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Steroid Hormones and their Synthetic Pathways in the Vitellogenic Ovarian Follicles of Yellowtail, *Seriola quinqueradiata*

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In the present study, we clarified the steroid hormones produced and their synthetic pathways, particularly focusing the estradiol–17β (E2) synthesis, in the vitellogenic ovarian follicles to provide information on the endocrine control of vitellogenesis in yellowtail, *Seriola quinqueradiata*. Intact vitellogenic ovarian follicles were isolated and incubated with radioactive [^{3}H]pregnenolone and [^{14}C]androstenedione and steroid metabolites were identified by thin layer chromatography (TLC) followed by recrystallization to constant specific activity. Results obtained clearly indicated that testosterone (T) is the substrate precursor of E2 synthesis. In the vitellogenic ovarian follicles, the steroid metabolites produced and major pathway followed were pregnenolone, 17–hydroxypregnenolone, dehydroepiandrosterone, androstenedione (AD), T and E2. AD, T and E2 were also present in the serum of fish during vitellogenesis, and serum level of T was the highest (3.7 ng/ml), followed by E2 (2.3 ng/ml). Thus, this study demonstrated the complete steroidogenic pathway of E2 synthesis in the ovarian follicles of yellowtail, and revealed that T is the major precursor of E2.

INTRODUCTION

In Japanese aquaculture, the total production of yellowtail, *Seriola quinqueradiata*, has always contributed a major share of the marine finfish production, approximately amounting to 140,000 tons per year. Despite the great importance of this species in marine finfish aquaculture, all juveniles needed have been caught from the wild stock due to difficulties in obtaining fertilized eggs under the captive conditions. However, the catch of wild juvenile is unreliable and unpredictable, in fact, for several years culture practices have been seriously affected due to a decrease in catch of wild seedstock. Therefore, a stable supply of yellowtail juveniles is highly desirable. Like hatchery–reared broodstock of many commercial fish species (Zohar and Mylonas, 2001), female Japanese yellowtail are unable to complete their reproductive cycle in captivity. The major endocrinological dysfunction involves lack of the pituitary gonadotropin (GtH) secretion responsible for inducing final oocyte maturation (FOM) through producing the maturation–inducing hormone (MIH) in the follicular cells after the completion of vitellogenesis. The use of human chorionic gonadotropin (HCG) to induce FOM in captive Japanese yellowtail (Matsuyama *et al.*, 1996) has been reported. More recently, we succeeded in inducing maturation and artificial fertilization of cultured yellowtail by

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means of single injection of HCG (Chuda *et al.*, 2001a, b). In these studies, we obtained the quality fertilized eggs from the cultured broodstock two months prior to natural spawning season by HCG treatment combined with high water temperature and long photoperiod. For industrialization of yellowtail aquaculture, however, a steady supply of fish seed produced by off–season spawning should be established. To ensure and stabilize the seed production of yellowtail by off–season spawning, detail information of endocrine kinetics regulating the oocyte growth and maturation, especially endocrine mechanism that regulates onset of vitellogenesis or puberty, is needed.

Ovarian development and recrudescence in teleost are regulated by pituitary GtH through the production of ovarian steroid hormones. During oocyte vitellogenesis, estradiol–17β (E2) is synthesized by the follicular layer and stimulates the hepatic production of vitellogenin as a yolk precursor (reviewed by Specker and Sullivan 1994; Tyler and Sumpter 1996). To date, in teleost species many studies have been performed on the E2 production *in vivo* and *in vitro*. Results obtained from these studies implicate that testosterone (T) is a substrate precursor of E2 in most teleost species. Recently, it has been shown that in teleost females serum E2 and T exert negative and/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 some important roles in endocrine control of oogenesis in many teleost species. However, determination of E2 synthetic pathway in ovarian follicles has been conducted in a limited number of species. Of the species studied, the complete steroidogenic pathway from pregnenolone (P5) to E2 has been determined only in medaka, *Oryzias latipes*, where E2 is converted from T (Kobayashi *et al.*, 1996). More recently, we demonstrated the complete steroidogenic pathway in the ovarian follicles of bambooleaf wrasse, *Pseudolabrus sieboldi*, and red seabream, *Pagrus major*, in which we revealed that E2 is synthesized via estrone (E1), but not via T (Ohta *et al*, 2001a, b). These findings indicate that steroidogenic pathway for E2 production differs among teleost species, and further suggest a possibility that intermediate product(s) to E2 synthesis may play a role in feedback control for GtH synthesis and secretion.

