# Maturation-inducing Hormone of the Tiger Puffer, Takifugu rubripes (Tetraodontidae, Teleostei): Biosynthesis of Steroids by the Ovaries and the Relative Effectiveness of Steroid Metabolites for Germinal Vesicle Breakdown In Vitro

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# Maturation-inducing Hormone of the Tiger Puffer, *Takifugu rubripes* (Tetraodontidae, Teleostei): Biosynthesis of Steroids by the Ovaries and the Relative Effectiveness of Steroid Metabolites for Germinal Vesicle Breakdown *In Vitro*

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**ABSTRACT**—Yolk-free follicle cells during final meiotic maturation were incubated with a radioactively labeled steroid precursor, and the maturation-inducing activity of the steroid metabolites produced was tested in an *in vitro* assay based on GVBD. Of the metabolites obtained by TLC, whose purity and final characterization were confirmed by recrystallization to constant specific activity, two steroids, 17,20β-P and 20β-S, exhibited maturation-inducing activity *in vitro*. However, 20β-S was overwhelmingly more effective and faster at inducing GVBD *in vitro* than 17,20β-P. During final oocyte maturation in the tiger puffer, a shift in steroidogenic enzymes, a decrease in C17,20-lyase activity, and an increase in 20β-HSD activity, were found, as previously reported in salmonids and medaka. In addition to this shift in steroidogenic enzyme activity, however, high and increasing 21-hydroxylase activity throughout all the oocyte developmental stages was a distinctive feature in tiger puffer follicles. This 21-hydroxylase activity likely enables ovarian follicles to accumulate enough 11-deoxycortisol during vitellogenesis for 20β-S production on a massive scale during GVBD. Thus, this study provides not only evidence on the physiological role of 20β-S as a MIH in the tiger puffer, but also fact on enzymatic kinetics in 20β-S biosynthesis.

## INTRODUCTION

The primary hormone involved in the induction of oocyte maturation in vertebrates is gonadotropin, which is released from the pituitary gland. However, it is generally accepted that in lower vertebrates, such as teleosts and amphibians, the action of gonadotropin is not direct, but is mediated through the synthesis of a maturation-inducing hormone (MIH) by the ovarian follicles (Goetz, 1983; Masui and Clarke, 1979; Nagahama, 1987a). Oocytes respond directly to MIH by undergoing germinal vesicle breakdown (GVBD), a readily discernible step that can be used to determine the reinitiation of meiosis (reviewed by Nagahama, 1987b).

In teleosts, a number of $C_{21}$-steroids, such as progesterone and its derivatives, induce GVBD *in vitro*, but the potency of each steroid varies with the oocyte donor species, and is very dependent on the positions of specific functional groups on the steroid nucleus (Scott and Canario, 1987). Several investigations have indicated that ovarian tissue can synthesize a wide range of biologically active reduced and conjugated steroid metabolites around the time of oocyte maturation. However, naturally occurring MIH has not been conclusively purified and characterized. Two progestins have been identified as MIH in teleosts: 17α,20β-dihydroxy-4-pregnen-3-one and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S). 17α,20β-Dihydroxy-4-pregnen-3-one has been abbreviated to various terms such as 17α,20β-DP, DHP, or 17,20β-P by different authors. In this study, we use "17,20β-P" for 17 α,20β-dihydroxy-4-pregnen-3-one. The only definitive reports have been those on 17,20β-P as the MIH in amago salmon *Oncorhynchus rhodurus* (Nagahama and Adachi, 1985), Indian catfish *Clarias batrachus* (Haider and Rao, 1992), *killifish Fundulus heteroclitus* (Petrino *et al*, 1993), and medaka *Olyzias latipes* (Fukada *et al*., 1994), and on 20β-S as the MIH in Atlantic croaker *Micropogonius undulatus* (Trant *et al*., 1986; Trant and Thomas 1989), spotted seatrout *Cynoscion nebulosus* (Thomas and Trant, 1989; Patino and Thomas,

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1990), and striped bass *Morone saxatilis* (King *et al*., 1997).

In spite of several studies on MIH in teleosts, there is no available information on MIH in pufferfish (Order Tetraodontiformes, Family Tetraodontidae). Systematically, the order Tetraodontiformes is a rather isolated group that is divided into two suborders, the Triacanthoidei and Tetraodontoidei, with the latter comprised of four distinct families: the Tetraodontidae, Triodontidae, Diodontidae, and Molidae (Nelson, 1994). Recently, puffers have been used as a new model for genome studies because of their compact genome (Brenner *et al*., 1993; Crnogorac-Jurcevic *et al*., 1997).

This study was designed to obtain direct evidence for the true MIH of the tiger puffer, *Takifugu rubripes*. To this end, the following investigations have been made in the present study: (1) thin layer chromatography (TLC) analysis for radioactive metabolites of $^{14}$C-labeled 17α-hydroxyprogesterone (17-P) incubated with yolk-free follicle cells at four different stages of oocyte development, and (2) identification of steroid metabolites and their ability to induce GVBD in oocytes *in vitro*. In addition, in order to elucidate details of kinetics and pathways of MIH production relative to GVBD in tiger puffer, *in vitro* production of MIH by follicle-enclosed oocytes at three different developmental stages incubated with radioinert steroid precursor was performed. This study provides evidence that 20β-S is the naturally occurring MIH in tiger puffer.

## MATERIALS AND METHODS

### Animals

Three-year-old female tiger puffers (1.8–2.5 kg in body weight) were kept in square indoor tanks (10 tons) with running seawater, and fed a diet of scomber, sand eel, or blue sprat flesh daily. Since the wild stock of tiger puffer spawns between April and May around Kyushu, the photoperiod and water temperature were maintained at 14L:10D and 18°C, respectively between December 1995 and February 1996, for acceleration of vitellogenesis. Although cultured females commonly have ovaries that contain fully developed oocytes just prior to final oocyte maturation, the oocytes will not mature spontaneously in captivity. Therefore, a cholesterol pellet containing luteinizing hormone releasing hormone analog (LHRH-a 400 μg/kg body weight) was inserted into the dorsal muscle of the female fish according to Chuda *et al*. (1997) to induce final oocyte maturation by continuously releasing LHRH-a (des-Gly$^{10}$-[D-Ala$^{6}$] LHRH ethylamide, Sigma), which induces the discharge of innate gonadotropin from pituitary gonadotrophs. The present study was performed in February 1996. The egg diameter of each fish was monitored at intervals of several days by aspirating a small piece of ovarian tissue into a polyethylene tube (1.5 mm in diameter) introduced through the oviduct. During the spawning season, the tiger puffer ovary contains two clutches of oocytes; the majority of the ovarian oocytes are developing (vitellogenic) and there are a few residual pre-vitellogenic oocytes. After separating individual oocytes from the ovarian tissue, the diameter of ten developing oocytes was measured, and the remaining oocytes were fixed with Bouin's solution to investigate the developmental stage histologically.

