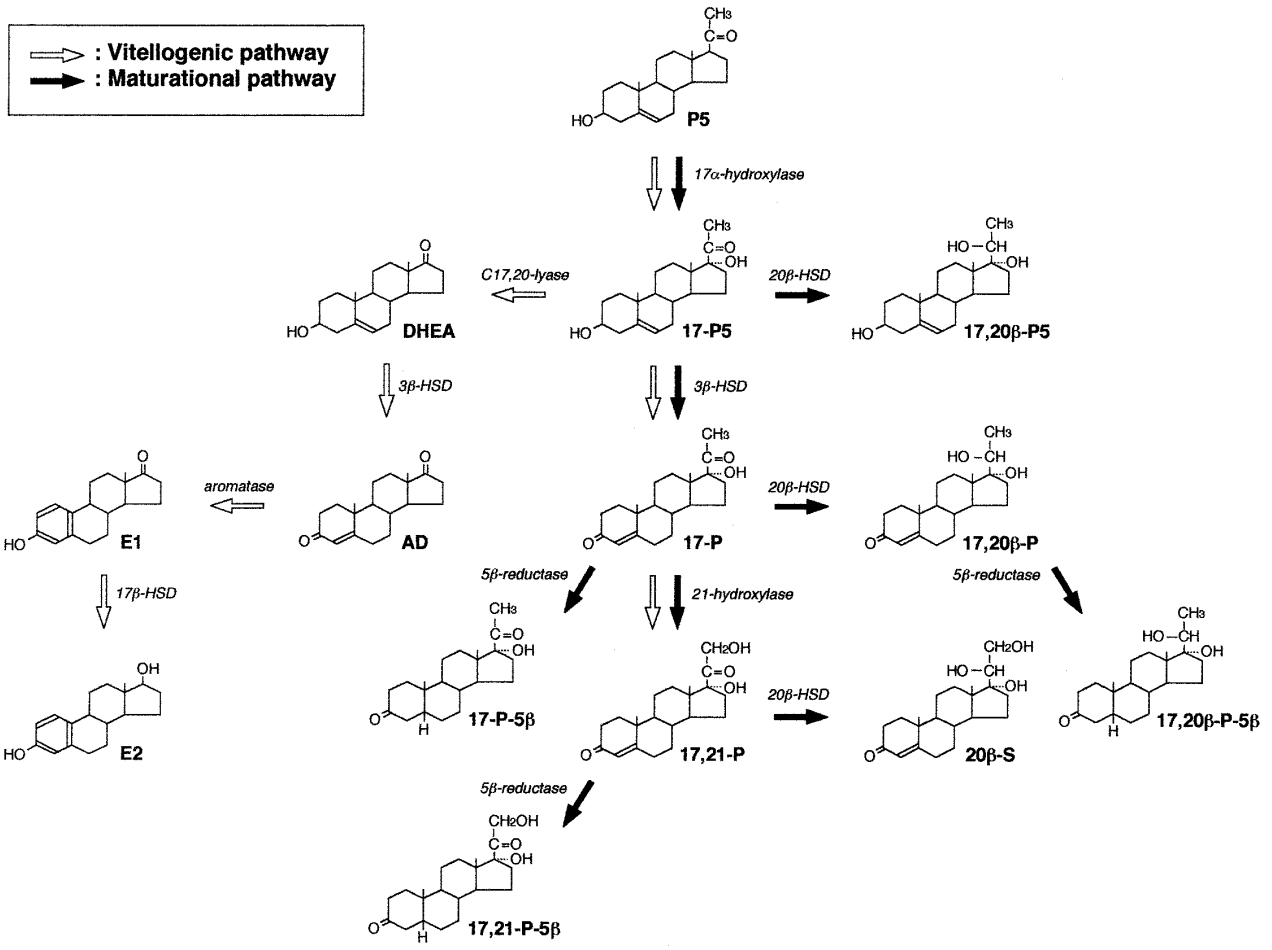

Fig. 4. Steroidogenic pathways in bambooleaf wrasse ovarian follicles during vitellogenesis (present study) combined with final oocyte maturation in our previous study (Ohta and Matsuyama, 2001).

into E2. The results suggest that E2 is synthesized from E1 in the vitellogenic follicles. Moreover, the serum steroid levels, in which serum E2 levels exhibited a diurnal rhythm that paralleled E1 levels but not T levels, support the *in vitro* data. To our knowledge, this is the first report that E1 is the major substratum of E2 in the ovarian follicles in a teleost.