In the present study, we clarified the biosynthesis of steroid hormones and their synthetic pathways, particularly focusing the E2 synthesis, in the vitellogenic ovarian follicles to provide information on the endocrine control of vitellogenesis in yellowtail. Additionally, serum levels of some important steroid hormones including E2 were assayed.

MATERIALS AND METHODS

Hormones and chemicals

Radioactive [7–^{3}H(N)]pregnenolone (P5, 780.7 GBq/mmol) and [4–^{14}C]androstenedione (AD, 2.0 GBq/mmol) were purchased from New England Nuclear (Boston, MA). The radiochemical purity of these steroids was repeatedly checked by TLC with two solvent systems immediately before use. Unlabeled steroids were obtained from either Sigma (St Louis, Mo) or Steraloids Inc. (Wilton, NH). Coenzymes, reagents and solvents were obtained from either Sigma or Wako (Tokyo, Japan).

Animals

Four-year-old male and female yellowtail (7.7–10.0 kg) were obtained from broodstock maintained at the sea pens of Nagasaki Prefectural Institute of Fisheries, Japan. Prior to the initiation of experiment, fish were moved to the concrete tank of this farm circulated with running sea water under a photoperiod of 18L: 6D at 18–19 °C during their spawning season in April, 1999. After anesthetizing with 2-phenoxyethanol (300 ppm), four females which had ovaries with vitellogenic oocytes of 680–720 μm in diameter were selected by ovarian biopsy. Blood samples were collected from the caudal vessel using syringes fitted with 18-gauge needles and centrifuged at 3,000 rpm for 20 min. The separated serum was stored at -30 °C until assayed for steroid level. Fish were sacrificed by decapitation and ovaries were removed. 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 stage of oocytes collected from four fish was late vitellogenic stage. Classification of oocyte stage of yellowtail has been previously described (Rahman *et al.*, 2001). For TLC samples, ovaries were placed in a Petri dish containing ice-cold Leibovitz's L-15 culture medium (GIBCO, Rockville, Maryland), buffered with 0.02 M HEPES, pH 7.6. Ovaries were then cut into small fragments and placed into small Petri dishes containing fresh cold medium. After separating the intact oocytes by forceps, twenty samples, each containing 200 oocytes collected from the most advanced group of oocytes, were frozen with liquid nitrogen and stored at -80 °C until use.

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

Ovarian follicles (200) were incubated with [7-^{3}H(N)]P5 (780.7 GBq/mmol, 58×10^{4} dpm) and [4-^{14}C]AD (2.0 GBq/mmol, 1.2×10^{4}dpm), 10 mM NAD, NADH, NADP and NADPH in 1.3 ml incubation buffer (250 mM sucrose, 20mM HEPES, with pH 7.5 adjusted by NaOH) at 18 °C for 2 h with constant shaking. At the end of incubation, steroids were extracted three times from the incubation mixture with 4 vol. of dichloromethane. The extracted solution was concentrated and applied on a glass TLC plate (Merck) with non-radioactive standard steroids, progesterone (P), AD, 17-hydroxyprogesterone (17-P), T, E1, E2, 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-P), 17, 20α-dihydroxy-4-pregnen-3-one (17, 20α-P) and 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), and developed in benzene : acetone (4:1) system. Standard (4-pregnene) steroids were detected with ultraviolet light (UV absorption at 254 nm). 5-Pregnene and reduced steroids were made visible by spraying the TLC plate with antimony trichloride in glacial acetic acid (1:1 v/v) and then heating at 95 °C for 10 min. Radioactive steroids were detected by autoradiography using a BAS 1000 bio-imaging analyzer (Fuji Film). The radioactive bands thus detected were scraped off the plate and extracted three times from the silica gel with 3 ml of diethyl ether. Steroid metabolites were identified by their chromatographic mobilities on TLC developed with three solvent systems using different combinations of solvent systems like benzene : acetone (4:1 by vol.), benzene : chloroform : diethyl ether : methanol (2:2:1:1 by vol.), chloroform: ethyl acetate (2:1 by vol.), and benzene : methanol (9:1 by vol.). Metabolites were confirmed by recrystallization to constant specific activity (Axelrod *et al.*, 1965). Eight milligrams of carrier steroids were

used for recrystallization, except for 17–hydroxypregnenolone (17–P5). Due to low solubility in solvents, only 2 mg of 17–P5 was used. Radioactivity was measured by a scintillation counter (Beckman LS–6500). There was a little difference in metabolite patterns of follicles sampled from four fish, therefore, we used follicles derived from one.