### Developmental stage of maturing oocytes

Histologically, the morphological events during final oocyte maturation in LHRH-a-stimulated tiger puffer ovaries can be described as follows: I) actively vitellogenic oocytes (from fish not injected with LHRH-a) with a central germinal vesicle at the secondary yolk (SY) stage, 750–850 μm in diameter; II) fully grown oocytes (from fish not injected with LHRH-a) with a central germinal vesicle at the tertiary yolk (TY) stage, 840–910 μm in diameter; III) the germinal vesicle migration (GVM) stage, with a germinal vesicle located between the center of the oocyte and the animal pole, 900–950 μm in diameter; and IV) the GVBD stage with a markedly hydrated cytoplasm and no germinal vesicle, 940–1,000 μm in diameter.

### Chemicals

The 20β-S was purchased from Steraloids, Inc. All the other unlabeled steroids, enzymes, LHRH-a, and other reagents were obtained from Sigma or Wako Chemicals Co. Rabbit anti-steroid hormone antibodies, and steroid hormones labeled with horseradish peroxidase were purchased from Cosmo-Bio. Radioactive 17-P was enzymatically derived from [4-$^{14}$C]-progesterone (NEN Research Products) using medaka, *Olyzias latipes*, P-450c17, as described below.

### Preparation of radioactive 17-P

The nucleotide sequence surrounding the first methionine of the cDNA was modified from CGATGG to CCATGG to generate an *Nco*I recognition sequence using conventional PCR. The modified cDNA sequences were confirmed by sequencing. P-450c17 cDNAs containing *Nco*I and *Xho*I were digested and ligated into the *Nco*I and *Xho*I sites of the pET15b expression vector (Novagen). *E. coli* BL21(DE3) was then transformed with the constructs and cultured in 40 ml of Luria-Bertani medium containing 200 μg/ml ampicillin. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of P-450c17. After a 3-hr incubation with agitation at 37°C, the bacteria were harvested, washed with 10 ml MOPS buffer (50 mM MOPS [3-(*N*-morpholino) propanesulfonic acid], 100 mM KCl, 1 mM EDTA and 1 mM dithiothreitol, pH 7.5), centrifuged, and resuspended in 5 ml of MOPS buffer. [4-$^{14}$C]-Progesterone was added to the resuspended cells, which were gently shaken at 28°C (Barnes *et al*., 1993). Metabolized steroids were extracted, fractionated, and identified by two-dimensional thin layer chromatography (TLC, Merck), with benzene:acetone (4:1, v/v) followed by cyclohexane:ethylacetate (1:1). Unlabeled progesterone, 17-P, androstenedione, testosterone, and 17,20β-P served as mobility markers and were detected under UV light.

### Ovarian follicle incubation for TLC

At the appropriate stage of development, the fish were killed by decapitation and their ovaries were removed and placed in Ringer's solution (135 mM NaCl, 2.4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $NaHCO_3$, 0.5 mM $NaH_2PO_4 \cdot 2H_2O$) at 4°C. Fragments of ovarian lamella were treated according to the experimental objectives. Follicle cell layers were obtained by squeezing the yolk from 200 mg (±0.2 mg) ovarian fragments in 1 ml Ringer's solution. After centrifugation at 3,000 rpm for 15 min, isolated follicle layers (mainly the follicle layers from the predominate clutch of oocytes, with some tissue from the small clutch of oocytes and ovarian stroma) were frozen in 1 ml buffer (250 mM sucrose, 1 mM EDTA, 20 mM HEPES, 0.2 mM p-ABSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], pH adjusted to 7.5 with NaOH) with liquid $N_2$, and stored at –80°C until use. The incubation mixture contained isolated follicle layers, $^{14}$C-17-P (18.5 GBq/m mol, $1.0\times10^5$ cpm), 10 mM NAD, NADH, NADP, and NADPH in 1.3 ml incubation buffer (250 mM sucrose, 20 mM HEPES, pH 7.5 with NaOH). The mixture was incubated at 16°C for 2 hr with constant shaking.

### Analysis of metabolites

Steroids were extracted from the incubation mixture with 8 volumes of $CHCl_3$ three times. The extracted solution was concentrated and applied to a TLC glass plate (Merck) with non-radioactive standard steroids (testosterone, androstenedione, progesterone, 17-P, 17α,20α-dihydroxy-4-pregnen-3-one, 17,20β-P, and 20β-S) and developed in a benzene:acetone (4:1, v/v) system. Radioactive ste-

**Table 1.** Steroid metabolites and solvent systems used for identification by their chromatographic mobilities

| Metabolite | Solvent system | | |
|---|---|---|---|
| | 1st | 2nd | 3rd |
| *17α,20β,21-trihydroxy-4-pregnen-3-one | (A) | (B) | (C) |
| *17α,20β-dihydroxy-4-pregnen-3-one | (A) | (C) | – |
| *11-deoxycortsol | (A) | (C) | – |
| *17α,20α-dihydroxy-4-pregnen-3-one | (A) | (B) | (C) |
| *androstenedione | (A) | (B) | (C) |
| testosterone | (A) | – | – |
| estradiol-17β | (A) | (D) | – |

*: further identified by recrystallization
(A) benzene: acetone=4:1, (B) chloroform: ethyl acetate=2:1, (C) benzene: chloroform: diethyl ether: methanol=2:2:1:1, and (D) benzene: methanol=9:1

roids were detected by autoradiography using a BAS 1000 bio-imaging analyzer (Fuji Film). Standard steroids were detected by UV absorption at 254 nm. Steroid metabolites were identified by their chromatographic mobilities in TLC using a combination of solvent systems (Table 1), followed by constant specific radioactivities of crystals after repeated crystallization of a radioactive metabolite with the corresponding authentic preparation termed as recrystallization. Recrystallization was performed according to Axelrod *et al.* (1965). The radioactive metabolites plus 10 mg of a steroid standard (chosen on the basis of identical mobility in TLC) were dissolved in a small volume of dichloromethane or chloroform in a sand bath at 42°C. After removing the tube from the sand bath, hexane or heptane was added to the saturated solution dropwise until crystals began to form. After centrifugation, the mother liquor was decanted into a second tube and the liquid was evaporated from both tubes under nitrogen. The tubes were then weighed in a Mettler M3 electrobalance. The crystals and mother liquor were redissolved in dichloromethane and an aliquot was removed for liquid scintillation counting. The dissolved crystals were recrystallized as described above. These procedures were repeated 4 times.