Daily changes in two clutches of oocytes, the largest and the second-largest oocytes, and serum levels of four steroids are summarized in Fig. 5. In this study, serum E2 levels peaked at 03:00 hr, when the developmental stage of the largest follicles was LMN. However, bambooleaf wrasse LMN follicles cannot produce E2, as reported in our previous study (Ohta and Matsuyama, 2001). The ovary of the bambooleaf wrasse has multiple oocyte clutches, and contains vitellogenic follicles throughout a day in the spawning season. In particular, follicles at secondary yolk stages, which have active vitellogenic capacity (present study; Ohta and Matsuyama, 2001), are mainly seen during midnight (Matsuyama *et al.*, 1998a, b), which results in the high serum E2 levels observed.

In vertebrates, GtH regulates gametogenesis partially through gonadal steroidogenesis, and gonadal steroids exert feedback effects on the brain-pituitary-gonad axis (Kalra and Kalra, 1983; Karsch, 1987; Pavgi and Licht, 1989; El Halawani

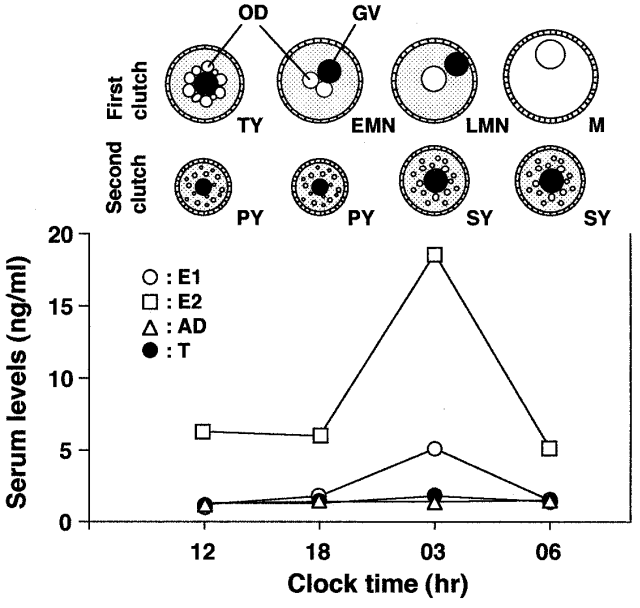

Fig. 5. Diagrammatic representation of diurnal changes of serum levels of E1, E2, AD, and T. PY, primary yolk stage; SY, secondary yolk stage; TY, tertiary yolk stage; EMN, early migratory nucleus stage; LMN, late migratory nucleus stage; M, mature stage; OD, oil droplet; GV, germinal vesicle.

et al., 1993; Tsai *et al*., 1994). In female teleosts, both serum E2 and T are elevated during vitellogenesis, which exerts negative or positive effects on GtH synthesis and secretion (reviewed in Linard *et al*., 1995; Schultz *et al*., 1995; Trudeau and Peter, 1995). In bambooleaf wrasse, however, serum T levels did not change throughout a day of spawning season, while serum E2 levels did. In contrast, serum E1 levels exhibited a diurnal change that paralleled E2. These results indicate that the potential of E1 feedback in female bambooleaf wrasse remains to be investigated.

Serum T levels were constant throughout a day of spawning season, and the maximum level was 10.9- and 3.0-fold lower than that of E2 and E1, respectively. These T levels are the result of secretion from extra-follicular tissues, because ovarian follicles do not produce T *in vitro*. This hypothesis is not contradicted by another study, which revealed that blood T does not disappear completely after castration in various species (Borg, 1994). In vertebrates, androgens play a variety of roles in reproductive and non-reproductive functions in both sexes, such as neuronal growth, muscle and bone development, immune reactions, epidermal gland development and function, and somatostatin release (reviewed by Staub and De Beer, 1997). Therefore, the low, stable serum T levels in the bambooleaf wrasse may reflect that T is produced in other organs or tissue, where it plays physiological roles other than oogenesis. In addition, T was synthesized from AD *in vitro* by head kidney and skin of rainbow trout *Salmo gairdneri* (Arai *et al*., 1969; Hay *et al*., 1976). In bambooleaf wrasse, AD was a precursor of E2 via E1 in the ovarian follicles. Unexpectedly, AD serum levels did not correlate with those of E1 or E2, and AD levels were constant (1.23–1.43 ng/ml) and similar to T serum levels. These constant levels of AD may result from rapid conversion of AD to E1 before its release into circulation from the follicles.