Steroid hormone measurement

Steroid hormones in serum were extracted two times with 10 volume of diethyl ether, the extracts redissolved and the steroid hormones measured by ELISA. Serum E2 and AD were measured by Enzyme Immunoassay Kits developed by Cayman Chemical (MI) and Oxford Biomedical Research Inc. (MI) respectively. The ELISA for T were as described by Ohta *et al.*, (2001a).

Statistics

Serum steroid hormone levels of E2, T and AD were represented by mean ± SEM (n=4), compared and analyzed by one–way ANOVA, followed by Tukey–Kramer test.

RESULTS

Identification of radioactive metabolites

After incubating late vitellogenic oocytes with [^{3}H]P5, six steroid hormones, including

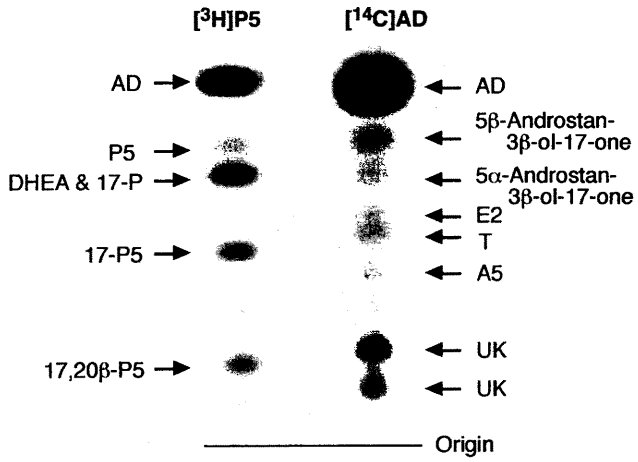

Fig. 1. Autoradiograms of steroid metabolites incubated with [^{3}H]pregnenolone and [^{14}C]androstenedione from the vitellogenic ovarian follicles of yellowtail. After 2 h incubation, steroids metabolites were extracted from media and follicular tissues and separated by TLC with benzene : acetone (4:1) solvent system. Autoradiogram of metabolite DHEA scraped from TLC plate and further rechromatographed on TLC and separated into two metabolites DHEA and 17–hydroxyprogesterone with chloroform : ethyl acetate (2:1) solvent system. AD, androstenedione; P5, pregnenolone; DHEA, dehydroepiandrosterone; 17–P, 17–hydroxyprogesterone; 17–P5, 17–hydroxypregnenolone; 17, 20β–P5, 5–pregnene–3β, 17, 20β–triol; E2, estradiol–17β; T, testosterone; A5, androstenediol; UK, unknown.

Table 1. Steroid metabolites and specific activity of crystals

Metabolites	Specific activity of crystals (cpm mg^{-1})				
	1st	2nd	3rd	4th	5th
Androstenedione (AD)	408	376	395	393	310
Pregnenolone (P5)	112	114	114	107	112
17–Hydroxyprogesterone (17–P)	135	128	128	127	102
17–Hydroxypregnenolone (17–P5)	671	650	583	688	689
Testosterone (T)	21	30	20	20	20

Solvent pairs: 1st, dichloromethane & n–heptane; 2nd, dichloromethane & n–hexane; 3rd, chloroform & n–heptane; 4th, chloroform & n–hexane; 5th, ethanol & water.

Table 2. Activity of metabolites recovered from yellowtail ovarian follicles incubated with [^{3}H]P5 and [^{14}C]AD during vitellogenesis

Metabolites	Activity (dpm)	
	[^{3}H]P5	[^{14}C]AD
Androstenedione (AD)	36,000	56,850
Pregnenolone (P5)	4,050	ND
Dehydroepiandrosterone (DHEA)	14,500	ND
17–Hydroxyprogesteone (17–P)	3,511	ND
17–Hydroxypregnenolone (17–P5)	5,650	ND
Testosterone (T)	ND	2,400
Androstenediol (A5)	ND	547
Estradiol–17β (E2)	ND	740
5β–Androstan–3β–ol–17–one	ND	3,073
5α–Androstan–3β–ol–17–one	ND	2,420
5–Pregnene–3β, 17, 20β–triol (17, 20β–P5)	6,700	ND