**Conversion of unlabeled precursors**

Fish were killed by decapitation, and their ovaries were removed and placed in ice-cold Leibovitz's L-15 culture medium (GIBCO), buffered with 0.02 M HEPES, pH adjusted to 7.5 with 1 N NaOH. Gentamycin sulfate (200 mg/l, Sigma) was added at the start of incubation. Ovarian fragments (40±1 mg) were placed in each well of a plastic tissue culture plate (24 wells, Falcon) containing 1 ml of medium in the presence or absence of 100 ng/ml of radioinert exogenous precursors of steroids, 17-P, 11-deoxycortisol or 17,20β-P. Steroids were dissolved in ethanol. Ten microliters of ethanol were added as control to some wells instead of the steroid hormones. The oocytes were incubated for 18 hr at 17°C in a temperature controlled incubator in an atmosphere of 100% air. All treatments were tested in triplicate. Following the incubation period, media were collected and stored at –20°C until examined by the ELISAs for 17,20β-P, 11-deoxycortisol and 20β-S.

**Measurement of steroids**

17,20β-P and 20β-S levels in serum and incubation medium were measured by specific ELISAs according to the methods of Matsuyama *et al.* (1998) for 17,20β-P and Asahina *et al.* (1995) for 20β-S. ELISA of 11-deoxycortisol used the same procedure and described below bliefly.

*Stock solutions*: Coating buffer, 0.05M carbonate buffer pH 8.4, containing 0.05% $NaN_3$; washing solution, 0.85% NaCl; blocking solution, 0.05M PBS containing 0.1% BSA, 3% sucrose, and 0.005% thimerosal; assay buffer, 0.05M borate buffer, pH 7.8, containing 0.1% BSA and 0.01% thimerosal; substrate solution, 0.2M citrate buffer, pH 4.5, containing 0.01% $H_2O_2$, added with 0.5% *o*-phenylenediamine immediately before use; stopping solution, 6N $H_2SO_4$. 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, CAPPEL, West Chester, PA). The plate was sealed and incubated at 4°C for 48 hr. After removal of unbound antiserum, 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*: Steroids in incubation medium and serum were extracted twice with 10 volumes of diethyl ether. The ether was evaporated, and the sample 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-11-deoxycortisol 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 antiserum was raised against 11-deoxycortisol-6β-Succinate-BSA. A steep standard curve covering 1.5–192 pg/well (30–3,840 pg/ml) for 11- deoxycortisol was obtained with the present ELISA (antiserum dilution, 1:100,000; labeled hormone dilution, 1:2,500). The intra- and interassay coefficients of variation were 7.1% (N=4, duplicate) and 14.5 % (N=4, duplicate)

**Table 2.** Cross reaction of various steroids measured in the specific enzyme immunoassay for 11-deoxycortisol

| Steroids* | Cross(%) |
|---|---|
| 11-Deoxycortisol | 100 |
| Cortisone | 1.00 |
| DOC | 0.75 |
| Cortisol | 0.35 |
| 6β-Hydroxycortisol | 0.25 |
| 17-P | 0.22 |
| Corticosterone | 0.12 |
| Progesterone | <0.10 |
| Pregnenolone | <0.01 |
| 17-P5 | <0.01 |
| DHEA | <0.01 |
| Testosterone | <0.01 |
| Androstenedione | <0.01 |
| Estradiol-17β | <0.01 |

*Abbreviations:
11-Deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione
Cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione
DOC, 21-hydroxy-4-pregnene-3,20-dione
Cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione
6β-Hydroxycortisol, 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione
17-P, 17α-hydroxy-4-pregnene-3,20-dione
Corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione
Progesterone, 4-pregnene-3,20-dione
Pregnenolone, 3β-hydroxy-5-pregnen-20-one
17-P5, 3β,17α-dihydroxy-5-pregnen-20-one
DHEA, 3β-hydroxy-5-androsten-17-one
Testosterone, 17β-hydroxy-4-androsten-3-one
Androstenedione, 4-androsten-3,17-dione
Estradiol-17β, 3,17β-dihydroxy-1,3,5(10)-estratriene

respectively, close to the 50% binding point. Competition curves for serum extract from tiger puffer were obtained. After logarithmic transformation, slope of regressions on each of the curve was confirmed to be parallel to the standard curve (ANCOVA. P>0.05). The sensitivity of this assay was 11.5 pg/ml. This antiserum cross-reacted with fewer than 1% of steroids other than 11-deoxycortisol (Table 2), and competition curves for serum collected from tiger puffer were almost parallel to the standard curve. These results indicate that the present ELISA is very specific for 11-deoxycortisol, and serum 11-deoxycortisol levels can be measured after simple collection.

### *In vitro* GVBD assay

The maturation-inducing activity of each metabolite was determined by an *in vitro* bioassay using radioinert steroids. One female fish, which had ovarian oocytes at the tertiary yolk stage, 890 μm in diameter, was injected intramuscularly with human chorionic gonadotropin (HCG, from Sankyo, 200 IU/kg body weight). This dose is sufficient to induce MIH receptors on the oocyte plasma membrane, but normally does not induce final oocyte maturation in tiger puffer females. Twenty-four hr after HCG injection, the fish was sacrificed and the ovary removed and placed in ice-cold Leibovitz's L-15 culture medium, buffered with 20 mM HEPES with the pH adjusted to 7.5 with NaOH. Gentamycin sulfate (200 mg/l) was added when the assay was set up. The ovary was cut into small pieces weighing approximately 20 mg, which were transferred separately to wells in a 24-well plastic dish (Sumitomo, Bakelite), each containing 1 ml of culture medium. Purchased radioinert steroids were dissolved in ethanol. Ten microliters of hormone solution were added to each well or 10 μl of ethanol was added as a control for the steroid hormones. The cultures were maintained for 12, 24, 36, and 48 hr at 16°C in a temperature-controlled incubator in air. All the treatments were tested in triplicate. In tiger puffer oocytes, clearing solution (ethanol : formalin : acetic acid = 6:3:1, v/v), which is commonly used for direct GVBD observation *in vitro* in teleosts, cannot effectively clear the cytoplasm because the chorion is too thick. Therefore, the response of each piece of ovary to different concentrations of hormones was judged histologically. After incubation, the ovarian fragments were fixed with Bouin's solution, dehydrated, and embedded in Technovit resin (Kulzer). For light microscopy, 4-μm-thick sections were cut and stained with 1% toluidine blue solution. Ten oocytes were selected randomly per ovarian fragment and scored for the occurrence of GVBD.