The difference in the steroidogenic pathway between species is probably due to differences in the substrate specificity of steroidogenic enzymes. In the ovarian follicles of the bambooleaf wrasse, aromatase exclusively catalyzed the synthesis of E1 from AD, suggesting that in this species aromatase has high substrate specificity for AD. This process is distinct from that of salmonids, goldfish, guppy, and medaka, whose aromatases usually convert T to E2 (Kagawa *et al*., 1984, 1985; Venkatesh *et al*., 1992; Kobayashi *et al*., 1996). The specificity of 17β-HSD activity is also different. In the ovarian follicles of the bambooleaf wrasse, 17β-HSD converts E1 to E2, while in the ovarian follicles of the other species that have been investigated 17β-HSD converts AD to T. Interestingly, the testes of the secondary male, which is the terminal phase of female bambooleaf wrasse, produce 11-tetotestosterone (11-KT), an androgen that is active in teleost spermatogenesis (Miura *et al*., 1994), and this steroid is likely metabolized from T (Matsuyama *et al*., 1995). Although the steroidogenic pathway to 11-KT in the testes of secondary males has not been completely determined, the production of 11-KT suggests the presence of another type of 17β-HSD, which may convert AD to T, or DHEA to androst-5-ene-3β,17β-diol (androstenediol). In this case, different types of 17β-HSD might be expressed time- (or phase-) specifically over the life of a fish. In mammals, nine 17β-HSD isoenzymes have been described and analyzed to date (Su *et al*., 1999). They differ in tissue distribution, kinetic parameters, substrate specificity, and preferred direction of conversion, reductive or oxidative. In humans, 17β-HSD type 1 is highly expressed in the ovary (Ghersevich *et al*., 1994; Sawetawan *et al*., 1994) and placenta (Maentausta *et al*., 1990), and catalyzes the reduction of E1 to E2. In contrast, 17β-HSD type 3, which converts AD to T, is predominantly expressed in the testes (Geissler *et al*., 1994). Although the diversity of 17β-HSD in teleosts is unknown, in the bambooleaf wrasse, distinct types of 17β-HSD could exist in the ovarian follicles and in the testes of secondary males. In order to verify this hypothesis, studies on molecular cloning and enzymatic characterization of 17β-HSD(s) in bambooleaf wrasse are underway. More recently, 17β-HSD type 1 cDNA was cloned from the ovary of the Japanese eel, suggesting that E2 would be produced via E1 in this species (Kazeto *et al*., 2000a).

This study and our previous work (Ohta and Matsuyama, 2001) completely describe the steroidogenic pathways in the ovarian follicles of the bambooleaf wrasse during vitellogenesis and oocyte maturation. During the FOM of this species, production of E2 decreases to zero, while two kinds of 20β-hydroxyprogestins, 17,20β-P and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), are produced by the follicles, and play a role as maturation-inducing hormone (MIH) (Matsuyama *et al*., 1998a, 1998b; Ohta and Matsuyama, 2001). The steroidogenic pathways to E2 and 20β-hydroxyprogestins branch from 17-P5. As reported in our previous study (Ohta and Matsuyama, 2001), production of E1, AD, and DHEA drastically decreases during FOM, indicating that the decrease in E2 is due mainly to a decrease in C17,20-lyase activity, which converts 17-P5 to DHEA. The substrate specificity of C17,20-lyase differs between species. C17,20-lyase in the ovarian follicles of medaka catalyzes 17-P to AD (Kobayashi *et al*., 1996). Recent molecular cloning and characterization of ovarian P450c17 cDNA in the rainbow trout *Oncorhynchus mykiss* (Sakai *et al*., 1992) and the Japanese eel *Anguilla japonica* (Kazeto *et al*., 2000b) revealed that eel C17,20-lyase P450c17 converts both 17-P5 and 17-P, while rainbow trout P450c17 favors conversion of 17-P5. These studies highly suggest that the mechanism of steroidogenic shift is different among species. Future study, utilizing molecular cloning and characterization of each enzyme cDNA, is necessary to understand the regulatory mechanisms of the steroidogenic shift in the ovarian follicles of the bambooleaf wrasse.

In conclusion, this study of the bambooleaf wrasse clearly reveals the steroidogenic pathway of E2 synthesis by the ovarian follicles, and is the first to report that E1, not T, is an intermediate in E2 production in a teleost. E1, not T, production in the ovary of bambooleaf wrasse may relate to following sex change in the life. Treatment of T and/or 11-KT induce body color change and/or gonadal reversal in protogynous wrasses (Reinboth. 1975; Kramer *et al*., 1988; Grober *et al*., 1991),

suggesting that no gonadal production and low blood levels of T during female phase may be essential to maintain the morphology, behavior and function as female. However, there is little information on E1 synthesis in the teleost ovary including sex reversal species, and its physiological role in fish gametogenesis is almost unknown. Studies on the distribution of E1 and of its function in different species may provide insights into the physiological diversity of steroid hormones in fish gametogenesis.

ACKNOWLEDGMENTS

We extend our sincere thanks to Captain Y. Shichida of the research vessel Wakasugi for providing generous support during fish sampling, and to the staff of the Fishery Research Laboratory of Kyushu University for assistance during the experiments.

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(Received April 16, 2001 / Accepted June 21, 2001)