ND, not detectable

four C21 steroids and two C19 steroids, could be detected (Fig. 1). During incubation, the steroid metabolites produced were AD, 17–P, P5 (precursor), dehydroepiandrosterone (DHEA), 17–P5 and 5–pregnene–3β, 17, 20β–triol (17, 20β–P5). During incubation with [^{14}C]AD, nine steroid bands were produced. Among the bands, only six were identified. The metabolites were AD, 5β–androstan–3β–ol–17–one, 5α–androstan–3β–ol–17–one, E2, T and androstenediol (A5). Due to isopolarity of DHEA and 17–P on TLC in benzene : acetone solvent system, these two steroids were separated using the chloroform : ethyl acetate (2:1) system. Identity of five metabolites was confirmed by recrystallization to constant specific activity (Table 1). Identifications of E2 and A5 were confirmed only by their chromatographic mobilities on TLC using a combination of solvent systems (benzene : acetone=4:1; chloroform : ethyl acetate=2:1; benzene : methanol=9:1). It was not possible to carry out their recrystallization due to lack of required radioactivity.

Of the major metabolites identified during incubation, AD showed the highest intensity for synthesis, followed by DHEA, 17–P5, P5, 17–P and T, whereas the lowest

intensity was found in the synthesis of E2 and A5 (Table 2).

Serum steroid hormone levels

E2 and its intermediate metabolites, AD and T, produced in the vitellogenic ovarian follicles of yelowtail *in vitro* were also present in the blood. Mean and SEM of serum E2, T and AD levels of four fish were 2.3±1.2 ng/ml, 3.7±1.3 ng/ml and 1.0±0.1 ng/ml, respectively (Fig. 2). The mean value of T was the highest, followed by E2 and AD ($P<0.05$).

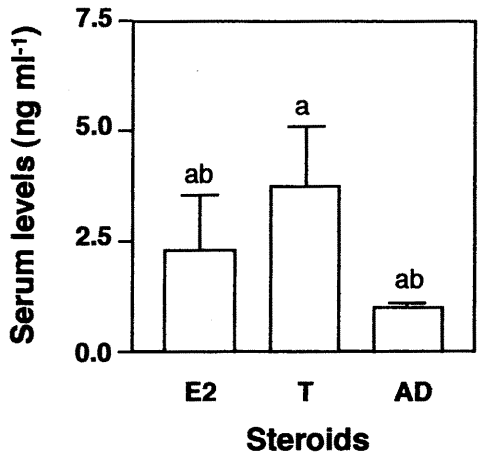

Fig. 2. Serum levels of E2, T and AD in female yellowtail during vitellogenesis. Each value is the mean±SEM (n=4). Statistically significant differences between groups are shown by different letters ($P<0.05$).

DISCUSSION

When vitellogenic follicles of yellowtail were incubated with [^{3}H]P5, the major metabolites were P5, 17–P5, 17–P, DHEA and AD. Progesterone (P) was not found in any incubate. These results indicate that there are two possible pathways from 17–P5 to AD production in the vitellogenic ovarian follicles of yellowtail, via 17–P or DHEA. The biosynthetic intensity of DHEA, however, was 4–times greater than that of 17–P (Table 2), it is likely that pathway mainly followed through DHEA to AD. When [^{14}C]AD was used as precursor, some metabolites including E2, T and A5 were produced. This result clearly indicates that AD was converted into E2 via T. Small production of A5 may be due to the reversible reaction by 3β–hydroxysteroid dehydrogenase (3β–HSD) from T to A5 in the *in vitro* condition. Thus, the present study identified all the steroid hormones produced and demonstrated that E2 was synthesized through a major pathway from P5 via 17–P5, DHEA, AD and T (Fig. 3).