### Statistics

Values below the minimum assay sensitivity (30 pg/ml serum for steroid assays) were treated as zero. Data on steroid levels in incubation medium were expressed as mean and SEM, and analyzed by Duncan's multiple range test. The Student-t test was used to compare means between treated and control groups in *in vitro* effects of eight steroids on GVBD assay.

## RESULTS

### Steroid transformation by follicular cells

Yolk-free follicular cells obtained from ovaries isolated at four different stages of development, stages SY, TY, GVM, and GVBD, were incubated with $^{14}$C-labeled 17-P for 2 hr and the steroid metabolites produced during incubation were separated by TLC with benzene-acetone (4:1).

The following six steroid metabolites were identified by

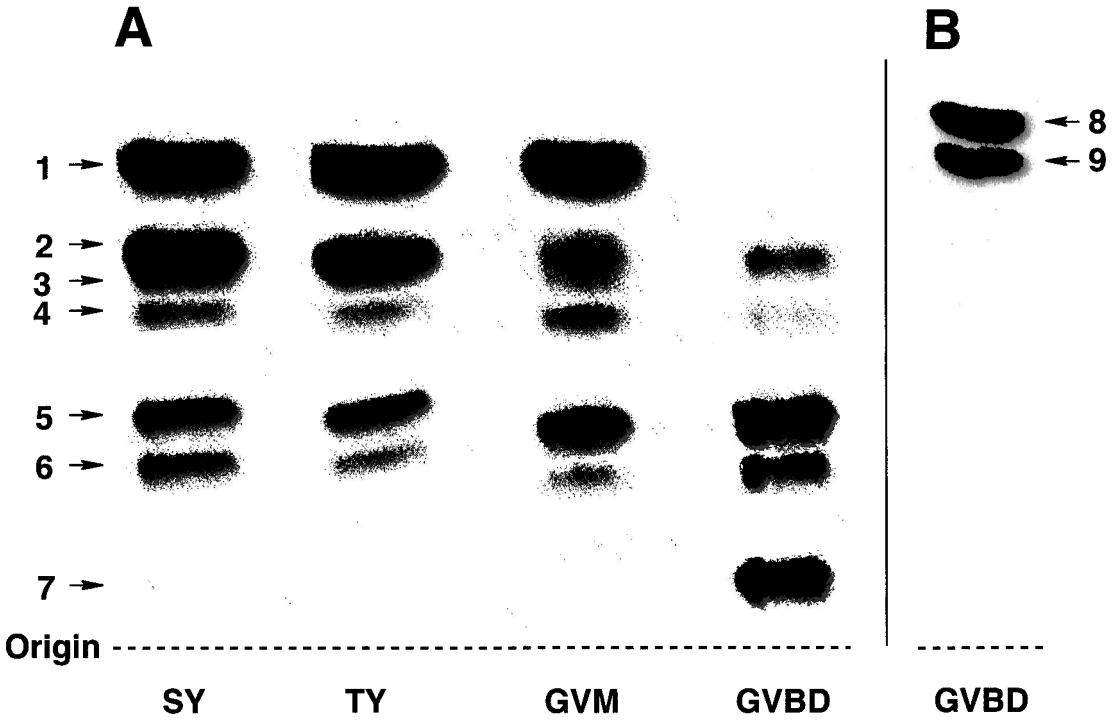


**Fig. 1.** Autoradiograms of the steroid metabolites produced by tiger puffer ovarian follicles incubated with $^{14}$C-labeled 17α-hydroxyprogesterone. A, Ovarian follicle cells were collected from fish at the SY (secondary yolk), TY (tertiary yolk), GVM (germinal vesicle migration), and GVBD (germinal vesicle breakdown) stages. The metabolites were separated by TLC with benzene-acetone (4:1). 1, androstenedione; 2, 17α-hydroxyprogesterone (precursor): 3, estradiol-17β; 4, testosterone; 5, 11-deoxycortisol and /or 17α,20β-dihydroxy-4-pregnen-3-one; 6, 17α,20α-dihydroxy-4-pregnen-3-one; and. 7, 17α,20β,21-trihydroxy-4-pregnen-3-one. B, Further chromatography of metabolite 5 from GVBD follicles in benzene:chloroform:diethyl ether:methanol (2:2:1:1) separated it into two metabolites. 8, 17α,20β-dihydroxy-4-pregnen-3-one; and 9, 11-deoxycortisol

**Table 3.** Steroid metabolite and specific activity of the crystal

| Metabolite | Specific activity of crystal (cpm/mg) | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| 17α,20β,21-trihydroxy-4-pregnen-3-one | 519 | 553 | 519 | 508 | 486[(b)] |
| 17α,20β-dihydroxy-4-pregnen-3-one | 728 | 794 | 853 | – | – |
| 11-deoxycortsol | 504 | 497 | 579 | 464 | 491[(a)] |
| 17α,20α-dihydroxy-4-pregnen-3-one | 275 | 249 | 252 | 281 | 319[(a)] |
| androstenedione | 350 | 338 | 330 | 332 | 310[(b)] |

Pair of solvents: 1st, dichloromethane & hexane; 2nd, dichloromethane & heptane; 3rd, chloroform & hexane; 4th, chloroform & heptane; 5th, ethanol & water (a), or methanol & water (b)

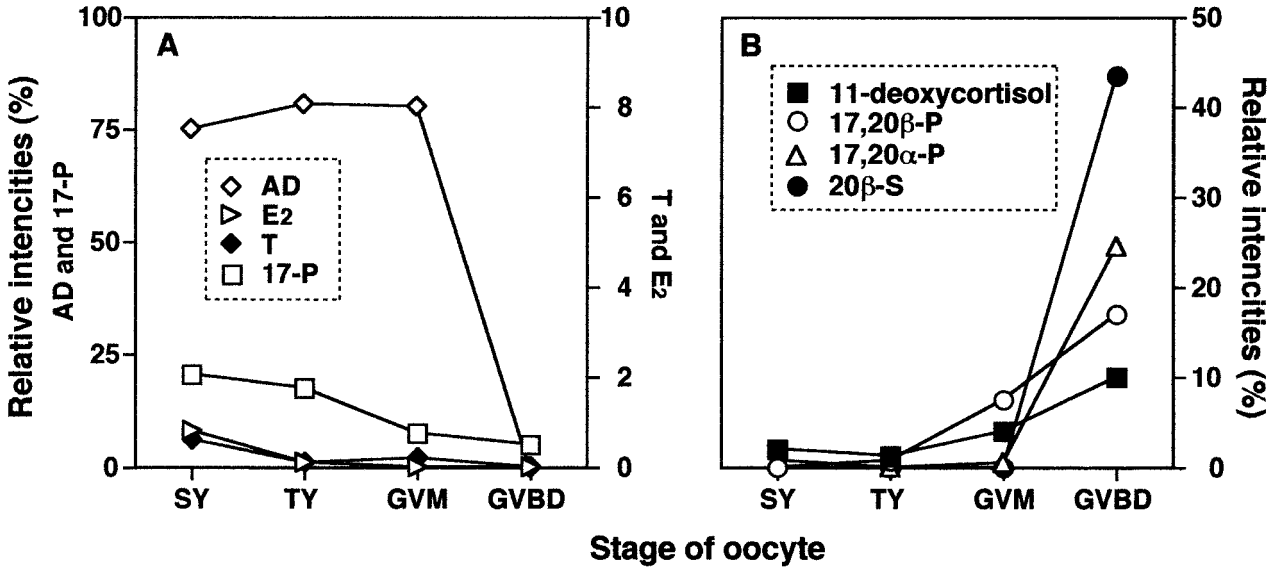


**Fig. 2.** Quantitative analysis of eight steroid metabolites, including the precursor 17α-hydroxyprogesterone, produced in ovarian follicle cells collected from vitellogenesis (SY) to final oocyte maturation (GVBD). A, Changes in the relative intensities (% radioactivity of each metabolite/total radioactivity) of four steroid metabolites showing a decline in intensity during the period from SY to GVBD. AD, androstenedione; E2, estradiol-17β; T, testosterone; and 17-P, 17α-hydroxyprogesterone. B, Changes in the relative intensities of the four steroid metabolites showing an increase in intensity during the same period. 17,20β-P, 17α,20β-dihydroxy-4-pregnen-3-one; 17,20α-P, 17α,20α-dihydroxy-4-pregnen-3-one, and 20β-S, 17α,20β,21-trihydroxy-4-pregnen-3-one.

their chromatographic mobilities in TLC: spot 1 to 6 corresponding to androstenedione, 17-P (precursor), estradiol-17β, testosterone, 11-deoxycortisol and 17α,20α-dihydroxy-4-pregnen-3-one (17,20α-P), respectively (Fig. 1A).

When mature follicle cells were incubated with $^{14}$C-labeled 17-P, one major polar metabolite, 20β-S (spot 7), was found. Spot 5 was further separated into 17,20β-P (spot 8) and 11-deoxycortisol (spot 9) by TLC in benzene : chloroform : diethyl ether : methanol (2:2:1:1) (Fig. 1B). The identities of all the metabolites, except for testosterone and estradiol-17β, were confirmed by recrystallization to a constant specific activity (Table 3). Quantitative analysis of the eight steroid metabolites including a precursor steroid, 17-P, was performed using the relative intensities (% radioactivity of each metabolite/total radioactivity) of the eight metabolite bands in the autoradiographs (Fig. 2).

There was a marked difference in the pattern of radioactive steroid metabolites between the GVBD stage and the first three stages (SY, TY, and GVM). SY, TY, and GVM follicles metabolized androstenedione extensively from 17-P (Fig. 2A). The second major metabolite produced by the same follicles was an 11-deoxycortisol (Fig. 2B). Follicles at the same stage also produced small amounts of estradiol-17β and testosterone; however, no radioactivity co-migrated with these two steroids and androstenedione at stage GVBD. In contrast, at the same stage, there was increasing production of three progestins, 20β-S, 17,20β-P, and 17,20α-P, and 11-deoxycortisol from 17-P. Particularly, 20β-S was not metabolized during stages SY to GVM, but intense 20β-S production occurred abruptly at stage GVBD.

### *In vitro* effects of eight steroids on GVBD

The substrate steroid (17-P) and seven metabolites (androstenedione, testosterone, estradiol-17β, 11-deoxycortisol, 17,20β-P, 17,20α-P, and 20β-S) produced in the follicle cells between stages SY (vitellogenic) and GVBD (mature) were tested for their ability to induce GVBD in tiger puffer oocytes *in vitro* using radioinert standard steroids. At a

concentration of 10 ng/ml, only two metabolites, 20β-S and 17,20β-P, were effective in inducing oocyte maturation. Of these two, 20β-S was the most effective inducer of oocyte maturation *in vitro*, inducing 40% and 83% GVBD after 36 and 48 hr incubations, respectively (Fig. 3). *In vitro*, 17,20β-P had much less effect on GVBD, inducing 3% and 10% GVBD after 36 and 48 hr incubations, respectively. The other six steroids were not effective in inducing GVBD at any concentration or incubation time.