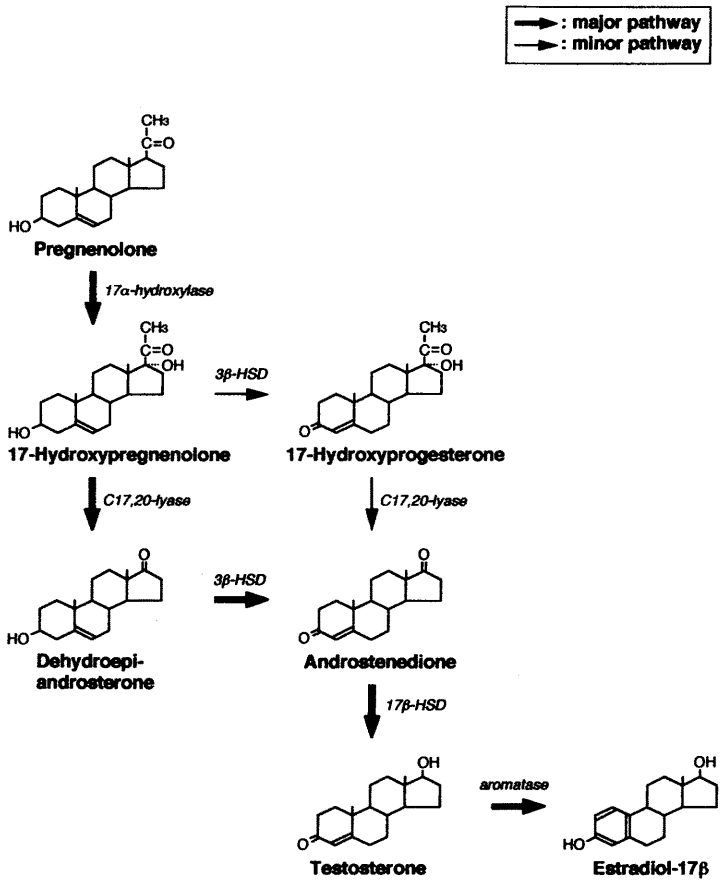

Fig. 3. Steroidogenic pathway of estradiol-17β biosynthesis in yellowtail ovarian follicles during the vitellogenic stage.

Of the teleost species studied, the complete steroidogenic pathway from pregnenolone (P5) to E2 has been determined only in three species, medaka, bambooleaf wrasse and red seabream. In the vitellogenic ovarian follicles of medaka, substrate precursor of E2 is T, although AD is converted from 17–P (Kobayashi *et al.*, 1996). In contrast, E2 is synthesized via E1, not via T, in the vitellogenic ovarian follicles of bambooleaf wrasse (Ohta *et al.*, 2001a) and red seabream (Ohta *et al.*, 2001b). Both medaka and yellowtail are gonochoristic species, while bambooleaf wrasse and red seabream have a bisexual period in their life. The bambooleaf wrasse exhibits diandric protogyny; functional females change the sex to secondary males with terminal–phase appearance (Nakazono, 1979). The sexual pattern of the red seabream is gonochorism with a bisexual juvenile stage; testes originate from the underdeveloped ovary via a bisexual gonad in the juvenile stage (Matsuyama *et al.*, 1988).

In general, gonadal steroid hormones are likely to play an important role in sex change. In protogynous species, androgen treatments have generally been shown to be effective in inducing female–to–male inversions. In protogynous wrasses, treatments of T and/or 11–ketotestosterone (11–KT, an active androgen in teleost male) induce body color change and/or gonadal reversal (Reinboth, 1975; Kramer *et al.*, 1988; Grober *et al.*, 1991). Treatments with synthetic, aromatizable 17α–methyltestosterone (MT) have been shown to be effective in different species of groupers (Baroiller *et al.*, 1999). These facts suggest a possibility that low or no production of T in the ovarian follicles in the fish like bambooleaf wrasse and red seabream may be related to sex reversal in their life cycles. Ovarian T production that exceeds a certain threshold may affect the maintenance of ovarian structure and function in the female phase of these species.

Recently, it has been shown that in teleost female serum E2 and T exert negative and/or positive feedback effects on GtH synthesis and secretion (reviewed in Linard *et al.*, 1995; Schultz *et al.*, 1995; Trudeau and Peter, 1995). It is unknown, however, whether the action of T is direct or indirect via conversion to E2 or other androgenic metabolites. The present study demonstrated that the substrate precursor of E2 is T in the vitellogenic ovarian follicles of yellowtail. Moreover, considerable levels of E2 and T were confirmed in the blood in which T levels were higher than that of E2. Therefore, also in yellowtail, E2 and T may exert negative and/or positive feedback effects on GtH synthesis and secretion as shown in some teleost species. Future study is necessary to examine whether any feedback effects of T on the brain–pituitary–gonad axis of yellowtail are present. If so, characterization of androgen receptor and the possibility of T conversion into other steroids in brain and pituitary have to be conducted in order to clarify whether the effect of T is direct or indirect. Molecular cloning of GtH–I and -II of yellowtail is underway.

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