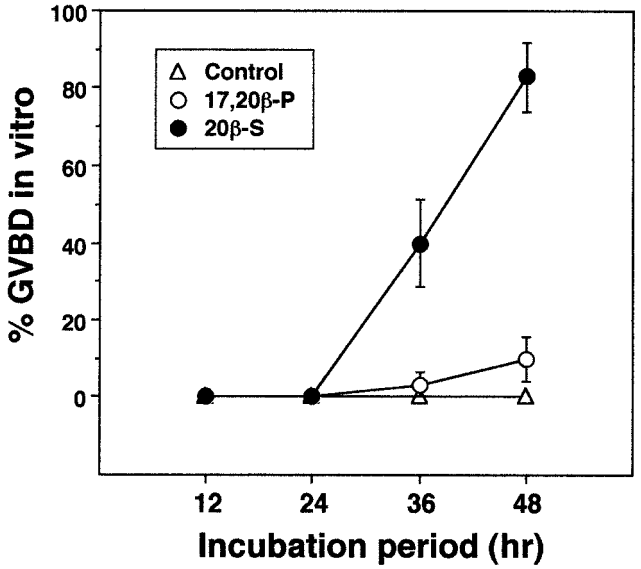


**Fig. 3.** Time course response curve for the effects of two steroid metabolites, 20β-S and 17,20β-P, produced by tiger puffer ovarian follicles on the induction of GVBD *in vitro*. Two of the eight metabolites, 20β-S and 17,20β-P, were effective in inducing GVBD at a concentration of 10 ng/ml, while the other six steroids did not induce GVBD *in vitro* at any concentration or incubation time. Data were expressed as mean and SEM for triplicate determinations.

### *In vitro* steroid production from radioinert precursor

*In vitro* production levels of 17,20β-P, 11-deoxycortisol and 20β-S from their radioinert precursors (100 ng/ml) by intact follicles of 40 mg are shown in Fig. 4. *In vitro* productions of 17,20β-P and 20β-S from 17-P and 11-deoxycortisol respectively reflect directly the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD) in the intact follicles, while those of 11-deoxycortisol and 20β-S from 17-P and 17,20β-P respectively reflect the activity of 21-hydroxylase.

*20β-HSD activity*: Any exogenous 17-P was hardly converted to 17,20β-P during vitellogenesis (TY) and GVM, while relatively low level of 17,20β-P (48 pg/ml/mg tissue) was produced at GVBD. *In vitro* 20β-S production level showed a drastic increase (401 pg/ml/mg tossue, $P<0.01$) at GVBD, although it was very low (under 19 pg/ml/mg tissue) during TY and GVM. These results indicate that 20β-HSD is highly activated at GVBD and 11-deocycortisol is largely converted to 20β-S compared with 17-P to 17,20β-P.

*21-hydroxylase activity*: Exogenous 17-P was largely converted to 11-deoxycortisol (225−468 pg/ml/mg tissue)

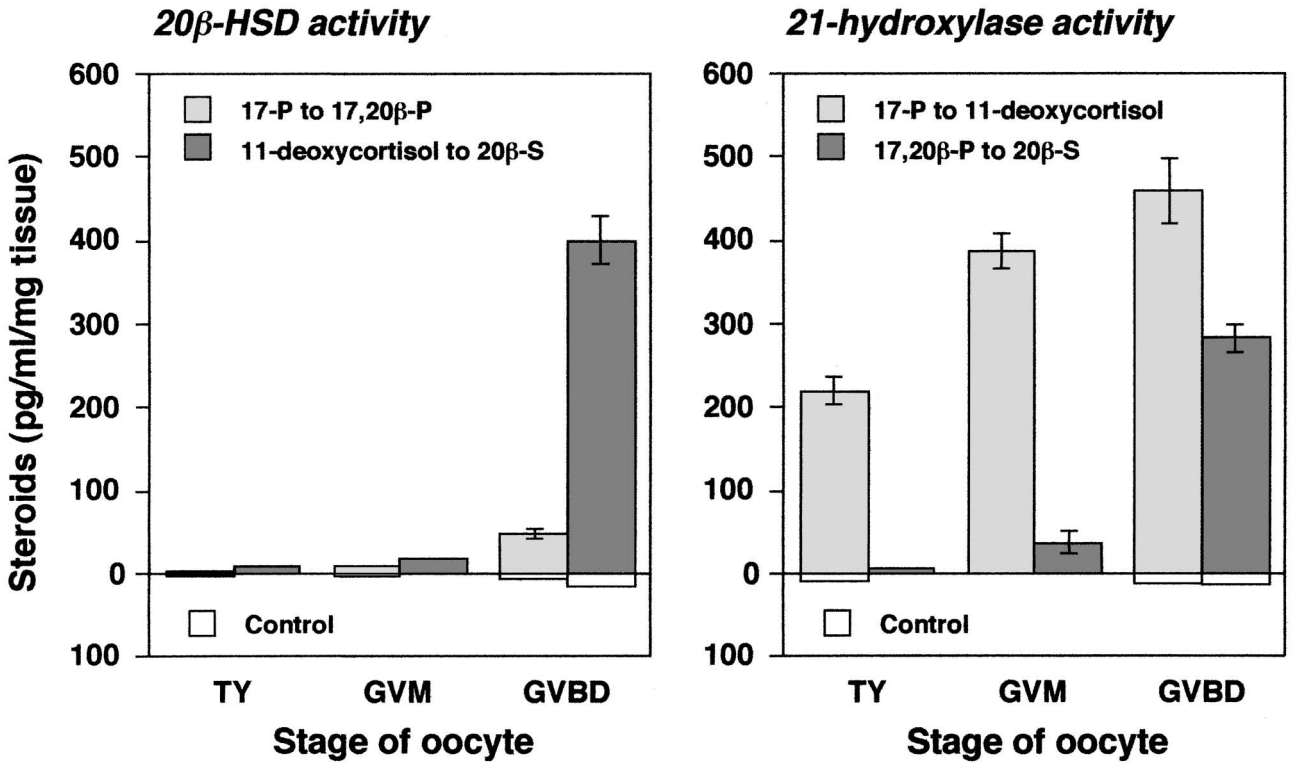


**Fig. 4.** *In vitro* 17,20β-P, 11-deoxycortisol and 20β-S production using radioinert precursors by intact follicles of tiger puffer sampled at three different developmental stages of oocyte. Each value is mean±SEM. TY, tertiary yolk stage; GVM, germinal vesicle migration; GVBD, germinal vesicle breakdown.

throughout the stages of oocyte, however, the 21-hydroxylase activity showed a tendency to increase from TY to GVBD by degrees. 20β-S production levels from 17,20β-P showed nearly zero at TY and a small increase (36 pg/ml/mg tissue, $P<0.01$) at GVM, following a dramatic increase (275 pg/ml/mg tissue, $P<0.01$) at GVBD. These results show that 21-hydroxylase is highly activated at GVBD, although its activity always present from vitellogenesis (TY) to GVBD, further suggesting the involvement of 21-hydroxylase in the conversion of 17-P to 11-deoxycortisol rather than 17,20β-P to 20β-S during TY and GVM.

## DISCUSSION

This study found that the final maturation of tiger puffer oocytes is induced primarily by 20β-S. The steroids tentatively identified on the basis of chromatographic mobility after incubating tiger puffer follicles with $^{14}$C-17-P were androstenedione, estradiol-17β, testosterone, 11-deoxycortisol, 17,20α-P, 17,20β-P, and 20β-S. The purity and final characterization of these metabolites were confirmed by recrystallization to a constant specific activity. Of all these steroids, only two, 17,20β-P and 20β-S, exhibited maturation-inducing activity *in vitro*. 20β-S was overwhelmingly more effective and quicker at inducing GVBD than 17,20β-P. The *in vivo* production of 20β-S has not been fully investigated in the tiger puffer due to lack of required facilities for collection of a large number of experimental fish. However, the correlation of 20β-S synthesis with final oocyte maturation, the fact that 20β-S is the major steroid produced by maturing ovarian follicles, and the demonstration of its high capacity to induce GVBD all provide strong evidence that 20β-S has a physiological role as an MIH in the tiger puffer.

This study found that there is a marked change in ovarian steroidogenesis in the tiger puffer in the production of MIH, coincident with final oocyte maturation. The chromatographic patterns of steroid metabolites at stages SY, TY, and GVM were similar. In particular, there was a two-step estradiol-17β (directly converted from testosterone by aromatase) synthesis pathway via androstenedione (from 17-P by C17,20-lyase) and testosterone (from androstenedione by 17β-hydroxysteroid dehydrogenase, 17β-HSD) (Fig. 5). Estradiol-17β promotes vitellogenesis in all non-mammalian vertebrates (Ho, 1991). At the same time, 11-deoxycortisol was actively converted from 17-P by 21-hydroxylase. On the contrary, a drastic change in the steroid metabolite chromatographic pattern was observed during GVBD. A massive amount of 20β-S (converted from 11-deoxycortisol by 20β-hydroxysteroid dehydrogenase, 20β-HSD) and relatively small amounts of 17,20α-P (from 17-P by 20α-HSD) and 17,20β-P (from 17-P by 20β-HSD) were produced in place of the metabolites for the synthesis of estradiol-17β. The conversion rate of 11-deoxycortisol increased further during GVBD, indicating increasing 21-hydroxylase activity following the vitellogenic stage.

In salmonid fishes and medaka, a distinct shift from estradiol-17β to 17,20β-P (the MIH of salmonids and medaka) has been reported to occur in the ovarian follicles immediately prior to oocyte maturation (Nagahama *et al*., 1994, Fukada *et al*., 1994). During vitellogenesis, in these fishes there are very high C17,20-lyase and aromatase activities, but 20β-HSD is undetectable, leading to the production of estradiol-17β. In contrast, during final oocyte maturation, the C17,20-lyase and aromatase activity is low, but 20β-HSD is highly active, leading to the production of 17,20β-P. Thus, in salmonids and medaka, there is a shift in steroidogenic enzyme activity that regulates the shift from estradiol-17β to 17,20β-P production. In the tiger puffer, a similar shift in steroidogenic enzyme activity (a decrease in the C17,20-lyase activity and an increase in 20β-HSD activity) occurs during final oocyte maturation. In addition to this shift in steroidogenic enzyme activity, continuously increasing 21-hydroxylase activity throughout all the oocyte developmental stages was also distinct in tiger puffer follicles in comparison with salmonids and medaka. During vitellogenesis, increasing 21-hydroxylase activity likely enables ovarian follicles to accumulate enough 11-deoxycortisol, which leads to 20β-S production on a massive scale by 20β-HSD during GVBD.

In this study, 11-deoxycortisol, which was produced as a precursor, was rapidly converted to 20β-S by 20β-HSD in the GVBD follicles, indicating 20β-HSD is a key enzyme in final oocyte maturation as in salmonids and medaka. 17-P was also converted into 17,20β-P due to sudden activation of 20β-HSD, but the total specific activity of 17,20β-P was considerably lower than that of 20β-S during GVBD. This result indicates the substrate specificity of 20β-HSD in the tiger puffer ovarian follicles, in fact, *in vitro* conversion experiment using radioinert precursors clearly showed the substrate specificity of 20β-HSD in the tiger puffer ovarian follicles, i.e., 20β-HSD selectively converted 11-deoxycortisol into 20β-S but not 17-P into 17,20β-P during GVBD. On the other hand, 21-hydroxylase exhibited high and increasing activity throughout the stages, resulting in the highest activity at GVBD period. The highest activity of 21-hydroxylase at GVBD period showed a possibility that ovarian follicles at GVBD period could convert 17,20β-P to 20β-S in a large scale, although it showed also substrate specific activity like 20β-HSD i.e., conversion rate of 11-deoxycortisol from 17-P was larger than that of 20β-S from 17,20β-P. A total volume of 17,20β-P endogenously produced in the GVBD ovarian follicles, however, might be small due to the substrate specificity of 20β-HSD. Consequently it is possible that a relatively small amount of 20β-S might be synthesized from 17,20β-P produced during GVBD by 21-hydroxylase. The later production and lower potency of 17,20β-P for GVBD induction *in vitro* supports this hypothesis. In order to clarify the function of 20β-HSD and 21-hydroxylase, including substrate specificity, studies on molecular cloning of the genes is now underway.

We cannot deny that 17,20β-P may act as another MIH for final oocyte maturation in the tiger puffer. Evidence from studies of teleost and amphibian oocyte maturation *in vitro* (Nagahama, 1987a; Smith and Ecker, 1971) indicates that

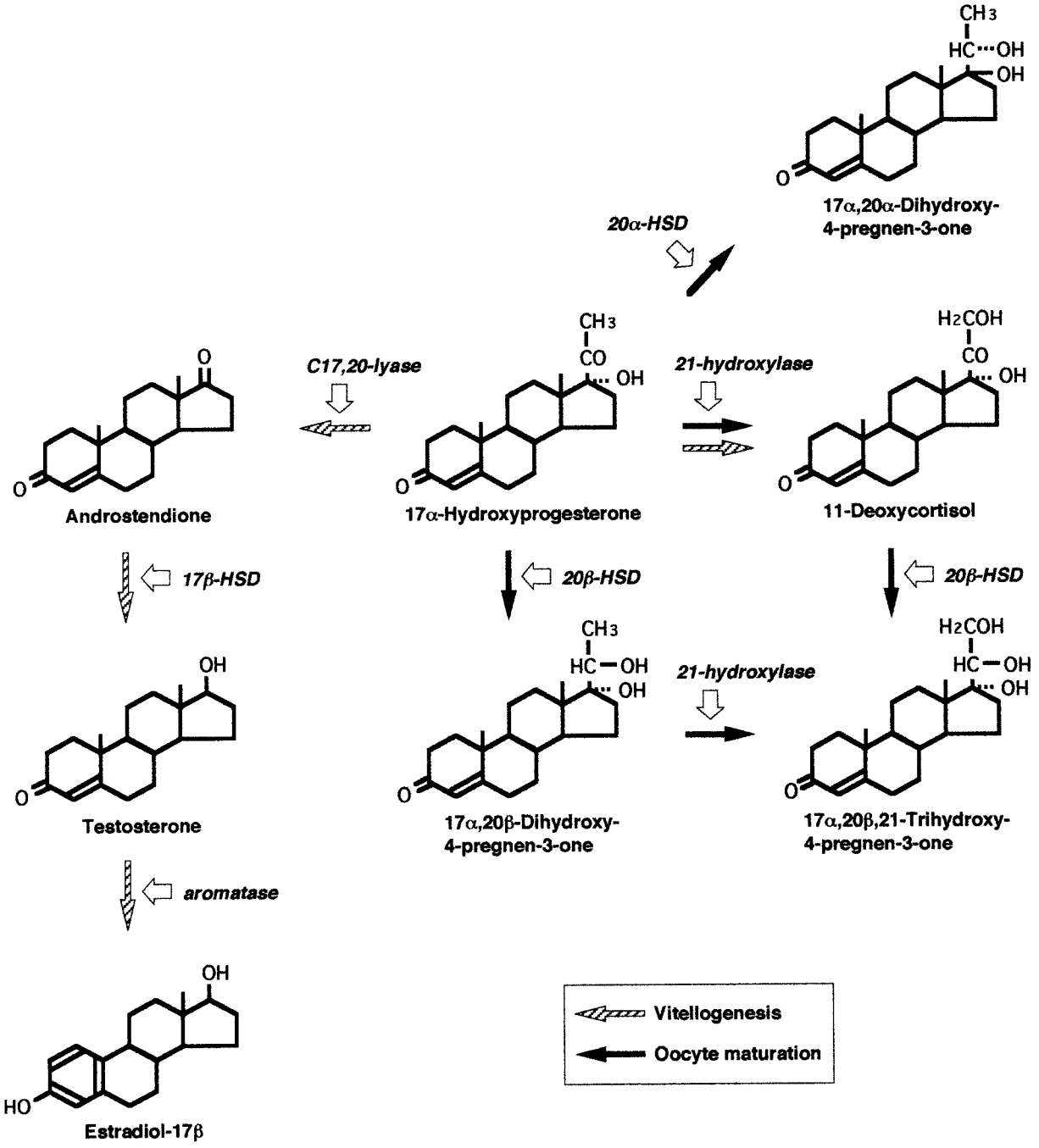


**Fig. 5.** Steroidogenic pathways for estradiol-17β and 17α,20β,21-trihydroxy-4-pregnen-3-one biosynthesis in tiger puffer ovarian follicles during vitellogenesis and oocyte maturation.

the mode of MIH action is quite different from the classic steroid hormone dogma, which states that steroids bind to intracellular or nuclear hormone receptors. An oocyte 17,20β-P receptor has been characterized on ovarian membranes prepared from rainbow trout *Oncorhynchus mykiss*. High levels of specific binding of 17,20β-P were found using plasma membranes prepared from defolliculated oocytes (Yoshikuni *et al.*, 1993). Similarly, ovarian 20β-S receptors have been characterized for the spotted seatrout (Patino and Thomas, 1990) and striped bass (King *et al.*, 1997). To clarify the involvement of 17,20β-P in final oocyte maturation in the tiger puffer, future studies characterizing the oocyte MIH receptor are required.

17,20β-P was first discovered in the plasma of the sockeye salmon, *Oncorhynchus nerka* (Idler *et al.*, 1960), and has been definitively identified as the MIH in amago salmon (Nagahama and Adachi, 1985). Fish that use 17,20β-P as the MIH are widely distributed taxonomically; the amago salmon belongs to the order Salmoniformes, the Indian catfish to the Siluriformes, and the killifish and medaka to the Atheriniformes.

In contrast, 20β-S was first discovered and simultaneously identified as the MIH in the Atlantic croaker in 1986 (Trant *et al*., 1986). Subsequently, it has been confirmed as an MIH in the three marine Perciformes species already mentioned. To date, 20β-S has not been identified as an MIH in other teleost orders. This study is the first to demonstrate that 20β-S also acts as an MIH in the Tetraodontiformes, which are distantly related to the perciform fish taxonomically, suggesting the possible overall importance of 20β-S in inducing final oocyte maturation in a large vertebrate group, the teleosts.

The production of 17,20α-P, an isomer of 17,20β-P, (converted from 17-P by 20α-HSD) was dramatically increased in tiger puffer GVBD follicles, and its peak was higher than that of 17,20β-P, but lower than that of 20β-S. A large amount of 17,20α-P has been found in the blood and ovarian incubates of mature flatfish species (Canario and Scott, 1990; Scott and Canario, 1987, 1990), cyprinids (Kime, 1992), and catfish (Zairin *et al*., 1992). The role of this steroid is still unknown. It is not a potent inducer of oocyte maturation, but it may be a behavioral steroid or pheromone (Scott and Canario, 1987)

In summary, this study provided strong evidence that 20β-S is the MIH of the tiger puffer, and is the first report of 20β-S acting as an MIH in a teleost other than a perciform fish. This study further clarified the 20β-S production mechanism during final oocyte maturation by analyzing the changes in each steroidogenic enzyme throughout ovarian development, i.e. a decrease in the C17,20-lyase activity and an increase in 20β-HSD activity during final oocyte maturation, combined with high, increasing 21-hydroxylase activity throughout all the oocyte development stages, lead to ample 20β-S production for GVBD. Regardless of whether the teleost MIH of is 17,20β-P or 20β-S, there may be common enzymatic kinetics in MIH production; the decrease in C17,20-lyase activity and increase in 20β-HSD activity and this shift seem to be a prerequisites for the final maturation of growing oocytes in teleosts.

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